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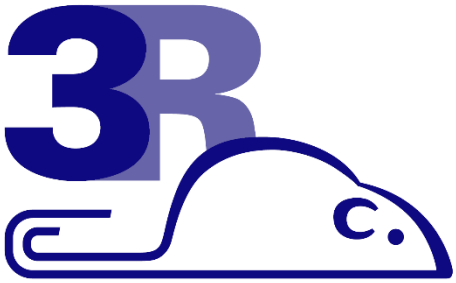
3R Research Foundation Switzerland



Abstracts of the research projects funded by the

3R Research Foundation Switzerland

1987-2017

The 3R Research Foundation Switzerland

The Aim of the Foundation
The aim of the 3R Foundation is to promote alternative research methods to animal experimentation through grants for research projects as well as to implement and promote the 3R principles. The organisation supports first and foremost projects aimed at developing new methods or refining accepted methods (validation) which offer improvements vis-à-vis standard animal experimentation in line with the 3R motto Reduce, Refine, Replace. Research projects are selected for support according to periodically defined principal areas for financial support.

How the Foundation is funded
The 3R Foundation is a cooperative institution set up in 1987 by the Parliamentary Group for Animal Experimentation Questions (public organ), Interpharma and the Foundation for Animalfree Research (animal protection). The organisation is supervised by the Federal Department of Home Affairs.

The Administrative Board of the Foundation is made up of nine members, two representing the Swiss parliament, two representing animal protection, two from Interpharma and two from the Federal Food Safety and Veterinary Office, as well as a representative of other interested circles.

The Board has appointed an Evaluation Committee to revue proposed research projects and applications for grants. This Committee at present comprises 14 scientists from the academic and industrial sectors.

The Foundation's Achievements
Research into experimental methods which do not involve live animals has played an important role in replacing animal experimentation by alternative methods. The Foundation also aims, however, at improving animal experimentation methods in line with increased animal protection. The research activities which have benefited from 3R's support have made a major contribution towards reducing the number of experiments involving live animals in Switzerland, as well as developing better or new methods.

Since it was founded, 3R has received around 482 applications for research grants and 146 of these projects have received some financial aid.

The funds used to support approved projects are provided in equal parts by the Federal Food Safety and Veterinary Office and Interpharma. An annual amount of approximately CHF 500,000 is available for grants.

Total funding for approved projects	CHF 18 798 418.70
Other Subsidies	CHF 834 533.00
Contributions from the Confederation	CHF 11 828 000.00
Contributions from Interpharma	CHF 11 998 000.00

The 3R Research Foundation Switzerland will end all activities after 30 years of tirelessly promoting the 3Rs and funding research projects aimed at replacing, reducing and refining animal experiments.

Münsingen, January 22, 2018

The Three Rs of Russell & Burch, 1959

Scope and Progress of Humane Techniques
The 3Rs refer to a study published in 1959. At the annual meeting of the former American Association of Laboratory Animal Science in Washington D.C., the late Major Charles W. Hume, the founder of the Universities Federation of Animal Welfare (UFAW), presented a study by two English scientists, William Russell, described as a brilliant zoologist, psychologist and classics scholar, and Rex Burch, a micro- biologist. They had carried out a systematic study of the ethical aspects and "the development and progress of humane techniques in the laboratory".

Humanity vs. Inhumanity
Russell and Burch based their study on the philosophical concept of humanity - in the sense of "humane-ness" - v.s. inhumanity in the context of animal experiments. They stated that true humanity, which distinguishes humans from all other species, was the capability for social cooperation, intimately linked to a compassionate and empathetic attitude towards other species. Although they assumed that biologists treated their animals as humanely as possible within the boundaries of then-current experimental protocols, Russell and Burch observed that some procedures were inhumane **per se**. By analysing and documenting the relative "humanity" or "inhumanity" of biological experiments, the authors hoped to promote the development of humane experimental techniques and reduce the amount of pain and fear inflicted upon laboratory animals. Russell and Burch stated clearly that they limited their definitions of "humane" and "inhumane" strictly to the experimental procedures under analysis; no criticism or moral judgement of the experimenters involved was intended or implied.

Criteria of Distress
How does one measure "inhumanity"? Russell and Burch used the criteria of pain, or more generally, "distress" experienced by the animals. Physiological and endocrine parameters offered objective measurements of stress. Any set-up using negative reinforcement (punishment) as motivation to train or condition responses qualified as a source of stress and fear. The behaviour of the animals towards the experimenters and each other served as a further indicator of well-being or distress (e.g. incidents of asocial behaviour such as biting and scratching, the need for restraints during experimental procedures vs. tameness, etc.).

Man-made Ecology
A final area examined by Russell and Burch was the environment, both social and physical, of the experimental animals. All laboratory animals are alike in this respect: their environment is manmade. The size and distribution of their social groups, and their physical surroundings, tended to be optimized for the convenience of the experimenters, rather than to meet the needs of the animals.

The Study of Russell and Burch
The first step in the Russell and Burch study was to gather data on animal experimentation: what numbers of which species were used for what types of experiments. Their sources included the L.A.B. survey of 1952 (Laboratory Animal Bureau of the British Medical Research Council), which provided the following information: animal species used, laboratory type, purpose of research and the number of animals. Russell and Burch then analyzed each experimental procedure for its degree of inhumanity. They examined the incidence of inhumanity and graded the severity of the distress experienced by the animals from mild to severe. Especially severe forms of distress, such as potentially lethal experiments or operations with accompanying post-operative pain were classified separately. The authors then combined the three variables to identify those procedures that were the most inhumane. This analysis provided the systematic basis upon which to launch a programme for humane procedures in experimental biology, termed the three R's: **R**eplace, **R**educe, **R**efine.

Replacement
Replacement was the most radical proposal: The use of nonsentient organisms rather than higher animals for experiments. Microorganisms, metazoan parasites, and higher plants were suggested as possible alternatives. Experiments using these materials were labelled **"absolute replacement"**, since no higher animals were required at any stage. In vitro techniques with cell cultures from animal tissues were defined as **"relative replacement"** procedures, since the experiments themselves were conducted on non-sentient material, but still depended upon animal materials.

Reduction
Reduction meant obtaining the best quality and most precise information with the smallest possible number of animals. Experiments that were well-designed and well-conducted would deliver reliable results, and eliminate the need for endless repetition of the same tests. This included close cooperation with statisticians and establishing in advance the required level of statistical significance. Similar gains could be achieved by careful dose-level selections in dose-response studies.

Refinement
Refinement was the most subtle approach. It referred to all changes in protocols that reduced the incidence or severity of distress experienced by laboratory animals.

Reference
Russell, W.M.S. and Burch, R.L.: The Principles of Humane Experimental Technique. Methuen, London, 1959. Reprinted by UFAW, 1992: 8 Hamilton Close, South Mimms, Potters Bar, Herts EN6 3QD England. ISBN 0 900767 78 2

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3R-Project 01-87

Verminderung von Tierversuchen in der Tollwut-Diagnostik durch Verbesserung der in vitro Diagnostik-Methoden

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Duration: 2 years **End of the Project:** 1989

Background and Aim
Die Diagnose "Tollwut" wird beim Tier aufgrund einer Untersuchung von Hirnmaterial aus dem verdächtigen Tier gestellt. Falls die Untersuchung des Hirnmaterials negativ ausfällt, setzt man damit einen Tierversuch an. Mit dem Projekt sollen 2 Ziele erreicht werden:
1. Laboratorien in Entwicklungsländern Antiseren für die Tollwutdiagnostik zur Verfügung stellen (für die Immunfluoreszenzmethode).
2. Abklärung, ob sich der Tollwutnachweis mittels Tierversuch unter Routinebedingungen durch die in der Literatur beschriebenen Zellkulturtechniken ersetzen lässt.

Method and Results
Zum Tollwutnachweis wird heute jungen Mäusen Hirnmaterial des verdächtigen Tieres ins Hirn injiziert. Dieses für das Versuchstier stark belastende Vorgehen sollte durch Versuche an Zellkulturen ersetzt werden. Die bisherigen Resultate haben ergeben, dass die Zellkultur-Methode den Anforderungen vorläufig nicht genügt. Es ist noch mit einer Fehlerrate von 20% zu rechnen, d.h. 20% falsch negative Ergebnisse im Vergleich zum praktisch unfehlbaren Mäuse-Inokulationstest. Die Untersucher sind jedoch zuversichtlich, dass die noch bestehenden Probleme durch methodische Änderungen gelöst werden können.

Reference
Gerhardt A. (1995), Teilvalidierung einer Zellkulturmethode als Alternative zum Tierversuch für den Nachweis von Tollwutvirus aus Hirnmaterial. (Veterinärmedizinische Dissertation, Universität Bern).

3R-Project 02-87

Menschliches Lebergewebe als Ersatz des Tierversuchs bei der Arzneimittelprüfung

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Biozentrum, Universität Basel, Basel

Duration: 3 years **End of the Project:** 1990

Background and Aim
Arzneimittel und Chemikalien müssen auf ihre Umwandlung im Körper geprüft werden. Für diese Abklärungen finden meist Ratten und Hunde Verwendung. Das Projekt sollte abklären, ob mit Hilfe der Verwendung von isoliertem, menschlichem Lebergewebe der Tierversuch ersetzt und die Aussagekraft solcher Untersuchungen verbessert werden kann.

Method and Results
Arzneimittel und Chemikalien müssen auf ihre Umwandlung im Körper geprüft werden. Für diese Abklärungen finden meist Ratten und Hunde Verwendung. Innerhalb dieses Projektes wurde eine Bank von menschlichem Lebergewebe für die Untersuchung des Abbaus neuer und bereits bekannter Arzneimittel entwickelt. Das Gewebe stammt von hirntoten Organspendern oder von Leberoperationen und wird gleich nach der Entnahme in flüssigem Stickstoff eingefroren und bei - 80 Grad C aufbewahrt. Die meisten arzneimittelabbauenden Enzyme bleiben unter diesen Bedingungen bei intakter Funktion erhalten. Die Bank enthält über 100 Leberproben.
Dieses Gewebe wird dazu verwendet, um die Umwandlung von Arzneimitteln zu pharmakologisch inaktiven, aktiven oder sogar toxischen (z.B. karzinogenen) Metaboliten vorauszusagen, bevor sie an Tier und Mensch erprobt werden. An mehreren Beispielen konnte demonstriert werden, dass dies möglich ist, beispielsweise mit den bekannten Arzneimitteln Midazolam, Cyclosporin A, Lidocain. Diese Methodik erlaubt die Einsparung von Tierversuchen auf drei Gebieten der pharmazeutischen Forschung.
1. Durch die Kenntnis des menschlichen Abbaumusters kann die geeignete Tierart unter den Nicht-Nagetieren für toxikologische Untersuchungen ausgewählt werden.
2. Die Verwendung von menschlichem anstatt tierischem Gewebe soll in Zukunft die Aussagekraft von in vitro Testsystemen zur Prüfung von mutagenen, tumorigenen und teratogenen Eigenschaften von natürlichen und chemischen Substanzen verbessern.
3. Durch die Verwendung einer Serie von Gewebeproben von verschiedenen Spendern (Raucher, Arzneimittelexponierte, Alkoholiker etc.) können interindividuelle Unterschiede in der Wirksamkeit und Toxizität von Arzneimitteln frühzeitig abgeschätzt werden.
Diese auch den Forschern in der Basler Industrie frühzeitig zugänglich gemachten Erkenntnisse werden zu einer Reduktion von Tierversuchen vorab in der Toxikologie führen.

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3R-Project 03-87

In vitro Test zum Nachweis des LCM-Virus

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Duration: 2 years **End of the Project:** 1989

Background and Aim

Weil das lymphozytäre Choriomeningitis Virus (LCM) die Zellen, die es befällt, nicht oder nur selten zerstört, fehlt ein leicht zugänglicher Virusplaque Assay, mit dem alle LCMV-Isolate zuverlässig nachgewiesen werden können. Um die LCMV-Titrationen in vivo in Mäusen zu ersetzen wird vorgeschlagen, einen allgemein in vitro anwendbaren Assay zu etablieren: LCMV - Verdünnungen werden auf Gewebekulturzellen in flache 96-Mikrowellenplatten während 48 Stunden inkubiert, fixiert und mit einem Enzym-gekoppelten monoklonalen oder polyklonalen Antikörper gegen LCMV angefärbt. Dieser Test sollte allein dem eigenen Labor ermöglichen, eine Reduktion von 1000 bis 3000 Versuchstieren (Mäuse) im Jahr zu erreichen.

Method and Results

Zum Studium der Immunologie und Pathogenese des lymphozytären Choriomeningitis-Virus (LCM-Virus), einem Verwandten des menschlichen Hepatitis- und Aids-Virus, werden Mäuse verwendet. Zum Nachweis des LCM-Virus wurde ein in vitro-Test entwickelt, mit welchem 90% der in diesem belastenden tödlichen Versuch eingesetzten Mäuse eingespart werden können.

Reference

S. Battegay, S. Cooper, A. Althage, J. Bänziger, H. Hengartner and R.M. Zinkernagel (1991) Quantification of lymphocytic choriomeningitis virus with an immunological focus assay in 24- or 96-well plates. J Virol Meth 33: 191-198.

3R-Project 04-87

Erkennung erbschädigender Substanzen an der Taufliege Drosophila

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Duration: 2 years **End of the Project:** 1989

Background and Aim

Eine Grundaufgabe der genetischen Toxikologie ist die Erkennung von Substanzen mit erbschädigender Wirkung. Für wenige hundert Substanzen liegen bisher (1987) Ergebnisse über ihre Wirkung auf eines oder mehrere Gewebe im lebenden Säugetier vor. Bei der Taufliege Drosophila sind in den letzten Jahren rasche und flexible Tests zur Erfassung genotoxischer Wirkungen in somatischen Zellen entwickelt worden. Am Säuger gut untersuchte Substanzen sollen nun mit einem solchen Test geprüft werden. Diese vergleichenden Untersuchungen, in kritischen Fällen ergänzt durch bakterielle Tests, sollen zeigen, für welche Substanzen oder Substanzgruppen Drosophila-Tests Untersuchungen am Säuger ersetzen können.

Method and Results

Bei der Taufliege Drosophila melanogaster wurde ein Testsystem entwickelt, das neben dem Nachweis der Induktion von Genmutationen auch die Erfassung von rekombinogenen Wirkungen erlaubt. In diesem System (Somatic Mutation And Recombination Test, SMART) zeigte es sich, dass in der Kanzerogenese das Phänomen des Heterozygotie-Verlusts eine wichtige Rolle spielt, und dass dabei somatische Rekombinationsereignisse von grosser Bedeutung sind. Eine induzierte genotoxische Veränderung z.B. durch Bestrahlung der Anlagen künftiger Organe in den Drosophila-Larven führt zur phänotypischen Ausprägung dieser Markier-Gene auf der Flügeloberfläche oder am Auge.
Ausgehend von einer im Rahmen dieses Projektes weiterentwickelten Computerdatenbank GEN (sie enthält die bekannten Versuchsergebnisse für ca. 3000 Chemikalien) wurde eine Reihe von 21 Substanzen geprüft. Die Daten zeigen, dass sich das Testsystem mit somatischen Zellen der Taufliege Drosophila als einfache und zuverlässige Nachweismethode für genotoxische Substanzen eignet.

References

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3R-Project 05-87

Kältekonservierung von Wurmparasiten

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Institut für Parasitologie, Universität Zürich, Zürich

Duration: 3 years End of the Project: 1990

Background and Aim

In der parasitologischen Forschung und Diagnostik müssen weltweit zahlreiche Parasiten - Arten eingesetzt und unter Laborbedingungen lebensfähig gehalten werden. Die Haltung solcher “Parasiten-Isolate” erfolgt zum Teil durch sogenannte “Serienpassagen” in Wirbeltieren. Sollen bestimmte Parasiten-Isolate jederzeit verfügbar sein, so müssen ständig Serienpassagen durchgeführt werden, auch dann, wenn die Forschungen vorübergehend unterbrochen werden oder nur geringe Mengen des Parasitenmaterials benötigt werden. Dies führt zu einem unnötigen Verschleiss von Versuchstieren.
Daher ist es für die Zukunft anzustreben, die benötigten Parasiten - Isolate in künstlichen Nährmedien ausserhalb von Tieren (in vitro) zu halten oder sie einzufrieren und nur dann auf Versuchstiere zu übertragen, wenn dies bestimmte Fragestellungen erfordern.
Die Kältekonservierung einzelliger Parasiten (Protozoen) und der mehrzelligen Wurmparasiten (Helminthen) wurde in den letzten Jahren als eine aussichtsreiche Methode angesehen, die Anzahl der zur Haltung von Parasiten-Isolaten benötigten Versuchstiere zu senken. Im Rahmen dieses Projektes werden diese Arbeiten mit Helminthen fortgesetzt.

Method and Results

Zum Studium von Parasitenerkrankungen müssen die Einzeller oder Würmer in Wirbeltieren "fortgepflanzt" werden. In diesem Projekt gelang es, verschiedene der benötigten Parasiten durch Einfrieren lebend zu erhalten. Insbesondere gelang es, für verschiedene Parasitenarten (Metazestoden von Echinococcus multilokularis, d.h. ein Zwischenwirtstadium von Bandwürmern, Sporozoiten von Eimeria tenella und Eimeria nieschulzi, d.h. Coccidien) geeignete Gefriertechniken zu erarbeiten. Damit ist es möglich geworden, gewisse Parasiten jahrelang lebensfähig zu erhalten.
Für andere Metazestoden sind bis jetzt alle Versuche zur Kältekonservierung fehlgeschlagen. Teilerfolge konnten bei den Versuchen zur Kultivierung von Eimeria-Arten auf verschiedenen Zellarten und im bebrüteten Hühnerei erzielt werden. Die Ursachen für die bisher sehr unterschiedlichen Züchtungserfolge werden noch weiter abgeklärt.

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3R-Project 06-88

Tierschonender Einsatz des Beagle Hundes in der Toxikologie

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Institut für Toxikologie, der ETH und Universität Zürich, Zürich

Duration: 5 years **End of the Project:** 1993

Background and Aim

Standard toxicological studies in dogs using high doses of vasodilators and positive inotropic / vasodilating agents give rise to a species-specific cardiotoxicity. The reason may be the extreme sensitivity of the dog to the pharmacological effects of these drugs; exaggerated pharmacodynamic effects and prolonged disturbance of homeostasis mechanisms often are responsible for the observed organ lesions. An assessment of the toxicological relevance and the risk for patients taking the drugs at therapeutic doses cannot be made without taking into account their pathomechanisms and the pathophysiological basis of the exceptional reaction patterrns occurring in dogs.

Method and Results

A large series of vasodilating and positive inotropic agents are presented, their pharmacological properties are described, and toxicological effects in dogs are compared. In view of the poor correlation between the distinct cardiac lesions induced in dogs and a lack of comparable toxicity in humans, it appears desirable to reassess the adequacy of the standard toxicological approaches for these substances.

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3R-Project 07-88

Erregungsleitung und Transporteigenschaften in Nerven und Epithelien

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Institut de Physiologie, Université de Fribourg, Fribourg

Duration: 1 year **End of the Project:** 1990

Background and Aim

Es wurde vorgeschlagen, für zwei Praktikumsplätze (Erregung im peripheren Nerven und Epitheltransporte) Kenntnisse und Konzepte anhand von Untersuchungen an Mitstudenten, bzw. an Gewebekulturen zu übermitteln. Das Ziel ist, ein (Replace-) Vorgehen zu entwickeln, bei dem auf die bisher eingesetzten Frösche verzichtet wird, und zwar ohne Einbusse didaktischer Inhalte. Mit diesem Projekt sollte unter Einbezug moderner Unterrichtsmethoden modellhaft ein tierschonender praktischer Unterricht in Physiologie verwirklicht werden.

Method and Results

Im Rahmen des Projekts wurden modellhafte Praktikumsplätze für Medizinstudenten geschaffen für einen tierschonenden, praktischen Unterricht in Physiologie, bei dem auf die bisher eingesetzten Frösche verzichtet werden kann. Anstelle von Fröschen werden zur Demonstration physiologischer Grundmechanismen - Erregungsleitung in peripheren Nerven, Transporteigenschaften von Epithelzellen - Menschen und Gewebekulturen eingesetzt. Die Anleitung zu den Praktika wird an interessierte Lehrer im In- und Ausland abgegeben. Sie kann bei den Projektleitern oder bei der Stiftung bezogen werden.

Reference

Kursanleitung für Praktikumsplätze "Transporteigenschaften von Epithelien" und "Erregung in peripheren Nerven" mit Resultat-Beispielen, Universität Freiburg, 1990.

3R-Project 08-88

Remplacement des animaux dans les études sur Toxoplasma gondii I

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Duration: 2 years **End of the Project:** 1990

Background and Aim
Toxoplasma gondii, un parasite responsable d'infection graves chez l'immunodéprimé et plus particulièrement les malades atteint du SIDA, ne se cultive pas sur les milieux usuels. Le maintien des souches du parasite, et les étudues d'efficacité des médicaments s'effectuent traditionellement chez l'animal. Afin de remplacer les études expérimentales, la possibilité d'utiliser des cultures de tissu pour ces études est évaluée. Des macrophages et des phagocytes non professionnels (cellules HeLa) vont être testés à cette fin. La conservation des souches de T. gondii intracellulaires par congélation sera recherché. L'activité des molécules antitoxoplasmes sera mesurée par l'incorporation de (H3)Uracil et par des sous-cultures sur cellules HeLa. Est attendu de ces études non seulement le remplacement des animaux mais aussi une amélioration des techniques existentes.

Method and Results
L'utilisation de cultures cellulaires pour étudier certains aspects physiopathologiques des infections à Toxoplasma gondii et Salmonella typhimurium a été evaluéé, dans un but de diminuer l'usage des animaux, souris en l'occurrence. Une culture de macrophages d'origine murine (P388D1) a permis de découvrir le rôle dans la virulence de deux gènes des salmonelles, motA et myjS: ils sont impliqués dans la capacité des salmonelles à l'intérieur du macrophage. Avec la toxoplasmose, nous avons montré l'implication de cytokines, en particulier le TNF, dans la capacité du prasite a se multiplier dans le macrophage.
Dans ces 2 cas, les études animales, effectuées sur un petit nombre d'animaux ont confirmé la pertinence des expériences avec des cellules. On peut souligner qu'au plan économique les études cellulaires coûtent beaucoup moins cher que les études animales conventionelles.

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3R-Project 09-88

In vitro Bestimmung schutzinduzierender Antigene in der Tollwutdiagnostik

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Duration: 3 years **End of the Project:** 1990

Background and Aim
Die Wirksamkeit von Tollwutimpfstoffen für Mensch und Tier wird gemäss Richtlinien der WHO oder nach den Vorschriften der Europäischen Pharmakopöe in einem Mäuseschutzversuch bestimmt. Dieser Test verlangt, dass Mäuse mit verschiedenen Impfstoffverdünnungen immunisiert werden und anschliessend das Schutzvermögen der Vakzine gegen eine intercerebrale Tollwutvirusinfektion berechnet wird (NIH - Test). Diese Versuchsanordnung erlaubt eine direkte quantitative Aussage über das Schutzvermögen der Vakzine bei der Maus und indirekt bei der Zielspecies. Jedes Lot der verschiedenen Tollwutimpfstoffe wird auf diese Art und Weise beim Impfstoffhersteller und meist auch bei der Kontrollbehörde geprüft. In der Schweiz werden dafür jedes Jahr ca. 150'000 Mäuse verbraucht (Stand 1988).
Es geht in diesem Projekt darum, mit anderen Parametern eine gleichwertige Aussage über die Wirksamkeit einer Tollwutvakzine in Korrelation mit dem Schutztest zu etablieren.

Method and Results
Ein Antigen-Antikörper-Test (ELISA) zur Bestimmung des schutzvermittelnden Glykoproteins in Tollwutimpfstoffen wurde mit dem Ziel entwickelt, einen Ersatz für den heute üblichen Versuch mit Mäusen zur Prüfung der Wirksamkeit von Tollwutimpfstoffen zu finden. Dabei hat sich gezeigt, dass bei den in der Tiermedizin gebräuchlichen Impfstoffen, die wenig gereinigt sind und verschiedene Adjuvantien (unspezifische Immunstimulantien) enthalten, unerwünschte Reaktionen mit Gewebekulturprotein und dem Adjuvans auftreten. Durch Modifikation des Tests (Catching-ELISA) konnten die Probleme der unspezifischen Reaktionen mit dem Adjuvans verringert werden.
Der Test erscheint als ein geeignetes Mittel zur Bestimmung der Wirksamkeit einer Vakzine während seiner Herstellung. Er erbringt aber nicht die notwendige Genauigkeit zur Chargenprüfung bei der Impfstoffkontrolle, bei der zahlreiche Vakzine mit unterschiedlichem Reinheitsgrad und verschiedenen Adjuvantien geprüft werden.

Reference
ALTEX 12: 46 - 58, 1989

3R-Project 10-88

Entwicklung eines künstlichen Fütterungssystems zur Zucht von Schildzecken

Diehl P. A., , Guerin P.
Institut de Zoologie, Université de Neuchâtel, Neuchâtel

Duration: 5 years **End of the Project:** 1993

Background and Aim

Ziel dieses 3R-Projektes war es, Alternativen zur Zucht von Schildzecken auf lebenden Tieren zu finden. Diese sogenannte "künstliche Fütterung" von Zecken sollte vom Ergebnis her mit natürlichen Zuchten konkurrieren können. Beim Festsetzen von Schildzecken sowohl auf ihren natürlichen Wirten als auch auf künstlichen Membranen spielen Substanzen aus dem Fell der Wirtstiere (Kontaktchemostimuli) und Pheromone der Zecken eine entscheidende Rolle. Es wurde ein Membran - Arrestment-Test entwickelt, um die Wirksamkeit von Kontaktstimuli zu prüfen. Dieser in vitro-Fütterungs - Test sollte auch für das 'Screening' von Zecken - Repellenzien und Antifeeding - Substanzen benutzt werden können.

Method and Results

Zum ersten Mal ist es nun gelungen, den Lebenszyklus einer Schildzeckenart (Amblyomma hebraeum) allein durch Fütterung auf künstlichen Membranen zu vollenden. Zum Einsatz kam ein preiswertes und einfach zu erweiterndes Fütterungssystem (umgebaute Honiggläser mit einer Silikonmembran). Angeboten wurde defibriniertes Rinderblut vom Schlachthof. Auf diese Weise ernährte Jugendstadien erreichten mit natürlichen Zecken vergleichbare Körpermassen und Häutungserfolge. Die Gewichte künstlich saugender Weibchen lagen jedoch unter den von ihren auf Rindern gefütterten Kolleginnen erreichten Werten. Die Eiproduktion der Adulten war noch nicht ganz zufriedenstellend und musste verbessert werden. In einer von industrieller Seite mit grossem Interesse aufgenommenen Versuchsreihe mit A. hebraeum - Nymphen hat diese in vitro Methode auch ihre Eignung zur Bewertung systemisch wirkender Akarizide beweisen können.
Heute ist der Arrestment-Verhaltenstest etabliert und zeigte sich in einer Dissertationsarbeit mit dem Repellent DEET erfolgreich. Auch mit dem Bienenparasiten Varroa wurde der Arrestment-Test in zwei anderen Dissertationsarbeiten erfolgreich angewandt. CIBA verwendet den Fütterungstest zu Screeningszwecken. Mehrere Substanzen sind erfolgreich getestet worden. Auch sind heute Ixodes ricinus Adulte aufziehbar. Mit diesem künstlichen Fütterungssystem bei Zecken wird eine Verminderung der Zahl von Versuchstieren erreicht.

References

1. International Journal for Parasitology 25, 8, 887 - 896, 1995
2. ALTEX 13, 2, 76 - 87, 1996

3R-Project 11-88

Tierschutzgerechte Euthanasie von Labortieren

Schatzmann U.
Institut für Nutztiere und Pferde, Universität Bern, Bern

Duration: 3 years **End of the Project:** 1991

Background and Aim

Im Sinne eines "Refinement" sollen die gängigen Tötungsmethoden unserer Labortiere (inkl. Chinchillas und Eintagsküken) nach anerkannten und erprobten Methoden überprüft und wo möglich verbessert werden.
Das Ziel des Projektes ist ein auf breitester Recherche und nach eigenen Versuchen erarbeiteter Katalog der tierschützerisch anerkannten Tötungsmethoden.

Method and Results

Die Studie zur tierschutzgerechten Tötung von Labortieren, Kücken und Chinchilla enthielt neben einer eingehenden Literaturrecherche eigene experimentelle Arbeiten. Das Vorgehen und die Belastung der Tiere bei den praktizierten Tötungsmethoden sowie die Vertretbarkeit der Methoden für die Ausführenden wurden kritisch beleuchtet.
Die wichtigste sich aus der Studie ergebende Schlussfolgerung ist, dass für jede im Labor gebrauchte Tierart eine geeignete Tötungsmethode existiert, die dem Tier Angst und Schmerzen erspart.

Reference

Der vollständige Bericht "Tierschutzgerechte Tötung von Labornagetieren" sowie eine Zusammenfassung in deutsch, französisch oder englisch ist beim Bundesamt für Veterinärwesen erhältlich.

3R-Project 12-88

DNA-Replikationssystem zur tierfreien Wirkungs- und Toxizitäts-Testung

Neftel K.
Ziegler Spital, Bern

Duration: 3 years **End of the Project:** 1991

Background and Aim
Das Projekt strebte den Ersatz und die Verbesserung von Tierversuchen durch kombinierten Einsatz von Zellkulturen mit einem in vitro DNA - Replikationssystem an. Die bisherige mehrjährige Erfahrung zeigt, dass dies für die Untersuchung sowohl von pharmakologischen Wirkungen als auch von toxikologischen Problemen möglich ist. Die Arbeiten sollen in tierfreien Versuchen:
1. eine neu entdeckte Toxizität der Betalactam-Antibiotika abklären;
2. die damit zusammenhängende Schutzwirkung der Cephalosporine gegen die Knollenblätterpilzvergiftung genauer definieren und
3. die Anwendung der eigenen in vitro Systeme auf die präklinische Testung von potentiellen Anti - HIV - Substanzen ausdehnen.
Das Endziel der gesamten Tätigkeit im beschriebenen Bereich ist die Abklärung der Einsatzmöglichkeiten der eigenen kombinierten in vitro Systeme (Zellkulturen und in vitro DNA - Replikation) zur präklinischen, tierfreien Testung verschiedener pharmakologischer Substanzklassen.

Method and Results
In der Validierungsstudie konnte gezeigt werden, dass sich der kombinierte Einsatz von Zellkulturen und von molekularbiologischen Methoden zur präklinischen Prüfung der Wirkung und der Toxizität von neuen Betalaktam-Antibiotika und Anti-HIV-Substanzen eignet. Er dürfte somit zur Reduktion des Tiereinsatzes im Bereich der Entwicklung dieser Substanzen führen.

References
1. Neftel K, Hafkemeyer P, Cottagnoud P, Eich G, Hübscher U: Symposium: 50 years of penicillin application, Springer 1991, Berlin - Heidelberg.
2. Nucleic Acids Research 19, 15: 4059-4065, 1991

3R-Project 13-88

Endothelzellkulturen auf Cytodex-Microcarrier: Adhärenzmessung von neutrophilen Granulozyten in einem physiologischen System

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Sektion Blutprodukte, Bundesamt für Gesundheitswesen, Bern

Duration: 3 years **End of the Project:** 1991

Background and Aim
Endothelzellkulturen bovinen Ursprungs werden auf kollagenbeschichteten Microcarriers (Cytodex 3) gezüchtet. In Glassäulen kalibriert dient das System zur Beurteilung der Adhärenz von neutrophilen Granulozyten (PMN). Die Messung unter Strömungsbedingungen sowie die Möglichkeit der Bestimmung der Vitalität beider Zelltypen, der Sekretion lysosomaler Enzyme und der morphologischen Kontrolle der Zell-Zell Interaktion simulieren die im Ganztier herrschenden Bedingungen gut. Mit diesem System sollen die vielfältige pharmakologische Beeinflussung des Entzündungssystems mit Medikamenten auf ihre Wirksamkeit und allfällige zelltoxischen Effekte in vitro untersucht werden. Dadurch sollten Abklärungen am Ganztier zum einen ersetzt und zum anderen reduziert werden.

Method and Results
In vivo sind akute entzündliche Prozesse durch Anhaftprozesse (Adhäsion) von spezifischen Entzündungszellen an Gefässzellen charakterisiert. Mit Hilfe eines Durchflussystems wurden in vitro die Anhaftung isolierter menschlicher Entzündungszellen (neutrophile Granulocyten PMN) an humane Gefässzellen aus menschlichen Nabelvenen (HUVEC) auf Microcarrierkulturen untersucht. Vorbehandlungen der HUVEC mit einer entzündungsfördernden Substanz führten zu einer dosisabhängigen Zunahme der anhaftenden Entzündungszellen. Diese Zunahme konnte sowohl mit einem steroiden als auch mit einem nichtsteroiden Entzündungshemmer vermieden werden. Ein zusätzlich getestes Homöopathikum (Engystol), zeigte nicht unerwartet keinen signifikanten Einfluss auf die in vitro Adhäsion. Entzündungshemmende Medikamente werden häufig als Therapeutika bei Mensch und Tier eingesetzt. Der entwickelte Assay erlaubt es, Erkenntnisse sowohl im Bereich der Grundlagenforschung als auch der angewandten pharmakologischen und toxikologischen Untersuchungen zu gewinnen und somit die Anzahl der in diesen Versuchen eingesetzten Versuchstiere zu reduzieren.

References
1. ALTEX 18: 37-44,1993
2. Schweiz Arch Tierheilk 132: 438- 439, 1990

3R-Project 15-88

Validation of three in vitro toxicity / teratogenicity test systems using identical coded compounds (together with Swiss NSFP 17)

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Duration: 2 years **End of the Project:** 1991

Background and Aim

The aim of the present project is to reinforce the validation of 3 in vitro toxicity / teratogenicity test systems which were recently developed within the Program 17 of the Swiss National Science Foundatin (SNSF). These systems are: aggregating cell cultures, whole chick embryo in artificial egg and whole rat embryo in vitro.
The validation will be based on the analysis and comparison of results obtained by testing a series of compounds presenting similarities in chemical structure but having variable degree of toxicity / teratogenicity. The compounds represent various classes of chemicals and will be provided in a coded manner by independent experts.

Method and Results

Drei in vitro-Methoden an Hirnzellkulturen und an Hühner- bzw. Ratten-Embryokulturen, die im Rahmen des Nationalen Forschungsprogrammes 17 (Projekt 4017-11078) an zwei Instituten (Institut de Physiologie, Lausanne und Zyma S.A., Nyon) zur Prüfung der Toxizität und Teratogenität von Wirkstoffen entwickelt wurden, sind mit gleichen Prüfsubstanzen in einer breit angelegten Studie in Bezug auf Ihre Eignung zum screeningmässigen Einsatz miteinander verglichen und validiert worden.

References

1. Kucera P., Cano E., Honegger P., Schiller B., Zijstra J.A. and Schmid B. Validation of whole chick embryo cultures, whole rat embryo cultures and aggreating embryonic brain cell cultures using six pairs of coded conpounds. Toxic in Vitro 7: 785 - 798 (1993).
2.Mihalikova, K. Ujhazy, E. Braxatorisova, E. and Kucera, P. Comparative teratological study of stobadin in vivo and in vitro. Toxicology in Vitro 7: 803-807 (1993).
3. The use of whole chick embryo cultures in physiology and developmental toxicology. P. Kucera In: Methods in developmental toxicology and biology, ed. S. Klug and R. Thiel, Blackwell, Berlin, pp. 74-86 (1996).
4. In vitro teratogenicity Schmid B., R. Bechter and P. Kucera. In: In vitro methods in pharmaceutical research, ed. J.V. Castel and M.-J. Gomez-Léchon, Academic Press, San Diego, pp. 353-373 (1996).

3R-Project 16-89

Produktionseinheit zur Produktion von mAK

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Duration: 2 years **End of the Project**: 1992

Background and Aim
Entsprechend der Forderung aus Tierschutzkreisen ist die Produktion von monoklonalen Antikörpern aus Aszites auf das Minimum zu reduzieren.
Es steht ausser Zweifel, dass die in vitro Herstellung von grossen Mengen von monoklonalen Antikörpern ein Mehrfaches an finanziellem, materiellem und apparativem Aufwand bedeutet. Zudem stellt es auch technisch grosse Anforderungen, verglichen mit der konventionellen Methode.

Method and Results
Verschiedene Systeme wurden auf ihre praktische Eignung zur Herstellung von monoklonalen Antikörpern ausgetestet. Die Erfahrung zeigte, dass das getestete automatisierte Gerät (Cell-Pharm II) schwierig zu handhaben ist und technische Mängel aufweist. Dieser Gerätetyp kann deshalb für Forschungslabors nicht empfohlen werden.

3R-Project 17-90

Einführung des Cell-Pharm für die mAK-Produktion

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Institut für Pharmakologie und Biochemie, Universität Zürich, Zürich

Duration: 2 years **End of the Project:** 1991

Background and Aim
Mit der Arbeit soll sofort die versuchstierfreie Produktion von monoklonalen Antikörpern aufgenommen werden. Die praktische Erfahrung zeigt, dass auch neue gebrauchsfertige Systeme eine gewisse Zeit brauchen, bis sie allgemein anwendbar sein werden. Die Einführung des "Cell Pharm"-Zellsystems sollte es ermöglichen, auch anderen Forschungsgruppen die Piloterfahrungen mitzuteilen, damit bei einem allfälligen gesetzlichen Auftrag ohne Forschungsverzögerung vom Tierversuch sofort weggegangen werden könnte.

Method and Results
Dieses Projekt ergab Resultate, die mit denjenigen aus den Projekten 18-90 und 21-91 vergleichbar sind. Als Bioreaktor wurde das Gerät Opticell 6200 getestet und erfolgreich in Betrieb genommen. Dieser Gerätetyp eignet sich lediglich für die Produktion von Antikörpermengen, die grösser sind als 1 Gramm. Sein computergesteuertes Überwachungsystem arbeitet einwandfrei. Die Arbeitsaufwendungen sind nach einer intensiven Startphase klein. Kontaminationen kamen vereinzelt vor, weshalb nur gut qualifiziertes Personal, welches sehr sorgfältig arbeitet, eingesetzt werden sollte.
Das System produziert monoklonale Antikörper, die nicht nur immunologisch, sondern auch funktionell aktiv sind. Eine erste vorläufige Berechnung der Kosten (ohne Löhne) ergab allerdings, dass bei der Produktion von 100 - 500 mg Antikörper die Kosten mindestens drei- bis viermal so hoch sind, wie wenn sie im Labor des Autors in Rollerflaschen, oder acht- bis zehnmal so hoch, wie wenn sie in Aszites-Mäusen hergestellt würden.

3R-Project 18-90

In vitro Herstellung von mAK mit Bioreaktoren I

Fischer R.
Institut für Biochemie, ETH Zürich, Zürich

Duration: 2 years **End of the Project:** 1991

Background and Aim
Als Alternativen zur in vivo Produktion monoklonaler Antikörper (mAK) werden von verschiedenen Herstellern “Hollow Fiber Cell Culture Systems” angeboten. Das vorliegende Projekt untersucht die Möglichkeiten dieser Technik.
Im wesentlichen sollen die folgenden Fragen beantwortet werden:
1. Kann diese Technik Tierversuche ersetzten?
2. Wie gross ist der zeitliche und finanzielle Aufwand zur mAK-Produktion?

Method and Results
Es wurden sieben kommerziell erhältliche Adjuvantien (Poly-A-poly-u, GERBU, RIBI, Pam3, Specol, Freund und Titermax) auf ihre immunstimulierende Wirkung sowie auf die Belastung der Tiere hin untersucht. Es hat sich gezeigt, dass in der Belastung der Tiere grosse Unterschiede zu finden sind: Poly-A-poly-U und das Freund'sche Adjuvans (subcutan verabreicht) verursachen keine bzw. eine geringe Belastung. In der Handhabung hingegen schneidet das Freund'sche Adjuvans schlecht ab (Zweispritzenverfahren zur Erstellung der stabilen Emulsion, grosse Verluste). Aufgrund der vorläufigen Daten empfehlen wir das Poly-A-poly-U zur Herstellung monoklonaler Antikörper in der Maus. Poly-A-poly-U zeigt, zusammen mit dem GERBU eine gute Immunstimulation. GERBU könnte nach erfolgter Optimierung, mit dem Ziel die Belastung zu senken, auch empfohlen werden.
Mit potenten Adjuvantien kann der Tierverbrauch bei Immunisierung verringert werden (reduce). Wenig oder nicht belastende Adjuvantien verringern den Schmerz der Tiere und verbessern dadurch den Tierversuch (refine).

Reference
3R-Info-Bulletin No 2, Sept 1994

3R-Project 19-90

Remplacement des animaux dans les études sur Toxoplasma gondii II

Pechère J.-C.
Microbiologie CMU, Centre Médical Universitaire, Genève

Duration: 2 years **End of the Project:** 1991

Background and Aim
La toxoplasmose est une maladie universelle dont l'importance a pris une dimension dramatique avec l'explosion de l'épidemie de SIDA. Toxoplasma gondii ne se cultive pas sur les milieux acellulaires usuels, et son étude requiert traditionellement un grand nombre d'animaux (souris). Grâce au soutien de la Fondation Recherches 3R nous avons pu développer un système in vitro (culture de cellules) au cours des 2 dernières années qui nous a permis d'étudier l'effet de plusieurs médicaments.
Nous voudrions, dans les 2 prochaines années étendre ces premières investigations vers des études immunologiques, qui devraient permettre de mieux cerner les raisons de la survie du parasite dans nos cellules et plus particulièrement les implications d'une infection toxoplasmique dans le réseau des cytokines.

Method and Results
Im Rahmen eines ersten 3R-Projektes (8-88) ist es gelungen, eine Zellkulturmethode für Untersuchungen im Zusammenhang mit Toxoplasma gondii, einem Parasiten, der vor allem bei Immunschwäche Infektionen verursacht, zu entwickeln.
Die Untersuchung der Wirkungen von Medikamenten wurde auf immunologische Studien ausgedehnt. Um gewisse physiopathologische Aspekte bei der Infektion durch Salmonella typhi murium zu klären, wurde eine Zellkulturmethode mit Makrophagen der Maus (P388D1) eingesetzt. Sowohl bei der Infektion durch Salmonellen als auch bei der Infektion durch Toxoplasma gondii konnten Wirkungsmechanismen des Parasitismus aufgedeckt werden.

3R-Project 20-90

Verwendung eines menschlichen Darmzellkulturmodelles zur Reduktion und zum Ersatz von Tierversuchen auf dem Gebiet der Verdauungsphysiologie

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Duration: 3 years **End of the Project:** 1993

Background and Aim
Die im Jahr 1990 erst seit wenigen Jahren bekannte menschliche Darmzellinie Caco-2 wurde auf ihre Verwendbarkeit für das Studium von Verdauungsfunktionen des menschlichen Dünndarms getestet. Anhand dieses Zellkulturmodelles wurde die Regulation menschlicher Verdauungsenzyme untersucht
Bisher ist noch unbekannt, wie Verdauungsenzyme im menschlichen Dünndarm reguliert werden. Verdauungsphysiologische Vorgänge wurden bisher fast ausschliesslich an Tiermodellen (Nager und Kaninchen) studiert, doch sind die gewonnenen Erkenntnisse nur bedingt auf den Menschen übertragbar.
Es ist zu erwarten, dass durch diese Studie die Attraktivität der Caco-2 Zellinie für darmphysiologische Studien erhöht und der Verbrauch von Tieren dadurch reduziert wird. Ein Teil der Methodik bestand zur Zeit des Projektbeginns bereits.

Method and Results
Es wurde ein menschliches Darmzellkulturmodell (Caco-2) verwendet, um erstmals die hormonelle Regulation von drei Verdauungsenzymen (Laktase, Saccharase und Dipeptidylpeptidase) zu studieren.
Unsere Resultate zeigen eine gegensätzliche Regulation von Laktase und Saccharase. Laktase wird durch Hormone des zyklischen AMP-Weges und durch Corticosteroide stimuliert, währenddem Saccharase inhibiert wird. Dipeptidylpeptidase IV lässt sich mit Tyroxin stimulieren. Vergleiche mit bekannten Hormon- und Bürstensaumspiegeln im Menschen lassen vermuten, dass die an Caco-2 Zellen gewonnenen Erkenntnisse mindestens teilweise für die menschliche Darmphysiologie relevant sind. Weitere direkte Vergleiche sind nötig, um diese Vermutung zu erhärten.
Die Regulation der Laktase ist von besonderem Interesse, da ca. 10% der Schweizer und über 50% der Weltbevölkerung insgesamt im Erwachsenenalter zu wenig Laktase produzieren. Milchgenuss führt bei diesen Erwachsenen zu Durchfall, womit sie in ihrer Ernährung nicht von den wertvollen Milchbestandteilen profitieren können.

Reference
Hauri H.P., Sander B. and Naim H. (1994) Induction of lactase biosynthesis in the human intestinal epithelial cell line Caco-2. Eur. J. Biochem. 219, 539-546.

3R-Project 21-91

In vitro Herstellung von mAK mit Bioreaktoren II

Fischer R.
Labor für Biochemie I, ETH Zürich, Zürich

Duration: 2 years **End of the Project:** 1992

Background and Aim
Die im 3R-Projekt 18-90 (Methoden zur in vitro Produktion monoklonaler Antikörper als Alternative zur in vivo Produktion) aufgeführten Ziele sollen weiterverfolgt werden.
Zur Ergänzung der bereits beschriebenen Resultate für die Produktion grosser AK-Mengen werden für die Produktion mittlerer Mengen folgende Produktionsverfahren evaluiert: Zellkulturen in Cellspin- und Rührflaschen sowie ein einfaches automatisches System. Die gesammelten Resultate werden in einer Wegleitung für die AK-Herstellung zusammengefasst und so den Interessenten zugänglich gemacht.
Im weiteren werden die Verfahren zur Immunisierung der Tiere untersucht und optimiert. Dies mit dem Ziel, den Tierverbrauch zu senken.

Method and Results
Die sieben kommerziell erhältlichen Adjuvantien (Poly-A-poly-u, GERBU, RIBI, Pam3, Specol, Freund und Titermax) wurden auf ihre immunstimulierende Wirkung sowie auf die Belastung der Tiere hin untersucht. Es hat sich gezeigt, dass in der Belastung der Tiere grosse Unterschiede zu finden sind: Poly-A-poly-U und das Freund'sche Adjuvans (subcutan verabreicht) verursachen keine bzw. eine geringe Belastung. In der Handhabung hingegen schneidet das Freund'sche Adjuvans schlecht ab (Zweispritzenverfahren zur Erstellung der stabilen Emulsion, grosse Verluste).
Aufgrund der vorläufigen Daten empfehlen wir das Poly-A-poly-U zur Herstellung monoklonaler Antikörper in der Maus. Poly-A-poly-U zeigt, zusammen mit dem GERBU, eine gute Immunstimulation. GERBU könnte nach erfolgter Optimierung, mit dem Ziel, die Belastung zu senken, auch empfohlen werden. Mit potenten Adjuvantien kann der Tierverbrauch bei Immunisierung verringert werden (reduce). Wenig oder nicht belastende Adjuvantien verringern den Schmerz der Tiere und verbessern dadurch den Tierversuch (refine).

Reference
3R-Info-Bulletin No 2, Sept. 1994

3R-Project 22-91

Screening Methode zur Abklärung des Abbaus von Cholesterin in vitro

Niésor E.
Symphar SA, Versoix

Duration: 1 year **End of the Project:** 1992

Background and Aim
Mesure in vitro de l'activité d'une série de dérivés en utilisant une ligne de cellules intestinales d'origine humaine. La méthode sera validée par l'analyse de ces résultats et ceux obtenues avec des médicaments de référence qui ont déjà été administrés aux animaux de laboratoires et aux patients hypercholestérolémiques.
Ces études devraient conduire à une diminution significative du nombre de petits rongeurs (souris, rats, hamsters) utilisés à ce jour pour le screening de médicaments hypocholestérolémiants.

Method and Results
Die Studie umfasste die Validierung einer Screening-Methode zur Abklärung der hypocholesterolämischen Aktivität (Abbau von Cholesterin) von Substanzen in vitro. Unter Verwendung einer Darmzellinie menschlichen Ursprungs erfolgte die Validation anhand von Substanzen, deren hypocholesterolämische Aktivität am lebenden Organismus bekannt ist. Bestimmt wurde die pharmakologische Aktivität des Produktes Symphar (SR) auf die Synthese von Cholesterin durch menschliche Darmzellen der Linie Caco2. Die erarbeitete in vitro-Methode erlaubte eine spürbare Senkung der Zahl der in diesem Forschungsgebiet heute eingesetzten Tiere (um ca. 60%). Dennoch kann heute noch nicht ganz auf Tierversuche verzichtet werden.

3R-Project 23-91

Anästhesiemöglichkeiten bei der Ratte

Becker M.
Kantonales Veterinäramt Basel Stadt, Abteilung Tierschutz, Basel

Duration: 3 years **End of the Project:** 1994

Background and Aim
Über 90% aller Versuchstiere im Kanton Basel-Stadt sind Mäuse und Ratten (1991). Wegen ihrer kleinen Körpergrösse eignet sich die Maus jedoch nicht zum instrumentierten Versuchstier; hier stellt die Ratte die häufigste Versuchstierspecies dar (1991 ca. 180’000 Tiere in Basel-Stadt).
Deshalb ist auch bei dieser Tierart das Anästhesie- (und m.E. auch das Analgesie-)problem das vordringlichste. Die Zahl der Anästhesiemittel und -formen ist zwar gross, jedoch sind kaum Übersichtsarbeiten mit harten Daten verfügbar, vor allem aber fehlt es an systematischen Untersuchungen über Einsatz- und Anwendungsmöglichkeiten der heute am meisten verwendeten Substanzen bzw. Anästhesieformen.
Als Gegenstand dieses Projektes werden die breit gestreute Literatur zusammengetragen und gesichtet sowie daraus Standardverfahren abgeleitet.

Method and Results
Bei diesem Projekt handelte es sich um eine Auswertung von anästhesierelevanten Daten in der Literatur. In mehr als 6780 Literaturstellen waren nur in 466 für die Form der Inhalation- bzw. 599 Fällen für die Form der Injektionsanästhesie entsprechende Angaben zu finden. Zur Auswertung gelangten bei den Inhalationsanästhetika folgende Stoffe: Lachgas, Chloroform, Diethylaether, Methoxyfluran, Enfluran, Isofluran, Halothan und Kohlendioxid. Zu diesen Stoffen können nun differenziertere Angaben bezüglich ihrer Anwendung, Konzentration (Dosis), Hauptwirkung, Wirkdauer, aber auch ihre Nebenwirkungen und deren Auswirkungen gemacht werden. Für einzelne Stoffe oder Stoffgruppen konnten zusätzliche Informationen über den Einfluss einer allfälligen Prämedikation, Kombination mit anderen Stoffen zur Verbesserung oder Sicherstellung der Analgesie oder, soweit vorhanden, die Anwendung von Antidot zusammengetragen werden. Für die Anwendung volatiler Anästhetika wurden zahlreiche Hinweise für eine schonende und sichere Zuführung der Anästhesiegase (Maske, Inkubation) zusammengetragen.

3R-Project 24-91

Nachweismethoden für enteropathogene E. coli (EPEC) beim Kalb

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Duration: 2 years **End of the Project:** 1993

Background and Aim
Enteropathogene E. coli (EPEC) werden vor allem charakterisiert: i) durch ihre - Plasmid - kodierten - adhäsiven Eigenschaften und zum Teil auch ii) durch ihre - in lysogenen Phagen kodierte - Fähigkeit, sog. Verotoxine zu produzieren. Typische EPEC verursachen sowohl in vivo (sog. attaching and effacing" Läsionen an Darmepithelzellen) als auch in vitro (sog. lokale bzw. diffuse Adhäsion an HeLa Zellen) charakteristische Adhäsionsbilder. Allerdings ist z.Z. die Uebereinstimmung von in vitro- und in vivo-Adhäsionen noch ungenügend belegt.
Bei diesem Projekt geht es darum:
- In vivo und in vitro Untersuchungsmethoden zum Nachweis adhäsiver Eigenschaften von EPEC zu vergleichen mit dem Ziel, den Tierversuch womöglich ganz durch Zellkultursysteme zu ersetzen.
- Gensonden zum diagnostischen Nachweis der Virulenzfaktoren von EPEC sowohl in Kulturen als auch in situ einzusetzen.

Method and Results
Die Charakterisierung von enteropathogenen Eigenschaften (Hervorrufen von schwerem Durchfall beim Kalb) von Escherichia coli-Bakterien erfolgt über deren Eigenschaft, sich an Darmepithelzellen zu binden und gewisse Toxine zu bilden. Herkömmlicherweise werden diese Eigenschaften in einem belastenden Tierversuch abgeklärt. Ziel des Projektes war es, zu belegen, dass diese Eigenschaften auch an HeLa-Zellen ermittelt werden können, so dass weitgehend auf den Tierversuch verzichtet werden kann.
EPEC spielen bei vielen Tieren und Menschen eine wichtige Rolle in der Pathogenese von neonatalem Durchfall. In einem ersten Teil der Arbeit wurde die spezifische Haftung der EPEC in vivo beim Kalb und in vitro an HeLa-Zellkulturen mittels Licht- (LM) und Elektronenmikroskopie (EM) verglichen. Der Nachweis von EPEC im EM in vivo und in vitro erwies sich im Gegensatz zum LM als sehr spezifisch, aber auch als aufwendig. In einem zweiten Teil der Arbeit wurde daher untersucht, ob sich die neue Methode der DNA-Hybridisierung von entsprechenden EPEC-Genomen mit spezifischen EPEC-Gensonden in situ an Paraffin-eingebetteten Darmschnitten für den Nachweis von EPEC eignet. Parallel dazu wurde der Nachweis von EPEC mittels der Kolonie-Hybridisierung aus Kotproben etabliert und mit der in situ Technik verglichen.

Reference
J Vet Med B 39: 575-584, 1992

3R-Project 25-92

Organotypic cultures: A model for pharmacological analysis in the central nervous system

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Duration: 3 years **End of the Project:** 1995

Background and Aim
The major aim of the project was to determine whether organotypic slice cultures could be used as a model for pharmacological studies and analyses in the central nervous system. Two possible applications were proposed to be tested: one concerned mechanisms of neurite sprouting and synapse regeneration, one of the most important property used by the brain for functional recovery in case of neuronal death; the second application concerned mechanisms of excitotoxicity, a phenomenon which is implicated in many neuropathological conditions.
These characteristics were planned to be studied using immunohistochemical and electrophysiological techniques and via the development of an electrode array for chronic recordings of electrical activity.

Method and Results
The experiments that have been carried out have lead to the main conclusion that organotypic slice cultures represent a valid and interesting model for pharmacological studies in the central nervous system. In particular, we have provided evidence that this in vitro preparation can be successfully used for studies of the mechanisms of sprouting and synaptogenesis.
We have shown that producing a lesion in these cultures even several weeks after explantations is followed by a recovery period of about 1-2 weeks during which a scar is formed by glial cells, a sprouting reaction takes place which can be visualised using immunohistochemical techniques, new fibres grow and cross the lesioned area and new synapses are formed on the opposite side of the lesion (Stoppini et al., 1993). This process of lesion-induced recovery, as it is under in vivo conditions, becomes much less effective in older cultures. In recent experiments, we found that this is at least partly due to extrinsic factors which inhibit sprouting and regeneration, since recovery was markedly improved when older cultures were allowed to regenerate into younger tissue (manuscript in preparation; Stoppini et al., 1996).
Impact on testing procedures: This study and the validation of the model of interface-type of organotypic cultures has already had some impact on testing procedures since numerous research laboratories as well as a Japanese company (Shionogi LTD) has adapted this system for the analysis of pharmacological agents and the testing of compounds in the domain of excitatory receptors and ischemia.

References
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3R-Project 26-92

Produktion von monoklonalen Antikörpern mittels Repertoire Cloning

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Duration: 3 years **End of the Project:** 1995

Background and Aim

Die Methodik der Repertoir Klonierung erlaubt es heute, praktisch jede denkbare Antikörperspezifität in vitro herzustellen. Auf der Oberfläche von filamentösen Phagen werden die variablen Immunglobulin-Ketten zur Expression gebracht, wodurch “Phaben” entstehen, d.h. Phagen, die Fab Antikörper Moleküle tragen. Auf diese Art können Antikörper-Genbanken hergetellt werden, die 10^7 bis 10^8 verschiedene Spezifitäten enthalten.
Über das Antigen können die spezifische Phagen isoliert werden, um so oligoklonale oder monoklonale Antikörper zu erhalten. Es ist denkbar, dass diese Methodik die traditionelle in vivo Antikörperproduktion oder die Hybridomatechnik ablösen wird, um Antikörper für die Diagnostik oder die Therapie herzustellen.

Method and Results

In diesem Projekt konnte der Nachweis erbracht werden, dass es möglich ist, direkt aus dem menschlichen Repertoire Immunglobuline zu klonieren und diese auf der Oberfläche von Phagen zu exprimieren. Aus der Vielzahl der entstandenen menschlichen Antikörper war es möglich, Autoantikörper und Antikörper gegen bekannte Immunogene zu isolieren. Die Technologie wurde für das Labor des Projektleiters einerseits zur Routine, andererseits zu einer wichtigen Grundlage auf der jetzt verschiedene andere Projekte basieren.

References

1. Vogel M., Miescher S., Biaggi C., Stadler B. M. (1994) Human anti-IgE antibodies by repertoire cloning. Eur. J. Immunol. 24, 1200-1207.
2. Lang A. B., Vogel M., Viret J.F. and Stadler B.M. (1995) Polyclonal preparations of anti-tetanus toxoid antibodies derived from a combinatorial library confer protection, Biotechnology 13, 683-685, 1995.
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3R-Project 27-92

Entwicklung und Evaluation von Methoden zum Erfassen pyrogener Substanzen

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Duration: 3 years **End of the Project:** 1996

Background and Aim
Pyrogene sind Substanzen aus Bakterien, die in geringsten Mengen in Mensch und Tier Fieberreaktionen bis hin zu lebensbedrohenden Schockzuständen auslösen können. Solche Substanzen sind weitverbreitet, und es bedarf eines grossen Aufwandes, um bei der Herstellung von Medikamenten eine Kontamination mit Pyrogenen zu vermeiden. Deshalb gehört zu jeder Qualitätskontrolle von Medikamenten oder Infusionslösungen der Nachweis, dass diese pyrogenfrei sind. Der klassische Pyrogen-Test ist ein Tierversuch und beruht auf der Messung der Temperatur nach Verabreichung der zu testenden Substanz.
Eine wichtige Gruppe von Pyrogenen, die Endotoxine, können in vitro im sog. Limulus- Amoebozyten- Lysat- Test (LAL-Test) gemessen werden. Dieser Test, obwohl für gewisse Substanzklassen als Pyrogentest heute in vielen Ländern akzeptiert, ist störungsanfällig und widerspiegelt nur ungenügend das Vorliegen von Substanzen, die auf Mensch und Tier pyrogen wirken. Im vorliegenden Projekt werden menschliche Zell-Linien selektioniert oder durch Transfektion erzeugt, die ein in vitro-Korrelat der Pyrogen- Reaktion bei Mensch und Tier darstellen.

Method and Results
Es konnte gezeigt werden, dass sich gewisse menschliche monozytoide Zell-Linien als hochsensitive Indikatoren von Endotoxin (in der Praxis die wichtigsten Pyrogene) eignen. Ausgehend von zwei verfügbaren Zell-Linien konnten Sublinien kloniert werden, die (1) empfindlicher sind als die Ausgangszell-Linie, (2) über viele (>50) Passagen hinweg stabil sind. Das Kultursystem für diese Klone wurde optimiert, und als Pyrogen- Indikator wurde aus mehreren möglichen Parametern die Sekretion von Tumor-Nekrose-Faktor ausgewählt, da sich dies als empfindlichste Messgrösse erwies, mit der Pyrogen- Exposition nachzuweisen war. Ausserdem ist die Menge von hoher Relevanz für eine Pyrogen-Reaktion in vivo.
Die drei Klone wurden einerseits auf Ansprechen auf eine breite Palette von gereinigten Pyrogenen geprüft. Anderseits wurden Pyrogen- kontaminierte und nicht kontaminierte Proben aus der industriellen Produktion gemessen. Die erhaltenen Ergebnisse wurden mit verschiedenen Varianten des Limulus-Tests sowie mit dem Kaninchen-Pyrogen-Test verglichen. Es zeigte sich, dass sich der Zellkulturtest von der Sensitivität wie von der Spezifität her als Pyrogen-Test sehr gut eignet, mit der einzigen Ausnahme der Pyrogene von gram-positiven Erregern.

References
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(3) Jungi TW, Brcic M, Eperon S. Human macrophages respond to LPS in a serum-independent, CD14- dependent manner. Immunol Lett. 1996;54:37-43.
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3R-Project 28-92

Analysis of the applicability of in vitro immunological methodologies to the study of foot-and-mouth disease vaccine efficacy

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Duration: 4 years **End of the Project:** 1996

Background and Aim
The humoral response in sera against viruses is a well studied aspect of the immune response. A good example of these studies is that on foot and mouth disease virus (FMDV). Traditionally, the detection of specific antibody via titration and subsequent dilution of the serum antibody to eliminate background problems does not take into account the in vivo environment of the serum antibodies. It is therefore unclear how the characteristics of such reactions in a diluent buffer can be related to those in natural body fluid extracts such as serum. The reactivity of serum antibody, as an entity, is also influenced by the time of contact with the antigen; in most immunoassays, an incubation time of one hour is chosen. Using vaccinated (FMDV serotype O1) and non-vaccinated cattle, we have demonstrated that the majority of the specific reaction between antibody and antigen in sera occurs within 10 to 60 seconds. In contrast, aspecific and non-specific reactions are still at low levels within such a short time span. When dilutions of sera were employed and compared with the original reactivity of the sera, it was observed that the use of diluted sera provided an assessment of antibody activity resulting from the dilutions, and not as pertaining directly to the reactivity in the serum itself. Results from serum which can be related to its capacity to function under in vivo conditions, can only be obtained when the in vitro analytical methodology creates conditions close to those found in the serum itself.

Method and Results
The objective was to identify in vitro means for measuring immunological potential in animals vaccinated against FMDV. Serum antibody avidity did not always relate to protection, whereas 10 and 60 second incubation times for serum with antigen identified protected animals. This test presumably permitted an analysis of combined reactive capacities for specific and aspecific (innate) serum components, but the reactivities were sensitive to freezing and thawing. It would be necessary to continue these analyses, to determine if serum samples should not be frozen/thawed, or if dilution with fresh non-immune serum would circumvent the problem.
The characteristics of an in vitro immunization system, as a means to replace animal immunization, was also investigated. Therein, the problem of culture-dependent programmed cell death (PCD) was identified, a solution for which was the provision of accessory cells and plasma - derived factors. These deliver the required signals to prevent PCD. Future work should concentrate in this area, in order to build an in vitro immunization system, wherein the viability and responsive capacity of the cells is more akin and relevant to that found in vivo.

References
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3R-Project 29-92

Corticotrophin (ACTH) -Bestimmung in Arzneimitteln

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Duration: 2 years **End of the Project:** 1994

Background and Aim
Die derzeit in den Arzneibüchern (Ph.Eur., Ph. Helv., BP, DAB10 etc.) vorgeschriebenen Wertbestimmungen an Ratten des Arzneistoffes Corticotrophin soll ersetzt werden durch eine validierte HPLC-Methode, die es erlaubt, biologisch aktives Corticotrophin als Reinstoff und in Fertigarzneimitteln qualitativ zu bestimmen, und den Tierversuch zu ersetzen.

Method and Results
Ergebnis ausstehend

3R-Project 30-92

Toxikologische Untersuchung der Blutbildung in vitro

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Duration: 2 years **End of the Project:** 1994

Background and Aim
Ziel dieses Projektes war die Etablierung von in vitro Methoden zur Abklärung von Xenobiotika-Effekten auf das blutbildende Knochenmark. Für diese Untersuchungen werden einerseits etablierte Zell-Linien und anderseits Primärkulturen von Maus und Mensch eingesetzt. Für die Bestimmung allgemein-toxischer Endpunkte, wie z.B. Zytotoxizität, Protein und DNS Synthesen sind Zell-Linien geeignete Modelle.
Die komplexen Ereignisse der Blutbildung (Hämatopoiese), wie Proliferation von verschiedenen Vorläuferzellen, Differenzierung und Reifung wird an Primärkulturen von Maus- und Humanknochenmark mittels der sogenannten Kolonien-Assays durchgeführt. Substanzeffekte auf frühe und späte erythroide Granulozyten- Monozyten und gemische Vorläufer werden erfasst.
Diese Untersuchungen sind für in vitro Screening Tests von Substanzen mit vermuteter Toxizität auf das Knochenmark geeignet.

Method and Results
Es wurden im wesentlichen folgende Teilprojekte bearbeitet:
1. Etablierung der in vitro Kultur von Knochenmarkzellen: eine Flüssigkultur über 4 Tage gestattet Substanzeffekte auf sehr frühe Vorläuferzellen der Blutbildung.
2. Untersuchungen an Knochenmarkszell-Linien: Dabei lag der Schwerpunkt auf dem Effekt von Immunsuppressivsubstanzen, insbesondere auch Zytokin-Inhibitoren wie Rapamycin, Worthmanine, daneben auch Chemotherapeutika.
3. Nachweis von Wachstumsfaktoren: Neben dem Proteinnachweis wurde auch eine sehr sensitive Methode des mRNS Nachweises eingesetzt: die PCR mittels eines im Hause modifizierten Vektors P-mus, das den Nachweis fast aller Zytokine gestattet.
4. Flow- zytometrische Untersuchung des Knochenmarks: Mit einem Flowzytometer (FACScan) und mit einem Laserscanningmikroskop konnten mit Verwendung spezieller Antikörper die Zugehörigkeit von einzelnen Zelltypen im Knochenmark charakterisiert werden.
5. Rezeptoruntersuchungen an Knochenmarks-Zell-Linien, besonders an IL-3 und GM-CSF. Diese Untersuchungen am Mäuseknochenmark sind ein guter Ausgangspunkt für ein breites Screening von Effekten zur Unterdrückung der Blutbildung.

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3R-Project 32-92

A new model for in vitro corneal epithelial wound healing study

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Duration: 1 year **End of the Project:** 1994

Background and Aim
Currently, the ocular surface toxicity (of eye drops and other substances) is tested by creating a superficial corneal wound in rabbits and observing the healing process. With a new model, a large number of these experiments can be conducted under in vitro conditions. Thus, the number of rabbits used in ocular pharmaceutical research can be significantly reduced.
The purpose of this study is to set up an in vitro epithelial wound healing model without photography and defect area measurement.

Method and Results
A new model was developed for epithelial wound healing study in pig eyes, in which the excimer laser was used for ablation to create an epithelial wound with precise depth and size. A medium perfusion system in intact bulbus helps to maintain the eye ball. 28 pig eyes received photokeratectomy (PKT) with the different diameters of 2.0mm, 1.8mm and 1.5mm, and 70microm in depth. After PKT, the eye balls were placed in the incubator and perfused with TC-199 media by vitreous puncture.
(i) 8 eyes in each group were rinsed with TC-199. The average healing time for 2.0mm group was 31.8 hours; the 1.8mm group was 28.8. hours, and for the 1.5mm group was 25.5 hours.
(ii) - 4 pig eyeballs with PKT of 1.5mm and receiving rinsing with Benzalkonium chloride on epithelial wounds did not heal after 36 hours' observation.
(iii) - 12 eyeballs without PKT were perfused under the same criteria to evaluate the change of the intraocular pressure (IOP). After 24 hours, the IOP was on the average of 19.0mm Hg.
This study revealed the healing time is proportional to the wound diameter and the healing was retarded by the drug effect. With this model, the drug influences on corneal epithelial wound healing can be evaluated and the result is revealed around 24 hours.

3R-Project 33-92

Nachweis karzinogenese-relevanter Punktmutationen

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Duration: 2 years **End of the Project:** 1994

Background and Aim

Es sollen zwei neuartige Methoden entwickelt werden, welche das Potential haben, punkt-mutierte DNA in einem hohen Background unveränderter Moleküle zu erfassen. Die Methoden sind so beschaffen, dass sie für den Nachweis beliebiger Mutationen anwendbar sind. Das Endziel besteht darin, mindestens eine Methode so zu verfeinern, dass sie für ein schnelles und zuverlässiges Screening von Hot-spots in carcinogenese-relevanten Genen benutzt werden kann, d.h. 1 - 10 punktmutierte DNA-Moleküle in 10^6 Wildtypmolekülen erkennt und amplifiziert.
Die Methoden sollen erlauben, die Wirkung genotoxischer Substanzen auf Zellen in einem sehr frühen Stadium zu charakterisieren und somit Tierversuche massiv zu verkürzen oder sogar duch Zellkulturen zu ersetzen. Als Modell dient murine Leber-Tumor-DNA mit Punktmutationen im 61, Codon des Ha-ras-Gens.

Method and Results

Die thermostabile DNA-Polymerase von Thermus litoralis besitzt eine Exonucleasefunktion, durch welche bei der Strangverlängerung fehleingesetzte Nucleotide wieder entfernt werden. Dies wurde ausgenutzt, um eine neuartige Mutations-Detektionsmethode zu entwickeln. Durch Verwendung markierter Primer-Oligonucleotide können PCR-Produkte produziert werden, die nur dann markiert sind, wenn mutierte DNA vorliegt.
Dieses Verfahren wurde getestet mit DNA, bei der eine Mutation in Codon 61 des Ha-ras-Gens vorliegt. Nach PCR-Amplifikation konnten so 10 bis 100 mutierte Gen-Kopien pro Ansatz nachgewiesen werden.

Reference

Nucleic Acids Research, 23, 2, 311-312, 1995

3R-Project 34-92

Validierungsstudie zur tierfreien in vitro Produktion von monoklonalen Antikörpern zusammen mit 31 Teilprojektteilnehmern

Stiftung Forschung 3R
Stiftung Forschung 3R, Münsingen

Duration: 3 years **End of the Project:** 1996

Background and Aim
Monoklonale Antikörper (mAK) sind heute wichtige Instrumente in Forschung, Diagnostik und Therapie. Im kleineren Mengenbereich (weniger als 1 Gramm) wurden mAK bis zu Beginn dieser Studie meistens noch im Aszitesverfahren, einem hochbelastenden Tierversuch, hergestellt. Bei der Produktion von grossen Mengen (einige Gramm bis Kilogramm) wurden diese Antikörper bereits technisch in spezialisierten Labors mit viel Know How unter Einsatz moderner Fermentertechnologie (Massenzellkulturverfahren) hergestellt. In kleineren Forschungslaboratorien hingegen, wo verschiedene mAK gleichzeitig, in ausreichender Menge und ohne grosse Investitionen produziert werden mussten, waren Forschende an Hochschulen und in der Industrie auf den Tierversuch angewiesen.
Die Studie hatte zum Ziel, durch den Einsatz von zwei verschiedenen Typen von Minibioreaktoren in 20 bis 30 verschiedenen Forschungslabors die praktische Anwendung dieser in vitro mAK-Produktion als Ersatz für den Tierversuch zu unterstützen. Mit diesem aktiven Vorgehen sollte der Versuchung entgegengewirkt werden, die mAK-Produktion mittels Ascites-Maus ins Ausland zu verlagern. Die Studie sollte Klarheit über die tatsächlichen Aufwendungen in finanzieller, personeller und infrastruktureller Art geben.

Method and Results
Als Result dieser Studie liegen im wesentlichen folgende Ergebnisse und Erfahrungen vor:

- Beide evaluierten Hohlfaser-Bioreaktoren sind für die in vitro mAK-Herstellung geeignet.
- Die Technologie der Hohlfaser-Bioreaktoren liefert brauchbare AK in adäquaten Mengen.
- Obwohl beide Geräte zu Beginn der Studie noch nicht bis zur Marktreife entwickelt waren, konnten die Geräte dank der Zusammenarbeit zwischen Geräteproduzenten und Forschenden zum grössten Teil erfolgreich betrieben werden.
- Die Studie bewirkte in der in vitro mAK Produktion einen Innovationsschub bei den Geräteherstellern: heute sind wesentlich einfachere und günstigere Geräte auf dem Markt.
- Forschende konnten ohne grössere finanzielle Risiken ein Gerät in Betrieb nehmen und erhielten von den Geräteproduzenten, von der Stiftung und von anderen Studienteilnehmern fachlichen Support.
- Diese Studie war eher eine Machbarkeitsstudie als Validierungsstudie.
- Die tierversuchsfreie mAK-Produktion ist bei den meisten Studienteilnehmern willkommen. Weitergehende Massnahmen im Sinne der 3R (z.B. Einführung eines Gütezeichens für die tierfreie Produktion von mAK) werden begrüsst.

Reference
3R-Info-Bulletin No 2, mabs without mice? Stiftung Forschung 3R, Sept. 1994

3R-Project 35-92

Nouvelle méthode de quantification de toxicité in vitro basée sur la modification de stress cellulaire

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Duration: 2 years **End of the Project:** 1994

Background and Aim
Ce nouveau système de test permet de réduire et de remplacer l'expérimentation animale en toxicologie et en tératogénicité. Il se base sur l'exploitation de la réaction du stress cellulaire et couvre un champ d'application plus général que la plupart des autres systèmes de culture qui sont plus limités, car plus spécifiques. Le stress engendré sur les animaux, les embryons et les cultures cellulaires par un produit toxique conduit à l'expression d'un ensemble de gènes spécifiques. Ces protéines du stress restent intracellulaires et sont difficilement quantifiables.
Par le génie génétique, on remplace les gènes du choc thermique par d'autres produisant des enzymes et/ou autres protéines (comme, par example, l'hormone de croissance, le lysozyme ou le luciférase) qui sont sécrétées et sont plus facilement quantifiables. L'utilisation des promoteurs hsp23 ou hsp70 permet de différencier les produits tératogènes et ceux toxiques pour l'animal.

Method and Results
An in vitro test method for general metal toxicity screening was designed, based on the cellular response to stress. The expression of a transfected human growth hormone gene sequence driven by the human heat-shock protein 70 promoter in NIH/3T3 cells was used as marker of noxious contact with metal compounds. Out of a series of 31 metals, 17 were competent for inducing this stress response system. According to the effective concentration and to the intensity of the response, three different clusters of positive compounds emerged and were ranked as strong, intermediate strength and weak inducers.
These results correlated well with data from other in vivo and in vitro metal toxicity studies, including LD50 in mice. Apparently the positive/negative compounds also fitted well with data from genotoxicity and carcinogenesis studies on metal salts.
(abstract from Cell Biology and Toxicology, 9, 2, 1993, p177)

Reference
Cell Biology and Toxicology, 9, 2, 177 - 188, 1993

3R-Project 36-92

Ersatz tierischer Antikörper durch rekombinante antikörper-präsentierende Bakteriophagen

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Duration: 3 years **End of the Project:** 1996

Background and Aim
Das Projekt hat zum Ziel abzuklären, ob rekombinante Phagenbibliotheken, die das Immunsystem von Säugern repräsentieren, für die Selektion und Produktion von spezifischen Antikörpern geeignet sind, und ob sie damit, zumindest längerfristig, das Tier als Produzent von spezifischen Antikörpern ersetzen könnten.
Mit gentechnologischen Methoden können Bakteriophagen hergestellt werden, welche als vermehrbare Antikörper funktionieren. Zumindest theoretisch können grosse Populationen solcher Phagen das gesamte immunologische Repertoire eines Organismus (z.B. einer Maus oder eines Menschen) darstellen. Experimentell wurde bereits nachgewiesen, dass aus einer solchen Population Phagen selektioniert werden können, die als hochspezifische und beliebig vermehrbare Antikörper funktionieren.
Nach wie vor offen bleibt aber die Frage, ob diese Methode nur dann funktioniert, wenn bei der Konstruktion der Bibliothek von der cDNA eines bereits mit dem entsprechenden Antigen immunisierten Tieres oder Menschen ausgegangen wird, oder ob eine Bibliothek die Selektionierung von Antikörpern beliebiger Spezifizität erlaubt.

Method and Results
Die im Rahmen dieses Projekts erhaltenen Daten führen zu zwei Schlussfolgerungen.
1. Im Prinzip erlauben in-vitro mutagenisierte Bibliotheken von Antikörper- produzierenden Bakteriophagen die Selektion von Antikörpern mit beliebiger Spezifizität. Dies lässt optimistischerweise die Hoffnung zu, dass diese Technologie zumindest mittelfristig den Ersatz von tierischen Antikörpern erlauben wird.
2. Die Arbeiten haben aber auch aufgezeigt, dass der Weg von der theoretischen Möglichkeit über das experimentelle Resultat bis zu einer ausgereiften und vernünftig einfachen und praktikablen Technik noch sehr weit ist. Im heutigen Zeitpunkt ist es nach wie vor wesentlich einfacher, kostengünstiger und schneller, konventionelle monoklonale Antikörper herzustellen. Hier ist auch noch anzufügen, dass mittlerweile die Produktion menschlicher monoklonaler Antikörper grosse Fortschritte gemacht hat.

3R-Project 37-92

Nachweis von Clostridien-Toxinen ohne Tierversuch

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Duration: 3 years **End of the Project:** 1996

Background and Aim
Die Diagnostik und Identifikation von human- und veterinärmedizinisch relevanten Clostridien- Spezies beruht im wesentlichen auf ihrer Produktion von Exotoxinen, welche zum grössten Teil mittels Tierversuchen nachgewiesen werden müssen. Neue Erkenntnisse über die Struktur von Clostridien -Toxinen und ihrer Gene erlauben die Entwicklung von in vitro Nachweisverfahren.
Aufgrund bekannter Toxingene der verschiedenen Clostridium perfringens - Toxintypen wird in diesem Projekt ein Polymerase - Ketten - Reaktionsverfahren (PCR) zum Nachweis der alpha-, beta, sigma- und Enterotoxin entwickelt. Dadurch können die wichtigen Toxintypen von C. perfringens eindeutig ohne Tierversuche identifiziert werden. Ein wichtiges Clostridium-Spezies ist Clostridium chauvoei, bei welchem überhaupt keine Virulenzfaktoren bekannt sind. Mit biochemischen und genetischen Methoden soll versucht werden, das Hämolysin von Clostridium chauvoei zu isolieren und zu klonieren. Dieser Projektteil wird schliesslich ebenfalls zu einer spezifischen in vitro-Nachweismethode von C. chauvoei führen.

Method and Results
In order to get basic results which allow the phylogenetic localisation and identification of Clostridium chauvoei and the closely related species Clostridium septicum, we cloned and sequenced the rrs genes encoding the 16SrRNA for these species and determined their phylogenetic position in Clostridium cluster I (C. carnis, C. perfringens, C. botulinum, C. tetani). Based on DNA sequence data, we developed an identification system for C. chauvoei, using specific PCR amplification of the rrs gene with specific oligonucleotids. . The developed identification system was evaluated on clinical material during a recent outbreak of blackleg. Thereby C. chauvoei was identified as the etiological agent of the outbreak either directly from clinical samples of muscle, liver spleen and kidney or from primary cultures made with this material.
A comparison with the standard diagnostic tools for C. chauvoei showed that the newly developed method has advantages over the immunofluorescence. Moreover, this assay is a valuable tool for the phylogenetic identification of C. chauvoei which can substitute the fastidious traditional identification and replace a considerable amount of laboratory animals traditionally used for testing.

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3R-Project 38-92

CO2-Anästhesie beim Labortier

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Duration: 2 years **End of the Project:** 1995

Background and Aim
In einer Literaturrecherche und eigenen Versuchen wird geklärt, ob sich CO2-Gas zur Einleitung einer Kurznarkose für schmerzhafte Eingriffe (Blutentnahme, usw.) eignet. Besondere Aufmerksamkeit wird dem Verhalten der Tiere gegenüber CO2 und den verschiedenen Konzentrationen mit ihren Wirkungen und Nebenwirkungen geschenkt.
Die Durchführung einer Kurznarkose ist für viele Manipulationen bei kleinen Labortieren ein heute unabdingbares Erfordernis. Injektionsanästhesien sind nicht nur aufwendiger und länger dauernd, sondern führen wegen der Manipulation der Tiere auch zu recht erheblichem Stress. Aus diesem Grund hat sich die Inhalationsnarkose nach Einleitung in der Narkosekammer bewährt. Alternativen zu Aether, welches heute als nicht mehr akzeptabel erachtet wird, sind andere, modernere Inhalationsanästhetika.
Von den Resultaten wird erwartet, ob eine einfache und billige Kurznarkose für Labortiere möglich ist. Diese Kurznarkose soll weniger belastend sein für Tier, Personal und Umwelt als es die heutigen Methoden sind und somit im Sinne eines Refinements wesentlich beitragen.

Method and Results
Es kann davon ausgegangen werden, dass eine Kohlendioxid(CO2)-Konzentration von 80% CO2 mit 20% Sauerstoff (O2) während einer Expositionszeit von 60 Sekunden für eine 30 - 40 Sekunden anhaltende sichere und tierschutzgerechte Anästhesie bei Ratten sorgt.
Für eine chirurgische Anästhesie von 40 Sekunden - als Kriterium für die Tauglichkeit dieser Anästhesiemethode - bei einer Gaskonzentration von 80% CO2 und 20% O2 sind als Expositionszeiten beim Meerschweinchen 30 Sekunden, bei Ratten 60 Sekunden und beim Goldhamster 120 Sekunden erforderlich. Für Mäuse scheint die CO2-Anästhesie für einen Eingriff von 40 Sekunden nicht auszureichen.
Die Kohlendioxidinhalation erfüllt alle für eine Anästhesie notwendigen Erfordernisse, wie Analgesie, Hypnose und Relaxation bei voller Reversibilität. Die Analgesie überdauert zeitlich die Dauer des Eingriffs. Als Nachteil dieser Anästhesieform gilt es zu erwähnen, dass die CO2-Anästhesie nicht beliebig verlängert werden kann.

3R-Project 39-92

In vitro Isolation von pathogenen Trypanosomen aus Mensch, Haustier und Tsetsefliege

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Duration: 3 years **End of the Project:** 1995

Background and Aim
Die Isolation von Trypanosomen (pathogene Blutparasiten) aus dem Menschen oder aus erkrankten Haustieren ist wichtig für die Diagnose und nachfolgende Behandlung der Krankheit sowie zur Charakterisierung und dem Erkennen von Arzneimittel-Resistenzen. Als Alternative zur Vermehrung in Nagetieren soll ein Protokoll für eine in vitro Isolation der Parasiten erarbeitet werden.
Im Labor sollen zunächst die geeigneten Kultivationsbedingungen gefunden werden. Dann soll die Toxizität verschiedener Antibiotika und -myotika für Trypanosomen festgelegt und synergistische Wirkung untersucht werden mit dem Ziel, eine gegen Bakterien und Pilze wirksame Antibiotika-Mischung zu finden, die für die Trypanosomen jedoch untoxisch ist. Die Methode soll in Uganda unter Feldbedingungen erprobt und dort auch eingeführt werden.

Method and Results
Um Antibiotika oder Antimykotika optimal zur Verhinderung von mikrobieller Kontamination bei der Isolierung von Trypanosomen einsetzen zu können, wurde zuerst die Toxizität von Antibiotika auf die Parasiten bestimmt. Zwei Referenzstämme von Trypanosomen, STIB 345 (Trypanosoma brucei) und STIB 704 (Trypanosoma rhodesiense), 7 Antibiotika und 4 Antimykotika sowie zwei Inkubationszeiten (3 und 10 Tage) wurden ausgewählt. . Es zeigt sich, dass drei Antibiotika (Chloramphenicol, Neomycin und Kanamycin) ausserordentlich gut verträglich sind. Von den Antimykotika ist lediglich Fluorcytosin bedenkenlos einsetzbar.
Zur Isolation von Trypanosomenstämmen werden normalerweise Labortiere eingesetzt. Durch ein effizientes Isolationsprotokoll mit optimal dosierten Antibiotika/ Antimykotika in Konzentrationen, die für die Parasiten unschädlich sind, können Labortiere weitgehend eingespart werden (Reduce). Von Replace zu sprechen, wäre noch verfrüht, da wir noch nicht wissen, wie empfindlich die Kulturisolation im Vergleich zur Isolation im Labortier wirklich ist.
Der Einsatz der beschriebenen Antibiotika/-mykotika Mischung wird zur Zeit in Uganda für die Isolation von Trypanosomen aus Patienten erprobt. Darüberhinaus hat sich die Methode bereits im Labor in Uganda und in der Schweiz für die Adaptation der Parasiten an in vitro Kulturen zu Charakterisierungszwecken bewährt. Die gewonnenen Erkenntnisse lassen sich auch für die Isolation anderer Parasiten anwenden, z.B. bei der Diagnose auf Leishmaniose mittels Kultur.

Reference
Mäser P. Grether-Bühler Y., Kaminsky R. and Brun R. (2002) An anti-contamination cocktail for the in vitro isolation and cultivation of parasitic protozoa. Parasit. Res. 88: 172-174.

3R-Project 40-92

Verwendung von Fischzellkulturen als Testsystem zur Reduktion und zum Ersatz von Tierversuchen in der Oekotoxikologie

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Duration: 3 years **End of the Project:** 1996

Background and Aim
Der Einsatz von Fischen als Versuchstiere in der Oekotoxikologie ist stark steigend und Alternativen zu Tierversuchen werden bisher wenig verfolgt. Ziel des Projektes ist die Evaluation eines Bioassays mit Fisch-Hepatomazellen für die oekotoxikologische Beurteilung von neuen Chemikalien und Umweltproben sowie zur Beurteilung der Belastung von Organismen mit Umweltschadstoffen.
Als sensitive Messgrössen dienen die Zytotoxizität und die durch eine Reihe von Umweltchemikalien bewirkte Induktion von spezifischen hepatischen Enzymen, die für den Metabolismus von Fremdstoffen wichtig sind. Es sollen, ausgehend von bekannten Umweltgiften (PCBs), die Induktion von Cytochrom P450 -abhängigen Monooxygenasen über die Enzymaktiviät und mittels monoklonaler Antikörper evaluiert, quantitative Struktur- Aktivitäts- Beziehungen hergestellt, und auf die Analyse und Beurteilung von neuen Schadstoffen und Umweltproben angewandt werden. Die Ergebnisse sollen den Ersatz von Tierversuchen in der akuten und chronischen Toxizitätsprüfung ermöglichen.

Method and Results
Die Untersuchungen über die Zytotoxizität von 21 organischen Zinnverbindungen haben gezeigt, dass sich dieses in vitro-System gut eignet, die Toxizität von Umweltschadstoffen für Fische abzuschätzen. Da die untersuchte permanente Leberzelle metabolisch kompetent ist, eignet sich dieses System als Alternative zu Tierversuchen besser, als bisher verwendete Fibroblastenzellen. Diese Versuche zeigen, dass die Wirkung von Umweltchemikalien und Chemikaliengemischen auf wichtige Enzymsysteme von Zellen in vitro untersucht werden können. Damit lassen sich wichtige Schlüsse über die Wirkung von Umweltchemikalien ziehen, die von ökotoxikologischer Bedeutung sind. Dieses Zellsystem hat ein grosses Potential für den breiteren Einsatz in der Chemikalienprüfung und Beurteilung der Toxizität von Umweltproben und sollte weiter validiert werden. Unsere Resultate sind zudem vielversprechend für die Verwendung eines solchen in vitro-Systems als bioanalytisches Werkzeug zur Untersuchung von Umweltproben. Damit ist ein Werkzeug für die Forschung und Praxis gegeben, Schadwirkungen auf Fische in einem hervorragenden in vitro-System statt im lebenden Tier zu untersuchen.

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3R-Project 42-92

Herstellung von Antikörpern: Einfluss der Adjuvantien auf die Immunantwort und die Belastung der Tiere

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Duration: 3 years **End of the Project:** 1995

Background and Aim
Mit dem vorliegenden Projekt sollen die aus 2 Vorgängerprojekten gewonnenen Erkenntnisse über Auswirkungen der Adjuvantien auf die Schmerzbelastung der Versuchstiere vertieft werden. Im Vordergrund steht dabei die Frage: Welches kommerziell erhältliche Adjuvans eignet sich in bezug auf die Schmerzbelastung des Tieres und der Immunantwort am besten zur Immunisierung von Mäusen (monoklonale Antikörper, mAK) sowie für Kaninchen (polyklonale Antikörper, pAK). Dabei werden nach der Immunisierung der Tiere mit den verschiedenen Adjuvantien einerseits die Antikörpertiter im Serum bestimmt und andererseits in Zusammenarbeit mit dem Institut für Labortierkunde (Prof. Thomann) und dem Institut für Pathologie (Dr. P. Ossent), Universität Zürich, die Tiere auf pathologische Gewebsveränderungen untersucht.

Method and Results
Im Projekt wurden sieben kommerziell erhältliche Adjuvantien (Poly-A-poly-u, GERBU, RIBI, Pam3, Specol, Freund und Titermax) auf ihre immunstimulierende Wirkung sowie auf die Belastung der Tiere hin untersucht. Es hat sich gezeigt, dass in der Belastung der Tiere grosse Unterschiede zu finden sind: Poly-A-poly-U und das Freund'sche Adjuvans (subcutan verabreicht) verursachen keine bzw. eine geringe Belastung. In der Handhabung hingegen schneidet das Freund'sche Adjuvans schlecht ab (Zweispritzenverfahren zur Erstellung der stabilen Emulsion, grosse Verluste). Aufgrund der vorläufigen Daten empfehlen wir das Poly-A-poly-U zur Herstellung monoklonaler Antikörper in der Maus. Poly-A-poly-U zeigt, zusammen mit dem GERBU eine gute Immunstimulation. GERBU könnte nach erfolgter Optimierung, mit dem Ziel die Belastung zu senken, auch empfohlen werden. Mit potenten Adjuvantien kann der Tierverbrauch bei Immunisierung verringert werden (reduce). Wenig oder nicht belastende Adjuvantien verringern den Schmerz der Tiere und verbessern dadurch den Tierversuch (refine).

Reference
3R-Info-Bulletin No 11, 1999

3R-Project 43-95

Short term assay for liver cell activated neurotoxic drugs

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Keywords: *chicken; rat*; *brain; liver*; *toxicology*; *cell cultures: co-cultures; cell cultures: organ-specific; cell cultures: primary*; *reduction; replacement*; *toxicity testing: xenobiotics*

Duration: 3 years **Project Completion:** 1999

Background and Aim
The identification of chemicals with a potential neurotoxic activity is an important aspect in toxicity testing of drugs, agricultural chemicals and industrial products. Current risk assessment guidelines recommend testing organophosphorus compounds (OP) in vivo in hens, since this species is highly sensitive. The relatively high costs, low "throughput" and massive animal distress involved in testing compounds in whole animal experiments led to the development of in vitro tests making use of human and animal neuroblastoma cell lines. However, a severe limitation of these tests is that they are unable to detect compounds requiring metabolic activation in order to exert their neurotoxicity as is the case for most OPs. The aim of this project was to establish a cell culture model capable of performing this metabolic activation, with the view of establishing it as an *in vitro* screening system to detect both specific and non-specific neurotoxic compounds.

Method and Results
Primary rat hepatocytes were used as a metabolic activation system (1). After 3 days in culture (2), the hepatocytes were exposed to the test compound. Six hours later, cultured chicken embryonic brain cells (3), a sensitive target for neurotoxic chemicals, were exposed to the supernatant of the treated hepatocytes. Stable metabolites released from intact liver cells (4) and exerting their toxicity in the cocultured brain cells might also have the potential to reach the nervous tissue in an intact organism.
Cylophosphamide (CP: 10 - 300 µM, activated mainly by CYP 2B1/2) was used as a positive control to characterise the metabolic competence of the coculture system. Isofenphos (IF: 10 -100 Bayer AG) was used as a representative organophosphate with a high selective neurotoxicity in animals and humans. Brain cells directly exposed to the two chemicals showed no signs of toxicity. When exposed to the supernatant collected from hepatocytes treated with the parent compounds, a concentration-dependent toxicity was detectable, indicating that the chemicals were converted to stable toxic metabolites by the hepatocytes. A similar dose-dependent toxicity of hepatocyte derived metabolites detectable was found with IF.
However, bioactivated CP metabolites induced both cytotoxicity (MTT-assay) and a parallel inhibition of acetylcholinesterase (AchE) activity in a dose-dependent fashion, whereas IF was able to inhibit AchE activity even at nontoxic concentrations (5). This corresponds well to the specific neurotoxic activity of this compound in vivo and demonstrates that the inhibition of an organ-specific function (in this case function of the cholinergic nerves) can occur independently of general cytotoxicity.

Conclusions and Relevance for 3R
The established sequential use of hepatocytes (activation system) and chicken brain cells (target) is suitable for the detection of the activation of OP and other xenobiotics (e.g. CP). The cytotoxicity obtained with the supernatant from hepatocytes treated with the parent compounds indicates that stable metabolites are formed; in vivo these would have a high probability of reaching the brain. Furthermore, this experimental model can discriminate between the induction of nonspecific cytotoxicity (CP) and specific neurotoxic effects (inhibition of AChE activity without cytotoxicity as shown with IF). Accordingly, chemicals with a cholinotoxic activity can be identified, making further testing in animal models superfluous. Used as a primary screening procedure, this test has the potential of reducing the number of animals needed to assess the toxicity profile of a compound.
Clearly the sequential and the co-culture approaches do not provide a complete answer as to the on-going processes in an intact organism. In the case of organophosphates, it is already known that this group of chemicals is potentially neurotoxic. In the case of pharmaceuticals, it may be important to know whether the metabolites reach brain cells or whether their access is inhibited by the well established blood-brain barrier (BBB) and choroid plexus (CP). This question can be addressed using in vitro cell culture systems in which the specific transporters and activities of the BBB (e.g.

Cecchelli et al.2007) or the CP (Baehr, Reichel et al. 2006) are expressed. Either the original compound or its metabolites can be tested and new information can be obtained about molecular structures which allow or prevent access to the brain.

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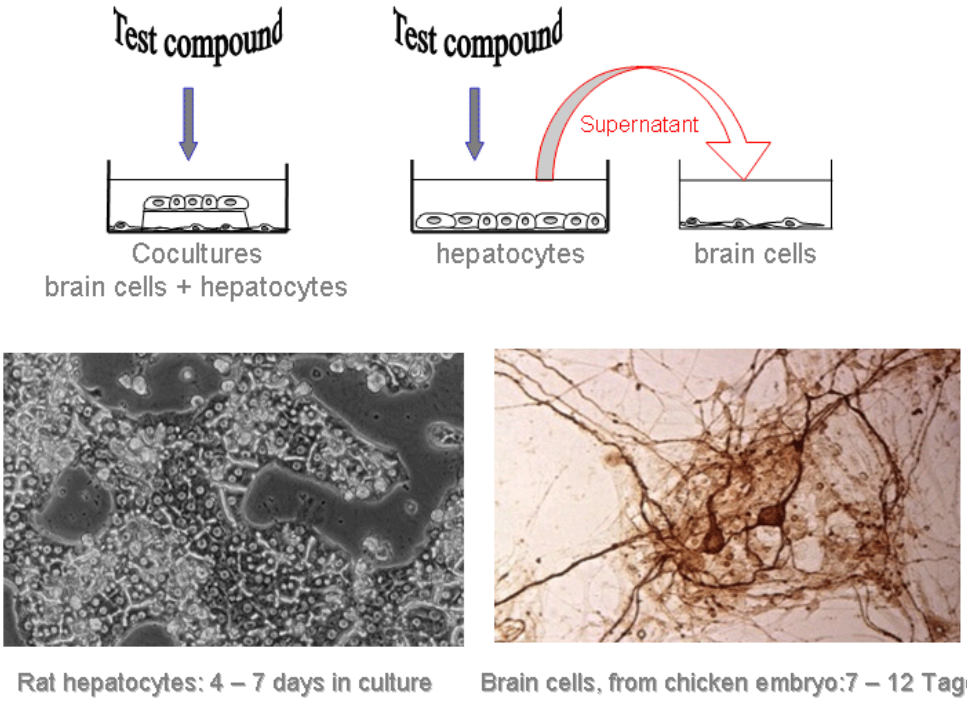

Figure 1: The sequential type of exposure mimics more closely the in vivo situation. The organ-specific (tissue-specific) toxicity in liver and brain cells as well as the toxicity of the parent test compound and its metabolites can easily be dissected in separate hepatocyte or brain cultures.

3R-Project 44-95

Establishment of a micro technique for corticosteroid receptor binding studies

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Keywords: *brain*

Duration: 1 year **Project Completion:** 1999

Background and Aim
The measurement of corticosteroid receptors in brain structures of the rat often requires a large number of animals due to the small size of some of the areas of interest. The objective of this study was to establish a micro-technique for measurements of both corticosteroid receptors (mineralo- and glucocorticoid) with much smaller tissue samples, so that fewer animals would be required for such investigations.

Method and Results
Tissue (hippocampus or anterior pituitary) from rats was homogenized in 1.5 ml buffer TEMDG (25 mM Tris-Cl, 1 mM EDTA, 20 mM molybdate, 5 mM DTT, 10% glycerol, pH 7.4) using a motor-driven glass-teflon homogenizer. The homogenate was first spun in a microfuge at 10,000 x g at 4°C for 15 min. The supernatant was then centrifuged at 105,000 x g at 4°C for 60 min in an airfuge (Beckman) to obtain the cytosol fraction. The mineralocorticoid receptor (MR) was measured by incubating 6 nM [^{3}H]-aldosterone in the presence of 0.5 microM RU-28362, a specific agonist that occupies glucocorticoid receptors. Non-specific binding was assessed by incubating 6 nM [^{3}H]-aldosterone in the presence of 2.5 microM corticosterone. The level of glucocorticoid receptor (GR) was given by the difference between the binding of 10 nM [^{3}H]-dexamethasone in the presence and in the absence of 0.5 microM RU-28362. The tubes containing a final volume of 100 microliters were incubated on ice for 24 h. Bound [^{3}H]-steroid was separated from unbound [^{3}H]-steroid by gel filtration on self-made Sephadex LH-20 mini-columns (1 ml). Binding levels were expressed as fmol/mg protein.

The results obtained show that the measurement in triplicate of both corticosteroid receptor types can be performed with 4 anterior pituitaries or 2 hippocampus, which is half the tissue required using the conventional standard method. The limit of detection of the micro-assay is 1.45 fmol/mg protein. Total bound radioactivity is 2% for MR and 6% for GR. Non-specific binding (in the presence of unlabeled ligand) is 10% of the total bound for MR and 5% for GR. The inter- and intraassay coefficients of variability are 6.3% and 4%, respectively (1).

Conclusions and Relevance for 3R
The refined technique developed for corticosteroid receptor binding in rat CNS tissue reduces the number of animals required per experiment by half.

Reference
Hügin-Flores M., Steimer T., Schulz P., Vallotton M.B. and Aubert M.L. (2000) Regulation of hippocampal corticosteroid receptors during chronic restraint stress ; effect of adrenalectomy and of CRH and AVP antagonists, submitted.

3R-Project 45-96

Replacement of mouse and rat antibody production test by polymerase chain reaction assays

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Keywords: *viruses*; *molecular biology: pcr*; *reduction; refinement*; *diagnostic approaches: viruses*

Duration: 1 year **Project Completion:** 1998

Background and Aim
Murine viruses may be transmitted from one animal to another other through contaminated biological material such as transplantable tumours, cell lines or hybridoma lines. Such inadvertent transmission may cause enzootic infections in the recipient colony resulting in clinical disease and/or compromised research results.

To avoid this, biological material is screened for pathogens before introduction into an animal colony. Historically, this has been done using the mouse antibody production (MAP) or the rat antibody production (RAP) test, by inoculating the material into naive mice or rats and subsequently testing the animals for seroconversion. The goal of this project was to establish an alternative assay using PCR technology that can detect all the relevant murine viruses directly in the biological material. Our aim is to replace the MAP/RAP test completely (see also 3R project 74-00).

Method and Results
PCR assays were developed to detect the Sendai virus, pneumonia virus of mice (PVM), minute virus of mice (MVM), Kilham rat virus (KRV), Toolan's H-1 virus, reovirus type 3, epizootic diarrhea of infant mice (EDIM), mouse adenoviruses type I and II, ectromelia virus, polyoma virus, K virus, Theiler's murine encephalomyelitis virus, lymphocytic choriomeningitis virus (LCMV) and the Hanta virus group. These tests, combined with PCR assays to detect the coronaviruses of mice and rats and the lactate dehydrogenase elevating virus (LDV) as published previously, cover the complete spectrum of agents detectable by the traditional MAP/RAP test. Routine samples submitted to our diagnostic lab for MAP testing, as well as deliberately spiked samples, were compared with the newly developed PCR screening test (1). The PCR approach was more sensitive and had a much shorter turnover time (two days) than the MAP test (one month to obtain seroconversion plus a few days for the serology results).

Conclusions and Relevance for 3R
This PCR screening assay eliminates the need of animals or animal tissue to screen for murine viruses in biological materials. It will eventually replace the MAP test. Additionally, the method contributes to the refinement of many other animal experiments by ensuring that research results are not compromised by inadvertent (subclinical) infections of experimental animals with viral pathogens. Since most diagnostic labs offering serology are equipped with the equipment necessary to perform PCR, this new assay can be easily implemented by other labs, and could become the regulatory standard in the future.

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3R-Project 46-96

Development and Validation of an In Vitro Model Simulating Mechanical Ventilation-Induced Inflammation

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Keywords: *lung*; *cytokines, growth factors*; *inflammation*; *cell cultures: organ-specific*; *reduction*

Duration: 3 years **Project Completion:** 2000

Background and Aim
Critically ill patients with sepsis and adult respiratory distress syndrome (ARDS) are frequently submitted to prolonged positive pressure mechanical ventilation (MV) to prevent pulmonary failure. It has recently been recognized that MV per se can induce significant lung injury. Evidence for this arises mainly from animal studies. This project was designed to develop an *in vitro* model to study MV-induced lung inflammation. In this model, lung cells were submitted to a prolonged cyclic pressure-stretching strain resembling that of conventional MV.

Method and Results
Primary human lung cells or cell lines were cultured on a stretchable silastic membrane forming the bottom of a 12-well plexiglas® box. The box was connected to an adult ventilator and "ventilated" for up to 36 hours at 20 cycles/min with a pressure-volume regimen resembling that of MV. Several lung cell types were tested in this model. The alveolar macrophage was identified as the main cellular source of key inflammatory mediators, such as tumor necrosis factor, the chemokine interleukin (IL)-8, and matrix metalloproteinase-9, produced during mechanical ventilation. Mechanical ventilation also induced low levels of IL-8 secretion by human alveolar epithelial type II-like cells. Other lung cell types such as endothelial cells, bronchial cells, and fibroblasts failed to produce IL-8 in response to mechanical ventilation (1,2).

Conclusions and Relevance for 3R
The described in vitro model provides mechanistic information at the molecular and cellular levels for the lung inflammation observed during mechanical ventilation of patients. It allows mechanical stress-induced signaling pathways to be explored without the use of animal models, and permits the testing of novel ventilatory strategies and adjunctive substances aimed at modulating cell activation induced by mechanical ventilation.

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3R-Project 47-96

Development of an in vitro system for the detection of estrogenic compounds (xenoestrogens)

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Keywords: *fish*; *toxicology*; *cell cultures: organ-specific; cell cultures: transgenic; reporter gene assay*; *reduction*; *toxicity testing: xenobiotics*

Duration: 4 years **Project Completion:** 2000

Background and Aim

An increasing number of compounds are being recognized as possessing estrogenic activity by interfering with the sex hormone system. Many of them are widely used chemicals that enter the aquatic environment via effluents from sewage treatment plants. Estrogenic compounds (xenoestrogens) mimic the natural estrogen 17beta-estradiol and may negatively interact with the sex hormone system and reproduction. These compounds compete for binding to the estrogen receptor (ER) and influence gene expression. The aim of this project is the development of in vitro systems using permanent fish cell lines to detect estrogenic activity of environmental chemicals, chemical mixtures and environmental samples (wastewater). The production of ER-mediated gene products (ER, yolk precursor vitellogenin: VG) will be used to identify and assess estrogenic potential.

Method and Results

Fish cell lines used include a hepatocarcinoma cell line PLHC-1 from *Poeciliopsis lucida* and two cell lines from rainbow trout, gonadal RTG-2 cells and liver RTL-W1 cells. PLHC-1 and RTG-2 cells were adapted to serumfree culture conditions showing similar growth rates as when thriving in serum-containing medium (1). RTL-W1 cells need 1% serum in order to thrive. A palette of techniques to measure estrogenic activity have been developed using antibody, PCR and transfection techniques. A monoclonal antibody against conserved sequences of fish VG proteins has been produced, along with a monoclonal anti-VG antibody which crossreacts with trout-VG. A competitive RT-PCR technique has been developed. To quantify levels of ER and VG mRNA induction in vitro and *in vivo*. Transfection of PLHC-1 and RTG-2 cells is performed in a dual luciferase assay using estrogen responsive reporter genes. In RTG-2 cells, an expressed estrogen receptor mRNA fragment was detected by RT-PCR. Several plasmids carrying different fragments of the VG promoter and fused to the firefly luciferase encoding cDNA were compared for their induction potential when transfected into fish cell lines. The quantity of expressed ER seems too low in these cells. Further experiments are aimed at the detection of estradiol-induced reporter gene activity .

Conclusions and Relevance for 3R

Results of this ongoing study indicate the potential of these in vitro systems for detecting xenoestrogens. The transfection system is most promising and will be further developed and optimized. Once established, the systems will allow the identification and assessment of potentially estrogenic chemicals prior to production and release into the environment, hence contributing to the reduction of in vivo fish tests (2).

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3R-Project 48-96

Utilization of a model of culture of human intestinal cell lines for the study of the pathogenicity of different strains of Clostridium difficile

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Keywords: *gastrointestinal tract*; *inflammation*; *reduction*; *diagnostic approaches: microorganisms*

Duration: 1 year **Project Completion:** 1998

Background and Aim
Clostridium difficile is recognized as the major etiologic agent of pseudomembranous colitis in humans. The microorganism produces two lethal toxins: toxin A and toxin B. The development of an in vitro model to assess the cellular cytotoxicity of both toxins will greatly reduce the use of animals which serve as models for the disease. These include mice, hamsters or rabbits. The in vitro model can be used further to evaluate the potency of chemicals and polyclonal or monoclonal antibodies as therapeutic agents.

Method and Results
We tested the direct effects of the two exotoxins A and B on intestinal permeability and epithelial integrity using human colonic T84 cells grown as monolayers on permeable substrates. When applied from the lumenal side at concentrations at which toxin A completely abolished transepithelial resistance, toxin B did not alter epithelial permeability nor morphological integrity. When added together there was a slight additive effect. In contrast, both toxins induced drastic and rapid epithelial alterations from the basolateral side. IgG and dimeric IgA monoclonal antibodies specific for toxin A were sufficient to prevent toxin-induced epithelial damages when added to the lumena compartment. In contrast, IgG did not block the action of toxin A when added basolaterally.These data establish that efficient immune protection against *C. difficile* infection requires the presence of secretory antibodies specific to toxin A in the intestinal lumen and serum antibodies directed against both toxins in the scrosal compartments. The results provide new molecular clues which should facilitate the design of an efficient mucosal vaccine and minimize the use of animal trials to determine the optimal conditions for an efficient vaccine.

Conclusions and Relevance for 3R
The development of an epithelial culture system base allowed to assess the cytotoxicity of two Clostridium difficile toxins and to establish the sidedness of their action. It is now clear that a vaccine has to elict a mucosal antibody response against toxin A and a systemic response against toxin B. Neutralization of toxin A is sufficient for protection since toxin A allows toxin B to act inside the mucosal barriers. This work can now be extended to other pathogens.

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3R-Project 49-96

Cours de formation continue universitaire intitulé “l’utilisation de l’animal en pharmaco/toxicologie et ses alternatives”

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Keywords: *3R education*

Duration: 1 year **Project Completion:** 1997

Background and Aim
Il s'agit d'un cours d'une durée de trois jours (début 1997) comprenant deux après-midi de démonstrations.
Responsables du projet: Pof. P. Kucera; Prof. J. Diezi, Université de Lausanne.
Collaborateures scientifiques: Prof. P. Honegger et Dr. F. Tschudi-Monnet, Université de Lausanne.

Method and Results
Le cours mentionné a eu lieu pendant 3 jours du 12 auf 14 mars 1997.
Les objectives: 1. informer sur les méthodes actuelles utilisées dans la recherche pharmaco / toxicologique; 2. illustrer, par des démonstrations, des méthodes alternatives actuellement utiliséees; 3. informer sur les bases légales et éthiques régissant les expériences humaines et animales dans le domaine bio-médical; 4. ouvrir un débat large et constructif entre les participants et les intervenants; 5. permettre aux représentants des mouvements de protection des animaux de s’exprimer à propos de leurs préoccupation.
Programme du cours:
1. aspect fondamental
2. aspect légal et éthique
3. aspects prospectifs
4. présentation de 4 méthodes in vitro de complexité croissante (cultures de bactéries, cultures cellulaires, culture d’organe, culture d’embryons entier).

3R-Project 50-96

Morphometric Analysis of human articular cartilage

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Duration: 3 years **Project Completion:** 1999

Background and Aim
Osteoarthritic and rheumatoid joint diseases are widespread in the Western population, especially among the elderly. Novel and improved treatment concepts are continually being developed in vitro and evaluated. Evaluations require both the availability of in vitro screening systems for the discovery of promising new drugs and therapeutic approaches. The usefulness of such in vitro studies depends heavily upon the extent to which they accurately mimic conditions within normal human tissue.
Although the basic composition of articular cartilage is known, its detailed structural organization in adult humans has not been extensively studied. It is therefore the purpose of this study to quantitatively analyze and provide a definition of the structural organization and variation within adult human articular cartilage tissue. This information would provide essential reference points for the development of in vitro conditions that closely simulate conditions in vivo and contributes to a database with the detailed structural organization of the human adult articular cartilage.

Method and Results
Adult human articular cartilage tissue was acquired from adult (age 20-40) human bodies within 48 hours of death of individuals without a history of joint disease. This tissue was then subjected to chemical fixation and morphometric analysis using confocal laser scanning microscopy. Morphological parameters quantified included tissue size, cellularity, cell volumes, and matrix volume per cell, all determined as functions of the zone (depth) within the articular cartilage. Nine different topographical sites of the human knee and ankle joints were analyzed for comparisons between anatomical locations.
It was found that the tissue thickness varies considerably between topographic locations in human joints. Moreover, the cellularity of the tissue was found to be, compared to animal tissues, extremely low (1.6 %). The cell-controlled matrix domains around individual cells varied on the order of 2 to 3 times between different zones in the joint cartilage, as well as between different regions in the joints. Mean chondrocyte cell volumes were found to vary between 1'200 and 20'000 cubic micrometers, whereas the mean matrix volumes per cell varied between 6'000 to 15'000 cubic micrometers. Significant differences in structural organization between different topographical sites were also encountered.

Conclusions and Relevance for 3R
The quantitative data gleaned from these studies allows precise simulation in vitro of the in vivo conditions of the human joint cartilage and will therefore improve the relevance of in vitro results, helping to reduce the necessity of animal experiments. Moreover, the data points out that the cellular and structural relationships in the human tissue are fundamentally different from those found in animal models. Thus future experimental designs will require appropriate adaptation. These results will help to optimize experimental screening conditions for new drug development for the treatment and prevention of articular cartilage diseases.

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3R-Project 51-96

Establishment of endotoxin limits in porcine vaccines: animal welfare aspects

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Keywords: *pig*; *cytokines, growth factors*; *refinement*; *diagnostic approaches: vaccines*

Duration: 3 years **Project Completion:** 1999

Background and Aim
Endotoxin (lipopolysaccharide, LPS) is a constituent of the cell wall of gram-negative bacteria. It is found in many vaccines produced from these bacteria. High levels of endotoxin may give rise to a range of adverse reactions often observed in animals following vaccination. For several porcine vaccines the maximum content of endotoxin is limited to 1 x 10^6 IU/dose by the *European Pharmacopoeia (Ph. Eur.)*; however, very few data are available to justify this limit.

Method and Results
In this, study pigs of various ages were vaccinated with 11 inactivated as well as 2 live attenuated vaccines. The endotoxin content of the vaccines was determined by means of the Limulus Amoebocyte Lysate Test (LAL) using the gel clot method according to the Ph. Eur. The animals were intensively monitored for 24 h after vaccination for the appearance of clinical symptoms: body temperature was measured and blood samples were taken for analysis (including blood cell count) prior to vaccination and 1, 2, 4, 6 and 24 h after injection. The chromogenic kinetic method of the LAL was used to determine the concentration of endotoxin in plasma. Release and pharmacokinetics of interleukin 6 (IL 6) and tumour necrosis factor (TNF) were determined in a bioassay by means of the murine cell line 7TD1 for IL 6 and the porcine kidney cell line PK 15 for TNF. Biologically active TNF was determined in a commercially available ELISA. Clinical symptoms including upregulation of body temperature and leucocytosis were observed during the first 24 h after inoculation of the vaccines. However, high doses of endotoxin (> 1 x 10^6 IU/vaccine dose) initially decreased the number of circulating white blood cells. The animals absorbed endotoxin from the vaccine in a dose-dependent manner. Depending on the kind of vaccine (inactivated or live attenuated) we found maximum endotoxin concentrations ranging from 1,40 to 6,5 IU/ml plasma 2h after immunisation. TNF reached a peak in concentration within the first hour (260-7000 U/ml) while maximal concentrations for IL 6 were seen after the second hour (24-500 U/ml). We found large differences in the individual reactions and severity of side effects due not only to the absolute endotoxin content of the vaccine but also to the application route (i.m. or s.c.), the age of the animals and the specifications of the vaccine itself. Therefore it would not be appropriate to establish one single maximum endotoxin limit and apply it to all vaccines. Control pigs never showed any abnormal reactions.

Conclusions and Relevance for 3R
The endotoxin limits for pig vaccines in the *Ph. Eur.* should be re-evaluated on an individual basis. Given the wide variation in the individual reactions, our results clearly demonstrate the need to establish an individually defined endotoxin limit for each porcine vaccine derived from gram negative bacteria. The method presented here offers parameters to investigate possible side effects more effectively. Regulations require that vaccines be "free of local or systemic adverse reactions". The clinical examination parameters presented here will serve to define more precise criteria for the safety evaluation of porcine vaccines. Improved clinical examination may help to optimise test procedures and result in a reduction of animal usage. Calculating possible side-effects in advance may avoid suffering not only of laboratory pigs.

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3R-Project 52-96

Development of an interleukin-1 (IL-1) assay with rabbit blood as an alternative to the rabbit pyrogen test

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Keywords: *rabbit*; *whole blood*; *cytokines, growth factors*; *fever*; *replacement*; *diagnostic approaches: pyrogens*

Duration: 3 years **Project Completion:** 2000

Background and Aim
In the 1940's drug testing for pyrogen contamination(fever-inducing impurities) was standardized using the rabbit pyrogen assay. Recently we proposed an alternative method using human whole blood (PyroCheck; the kit version was developed in conjunction with DPC Biermann, Bad Nauheim, Germany). This test is not restricted to endotoxins from gram-negative bacteria.

The goal of this project was to establish a rabbit IL-1-ELISA to validate the PyroCheck assay by comparing IL-1beta production in rabbit and human whole blood. This assay will potentially eliminate the need to test in rabbits.

Method and Results
The samples to be tested for contamination with pyrogens are incubated with human blood. The generation of the endogenous fever mediator interleukin 1beta (IL-1beta), indicative of a primary fever reaction in humans, is measured. This method detects the reaction of the targeted species. However, pronounced differences were reported in the potency of several bacterial pyrogens in different species. As part of the validation of the new method using human blood, discrepancies between this whole blood in vitro system and the rabbit in vivo test might occur. In these cases the rabbit blood assay will help to distinguish between an in vitro artefact and genuine species' differences.
The rabbit IL-1beta protein (produced by Dr. Reto Crameri, Schweizerisches Institut für Allergie- und Asthmaforschung Davos, Switzerland; SIAF) is currently being used at the University of Constance to immunize chickens and sheep into producing polyclonal antibodies. Likewise, murine monoclonal antibodies are being prepared.
After the production of murine monoclonal antibodies, they were tested in combination with a second antibody to detect the rabbit IL-1ß. In order to establish a new ELISA to detect rabbit IL-1beta, it is necessary to find the optimal combination of two antibodies. The first, a monoclonal mouse anti-rabbit IL-1beta antibody covers the surface of the microtiterplate, on which the antibody can bind selectively with the protein IL-1. The second, a polyclonal goat-anti rabbit IL-1 antibody, binds to this complex and can initiate a colour reaction necessary for the detection of the amount of the protein by a photometric method (ELISA). At this stage, we should be able to optimize this system to detect Interleukin in rabbit blood. With this method, we would have an instrument available, with which to compare the human and the rabbit reaction to pyrogen contamination. Therefore in the prevalidation phase we could differentiate between a species-specific reaction to exogenous pyrogens (rabbit-man).
Using a rabbit TNF-ELISA (another fever signal transmitter like IL-1) we could show that in principle special cytokines are produced and detectable in rabbit blood.

Conclusions and Relevance for 3R
After successful validation of the rabbit whole blood assay in combination with the human assay PyroCheck, it will be possible to distinghuish between in vitro artefacts and true species differences. With this information, the PyroCheck Test will replace the rabbit in vivo pyrogen test completely.

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3R-Project 53-96

Axenic cultivation of amastigote forms of Leishmania

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Keywords: *pharmacology; toxicology*; *reduction*; *toxicity testing: pharmaceuticals*

Duration: 2 years **Project Completion:** 1999

Background and Aim

Leishmaniasis is one of the major parasitic diseases in many tropical and subtropical countries. A safe and effective vaccine is not yet available. Some of the commonly used drugs have been known to cause severe adverse side effects; treatment failure is also common. The development of new effective drugs and reliable test procedures is therefore an urgent task.
The promastigote insect stages of leishmania parasites can easily be cultivated *in vitro*. These forms are used for drug testing although they do not reflect the situation in the host. Test systems which rely on the 'correct' vertebrate (amastigote) stage of the parasite require either *in vivo* infection experiments using laboratory animals or time consuming co-cultivation of the parasite with macrophages or macrophage-like cells *in vitro*. The present project had three goals: 1. Establishment of axenic amastigote cell lines of different *Leishmania* species *in vitro*. 2. Replacement of fetal bovine serum (FCS) in culture media and 3. Evaluation of axenically grown amastigotes as a screening system for leishmanicidal substances.

Method and Results

Ten Leishmania isolates (*L. infantum*: 6 isolates, *L. donovani, L. tropica, L. major, L. aethiopica*: one isolate each) have been successfully adapted to grow as axenic amastigote or amastigote-like forms. Promastigote forms were propagated in vitro (27°C, pH 7.2) in a defined liquid medium requiring no serum or serum substitutes at. Amastigotes forms were cultivated in a slightly modified medium [1] substituted with 1% of a commercially available serum fraction produced from abundantly available adult bovine serum (FC III, HyClone) which obviates the need for fetal bovine serum. Axenic amastigotes were produced from promastigotes by increasing the temperature stepwise to 36°C followed by decreasing the pH of the medium stepwise to 5.4 [2].
Drug sensitivities of log phase axenic *Leishmania infantum* (isolate 229/1/89, zymodeme MON1) promastigotes were then compared with those of the clinically relevant amastigote stage: the incorporation of [^{3}H]-thymidine in the presence of different concentrations of anti-leishmanial drugs was measured and the corresponding IC_{50} calculated. Three clinically important antileishmanial drugs (allopurinol, Glucantime®, ketoconazole) and a series of dinitroanilines [3] were tested in this system. Whereas promastigotes were completely unresponsive to one of the main antileishmanial drugs, Glucantime®, amastigote forms showed a promising sensitivity

Conclusions and Relevance for 3R

Amastigote forms are the more appropriate target. The finding that IC_{50} values obtained with axenic amastigotes are comparable to values obtained in intracellular systems shows that axenic amastigotes might have the potential to replace intracellular systems or animal models at defined stages during drug development. Furthermore, the possibility of producing large quantities of the clinical relevant amastigote stage *in vitro* allows detailed studies on the mode of action of antileishmanial drugs to be carried out, which in turn will promote the rational design of new leishmanicidal compounds.

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3R-Project 54-96

Elaboration of an in vitro screening method for testing the effect of drugs and other compounds on the renal transport of uric acid

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Keywords: *kidney*; *pharmacology; toxicology*; *cell cultures: organ-specific*; *reduction*; *toxicity testing: pharmaceuticals*

Duration: 1 year **Project Completion:** 1999

Background and Aim
Hyperuricemia is a risk factor for gouty arthritis, and renal failure. As many compounds interfere with the renal transport of uric acid, a screening test is necessary to detect if newly developed drugs affect urate transport and, consequently, urate plasma levels. At present such a drug screening is performed with an in vivo method (uric acid renal clearance measurements) in rats and Cebus monkeys. Recently, a cell line originating from human kidney, which has transport characteristics from proximal tubule, has been developped. By growing these cells on a porous support, an epithelium is formed.
It is intended to use such an epithelium (analogue of human proximal tubule epithelium) to measure the transport of uric acid, and to investigate the effect of drugs known to interfere with uric acid excretion, in order to obtain an in vitro model of uric acid transport.

3R-Project 55-96

Computer-aided identification of ochratoxin antagonists

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Keywords: *drug-design; pharmacology; qsar*; *in silico*; *reduction*; *toxicity testing: pharmaceuticals; toxicity testing: xenobiotics*

Duration: 2 years **Project Completion:** 1999

Background and Aim
This project aims at to discover an antidote for ochratoxin A (OcA), a mycotoxin released by moulds growing on grains, peanuts and vegetables. In animals, ochratoxin is nephrotoxic, genotoxic, teratogenic, carcinogenic and immunosuppressive. The compound has been linked to Balkan Endemic Nephropathy, a kidney disease frequently observed in the Balkan countries. The toxicity of OcA is thought to be due primarily to the inhibition of phenylalanine-tRNA synthetase (PheRS).

Method and Results
Simulating the molecular-dynamic behaviour of PheRS-OcA in aqueous solution, we have identified three quite different binding modes between the toxin and the enzyme, all of which suggest an affinity only in the millimolar range. This contradicts older toxicological findings, but is in agreement with more recent in vitro studies. In vivo, OcA binds preferentially to serum albumin, a plasma protein, with a corresponding effect on the toxicokinetics of the mycotoxin including a delay of the excretion rate. Antagonizing this effect under chronic low exposure to OcA might lead to the immediate elimination of the toxin by excretion and might prevent the accumulation of the mycotoxin in the body. Based on the three-dimensional structure of human serum albumin, we have simulated its interaction with OcA. The long-term goal of our study is to identify a synthetic antagonist with an affinity towards albumin between that of OcA and the endogenous ligands.

Conclusions and Relevance for 3R
Using computational technologies, we demonstrated that i) phenylalanine-tRNA synthetase is of questionable relevance in OcA action and ii) that its adverse effects may possibly all be eliminated by the design of a synthetic antagonist binding appropriately to serum albumin. Such computer-aided investigations of the structure-binding properties help to reduce the number of animal tests necessary to assess the toxicity of a given chemical.

updated version 2007 (pdf file)

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3R-Project 56-96

Development of a mimotope-based tetanus and diphtheria vaccine

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Keywords: *immunology: monoclonal antibody production*

Duration: 3 years **Project Completion:** 2000

Background and Aim
In Switzerland, many animal experiments are performed to ensure the quality of bacterial toxin vaccines. In the sense of "refine" and "reduce", we aim to develop an in vitro assay in order to reduce animal experimentation. Finally, the aim of our project is to develop synthetic tetanus and diphtheria vaccines.

Method and Results
Our first goal was the isolation of mimotopes (peptides that mimic antigen epitopes) recognised by human monoclonal anti-tetanus toxoid antibodies. Indeed we identified mimotopes for five protective antibodies by screening phage display libraries; their specificity was confirmed by in vitro assays. However, immunisation studies have shown that these mimotopes do not induce protection in vivo although a weak anti-tetanus toxoid response was observed in vitro.
We concluded that mimotope affinity to the human anti-tetanus toxoid antibodies is only weak. We therefore intend to screen additional phage display libraries displaying peptides of various lengths. Moreover, we aim to isolate mimotopes recognised by anti-diphtheria antibodies. As there are currently no monoclonal antibodies available, we purified polyclonal anti-diphtheria antibodies by affinity chromatography. So far we have shown that these antibodies have high toxin neutralising activity.

Conclusions and Relevance for 3R
We ultimately expect to completely replace animal experiments in the quality control of tetanus and diphtheria vaccine production. In addition, the project is expected to have a positive impact on the use of animals in the production of various other vaccines.

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3R-Project 57-97

Laboratory animal husbandry and welfare - Key strategic goals of future research

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Keywords: *ethology: laboratory animals*

Duration: 1 year **Project Completion:** 1997

Background and Aim
In 1995, the German Foundation for the Promotion of Research on Replacement and Complemen-tary Methods to Reduce Animal Testing (set), sent out a questionaire to laboratory animal scientists to assess (1) whether scientific evidence suggested that standard husbandry practice would interfere with the welfare of laboratory animals, (2) whether independent evidence suggested that impaired welfare would interfere with the validity of animal research and (3) whether there were alternatives in sight to current standard housing conditions. The highly diverse and sometimes contradictory responses raised concern as to the future allocation of research money in the area of laboratory animal welfare.
Therefore, the 3R Research Foundation Switzerland together with set decided to support an inter-national workshop where leading scientists from different research groups could discuss key strategic goals of future research on laboratory animal husbandry and welfare.

Method and Results
In 1997 this workshop was organized by the Center for Laboratory Animal Husbandry and Welfare (KLab) at the Institute of Animal Sciences, ETH Zürich. Participants were scientists, representatives of the two funding bodies, and legal authorities from the Swiss Federal Veterinary Office.
Participants agreed on the following priorities for future research: behavioural ecology of labora-tory animal species, mechanisms underlying behaviour, cognition, inter-individual differences, non-invasive indicators of impaired welfare, and husbandry effects on experimental results. Multidisciplinary and interdisciplinary approaches should be pursued to use present knowledge most effectively. However, it was also agreed that a framework was needed to facilitate research into how animals‘ behaviour in their natural habitat predicts their environmental needs, and how these needs are expressed as both suffering and pathologic changes when they are not met by the environment. As a result of a follow-up meeting with leading ethologists in 1998 (again organized and chaired by the KLab), a research strategy is currently being developed with the goal of identifying reliable behavioural indicators of impaired welfare. It is planned to publish the framework of this strategy as soon as possible.

Conclusions and Relevance for 3R
The relationships between suffering and pathology need to be understood from an evolutionary point of view. It is proposed that natural selection favours the conscious emotional experience of threats to survival and/or reproduction (e.g. pathological changes), if, and only if, such experi-ences are instrumental in avoiding fitness costs by means of behavioural changes. This view implies on the one hand that conditions leading to pathological changes do not necessarily induce suffering. On the other hand, suffering may occur under conditions that do not induce pathological changes. Thus, whereas some pathological changes may be irrelevant with respect to animal welfare, the mere absence of pathological changes does not guarantee good welfare. In consequence, behavioural changes associated with threats to fitness (e.g. avoidance behaviour) and behavioural signs that the animals´ attempts to adjust their behaviour have been stymied (e.g. stereotypies) represent the most direct indicators of suffering induced by inappropriate husbandry conditions.

3R-Project 58-97

Development of laboratory housing conditions preventing stereotypic behaviour in Mongolian gerbils *(Meriones unguiculatus)*

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Keywords: *gerbil*; *ethology: laboratory animals; ethology: enrichment*

Duration: 3 years **Project Completion:** 2000

Background and Aim
This project focuses on the influence of housing conditions on the occurrence of stereotypic behaviour and its physiological correlates in laboratory gerbils. Stereotypies are generally viewed as behavioural disturbances, since they neither achieve an obvious goal nor fulfill a clear function. Housing conditions may overtax the adaptability of the animal's behavioural repertoire, resulting in stereotypies, replacement activities, chronic stress and even bodily harm. So far, no consistent connections between housing conditions, stereotypies and chronic stress reactions have been found in laboratory rodents. The gerbil, although not one of the major laboratory rodent species, represents a suitable model to study these aspects.
The aims of this project are:
- assessment of the causes of stereotypic digging and bar-chewing in gerbils
- assessment of stress reactions induced by intensive housing conditions and/or stereotypies
- development of housing conditions which eliminate these causes and therefore prevent the development of stereotypies and chronic stress

Method and Results
Specific enrichment factors were added to standard housing conditions of laboratory gerbils. Their influence on the development of stereotypic behaviours and fecal cortisol levels was analyzed. Selected animals were observed weekly from their birth to adulthood, and frequency and duration of stereotyped behaviour bouts were protocolled. Fresh feces were noninvasively collected once weekly and assayed for Cortisol (RIA ^{125}Iod-Cortisol). The present status of the project is as follows:

1. Stereotypic digging develops under standard housing conditions when juvenile gerbils are motivated to retreat into a burrow but cannot do so. An artificial burrow consisting of a darkened nest chamber outside the cage, accessible through a tunnel at the back of the cage, significantly reduced stereotypic digging in juvenile gerbils. The same burrow structure, not darkened and presented within the cage, however, failed to reduce stereotypic digging in juvenile and adult gerbils. Up to this point, no correlation was found between the measured fecal cortisol levels (ranging from 80-120 ng/mg feces dry matter) and age, housing conditions or stereotypy. An artificial burrow system was developed which significantly reduces the development of stereotypic digging and can easily be integrated in a standard laboratory cage type 4, covering one third of the available space. It consists of an opaque nestbox with angled access tube, a transparent separation wall and for stability reasons of an additional, transparent box accesible through a hole.

2. Bar-chewing cannot be viewed as a replacement activity caused by a lack of chewable nesting material nor does it result from reinforced bar-manipulation caused by the vicinity of food pellets and cage-lid bars in the food hopper. Neither the presence of nesting material (straw, paper towels) nor the location of the food, whether contained in the food hopper or scattered on the bedding, had an influence on the duration of bar-chewing. A significant rise in bar-chewing occurred after juvenile gerbils were separated from their families at the age of 35 days and transferred to a similar cage with fresh bedding in same-sexed littermate pairs. High levels of bar-chewing then persisted up to adulthood. This rise in bar-chewing might reflect the animals' motivation to return to their families and home cages. We tested whether social separation from the family or transfer to a new cage caused the rise in bar-chewing. A significant rise in bar chewing occured in groups that were separated from their family before younger siblings are born. Transfer to a new cage had no

significant influence on the development of bar-chewing. These results imply that laboratory gerbils should not be prematurely separated from their parents, at least not until a new litter of young siblings is born.

Conclusions and Relevance for 3R

Refinement of experimental procedures calls for animals free from chronic stress and abnormal, selfmutilating behaviour. Appropriate enrichment factors added to standard housing conditions combined with certain animal management measures can prevent the development of stereotypies in gerbils. At present it is still unclear whether stereotypic behaviour is a marker of chronic stress or whether it represents a coping strategy with a stress reducing effect.

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3R-Project 59-97

Phenotype characterisation and health monitoring of transgenic mice

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Keywords: *mice*; *ethology: laboratory animals; ethology: transgenic animals*; *refinement*

Duration: 1 year **Project Completion:** 1999

Background and Aim
When ‘creating’ new transgenic animals by genetic engineering technology or by breeding (crossing of different existing transgenic strains), it is not always possible to predict the phenotype with certainty, particularly with respect to animal welfare problems. The generation of animal models for (human) diseases often deliberately implies that the health of the animals will be compromised to some degree. The effects range from minor to lethal. To protect animal welfare and apply the criteria of the 3R, there is a need for careful phenotype monitoring and documentation of transgenic animals. Results will influence not only ethical and legal decisions surrounding transgenic strains (e.g. humane experimental endpoints, cryoconservation), but also have ramifications for breeding and maintaining transgenic animal colonies.

Method and Results
To accurately characterise phenotype, i.e. recognise and assess genetic burden or adverse effects, two steps are necessary: 1) comprehensive monitoring and documentation of a representative sample of individual animals in the sense of "life histories", and 2) the characterisation of a strain as a whole, including: i) reproduction data at the population level, ii) statistical frequency of health problems and mean age of their occurrence, iii) differences between hetero-/ hemizygote and homozygote genotypes, iv) between males and females.
Two protocols have been developed to carry out this phenotypic characterisation with mice. They are available on the web at the following site: *www.bzl.unizh.ch*. They include 1) score sheets for individual and litter-wise phenotype and health monitoring (from birth until spontaneous death or euthanasia), and 2) an extensive and comprehensive form for the strain characterisation. The latter is subdivided into basic and detailed information. It can be kept up to date continuously in the form of a computerised database, incorporating growing knowledge and experience of the strain, beginning with the first two generations (F1, F2) of a new strain

Conclusions and Relevance for 3R
The idea of the authors is that transgenic animals, whether commercially distributed or transferred to new facilities, should always be accompanied by their score sheet and strain characterisation form. It is hoped that the newly created forms will have an impact on the procedures in use in Switzerland since 1998 as part of the current legislative programme for a standardised documentation and characterisation of genetically modified vertebrates (Bundesamt für Veterinärwesen 1998, Switzerland). The drawbacks of the presently used form are that it mainly serves official purposes (i.e. statistics) and asks for phenotype deviations from the ‘normal’ in only a very broad manner.

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3R-Project 60-97

Transgenic protozoa (Trypanosoma spp.) as an alternative to transgenic animals

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Keywords: *parasites, ectoparasites: trypanosoma*; *cytokines, growth factors*; *transgenic models*; *cell cultures: parasites*; *cell cultures: transgenic*; *molecular biology: recombinant proteins*; *replacement*; *toxicity testing: pharmaceuticals*

Duration: 2 years **Project Completion:** 2001

Background and Aim
Unicellular organisms (protozoa) can provide an attractive system for medium-scale production of biologically active recombinant proteins. The production of transgenic trypanosomes expressing foreign proteins has several advantages compared to transgenic animals as shown with a series of recently developed vectors in our laboratory. These include stable and precisely targeted integration into the genome by homologous recombination , a choice of integration into several defined sites, allowing expression of multi-subunit complexes, and easy maintenance of cells in a semi-defined medium and growth to high densities (>2 x 10^7 ml^{-1}). The aim of this study is to exploit transgenic trypanosomes, firstly as a source of recombinant proteins that might otherwise be produced in animals, and secondly as a tool for drug-screening in culture, thereby replacing animals as a source of parasites.

Method and Results
1) Expression of Theileria parva sporozoite antigen p67
Background: More than half a million cattle a year die of East Coast Fever, a disease caused by tick-borne transmission of the protozoan parasite *T. parva*. The infection is initiated when sporozoite forms of the parasite invade host lymphocytes and cause them to proliferate in an uncontrolled manner. More than ten years after its discovery, the sporozoite surface antigen p67 is still the only vaccine candidate against East coast fever. It is not possible to obtain sporozoites from infected ticks without allowing them to feed on experimental animals (cattle and rabbits). The application of ticks to rabbit ears can cause severe irritation, especially when larger numbers are used, and is sometimes accompanied by inflammatory responses that affect large areas of the ear epidermis.
Recombinant p67 that has been produced in either bacteria or baculoviruses cannot replace *Theileria* p67, since it is not recognised by antibodies against the native protein. Up to now we have expressed three different forms of p67 in trypanosomes. The most promising is a hybrid protein that contains the signal sequence and GPI-anchor addition sequence of an endogenous trypanosome surface protein (procyclin). A further set of experiments will establish whether this form of p67 can substitute for the *Theileria* protein.

2) A new system for testing drugs in vitro
Background: Bloodstream forms of African trypanosomes survive and replicate in the blood of the mammalian host whereas procyclic forms (which normally first appear in the insect vector) lyse immediately upon contact with serum. Identifying compounds that can induce premature differentiation and the subsequent destruction of trypanosomes in the bloodstream, could be of therapeutic value. To this end we have produced transgenic bloodstream form trypanosomes that synthesise the bacterial enzyme beta-glucuronidase when they differentiate into procyclic forms. Enzyme activity can be detected by adding a substrate which results in a colour change. This assay is so sensitive that it can be performed with small numbers of trypanosomes that are easily obtainable in vitro (whereas larger numbers would require the infection of mice or rats). Thus drugs which induce premature differention can be detected with this approach which is suitable for rapid screening of chemicals.

Conclusions and Relevance for 3R
The use of transgenic trypanosomes, firstly for the expression of recombinant proteins, and secondly as a readout for the detection of novel drugs with antitrypanosomal activities, provides alternatives to transgenic animals for the production of biologically active eukaryotic proteins and reduces the requirement for animals as a source of parasites in drug and vaccine development.

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3R-Project 61-97

An in vitro model to study mechanical forces on human venous coronary bypass graft stenosis: Molecular targeting by pharmacological intervention

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Keywords: *human*; *smooth muscle*; *bypass graft stenosis*; *cell cultures: organ-specific*; *reduction*

Duration: 1 year **Project Completion:** 2000

Background and Aim
Coronary venous bypass graft stenosis is the most important complication limiting long-term clinical improvement in patients after bypass surgery. In addition to vascular smooth muscle cell (SMC) proliferation and growth factors such as PDGF, beta-FGF andthrombin, mechanical force (namely pulsatile stretch) plays an important role in the development of stenosis (1). Although much is known about the response of signa transduction pathways to growth stimuli, the possibility of targeting these mechanisms to prevent deleterious human venous graft cell growth remain largely unexplored. Studies with human grafts in culture combined with the simulation of pulsatile streches off the possibility of reducing, or in the case of mechanistic studies even replacing the use of animal models.

Method and Results
In this project we investigated the effects of several cardiovascular therapeutics and specific inhibitors of the intracellular signalling pathways Rho, GTPase, MAPK, S6K, etc. on human SMC proliferation when the cells were subjected to pulsatile stretch or growth factors such as PDGF. SMC were isolated from human saphenous veins as previously described (1) and the cells were seeded onto Flex I culture plates coated with type-1 collagen. The plates were then placed on a computerized Flexercell Strain Unit (Flexcell Corp., McKeesport, Pa., USA) in an incubator (37°C, 5%CO_2/95% air) and subjected to mechanical stretch (60cycles/min; 25% elongation) in the presence or absence of the test substances substances. Cell proliferation was assayed by means of [^{3}H]-thymidine incorporation as described previously (1).
We showed that pulsatile stretch significantly stimulated [^{3}H]-thymidine incorporation in SMC; this effect was markedly enhanced by heparin. In contrast, [^{3}H]-thymidine incorporation induced by the peptide growth factor PDGF-BB (5ng/ml) was inhibited by heparin (2). Statins prevented stretch-induced SMC proliferation. Rho-kinase inhibitors (Y-27132 and hydroxyfasudil) or transient transfection with a dominant negative RhoA mutant also inhibited proliferation. Co-treatment with mevalonate in the presence of the statin restored PDGF-induced proliferation but had only slight effects on stretch, although mevalonate restored RhoA activation. Stretch induced hyperphosphorylation of pRb and downregulation of p27 expression. Cerivastatin or Rho-kinase inhibitors prevented hyperphosphorylation of pRb but not downregulation of p27. Mevalonate had a small restorative effect on pRb but not on p27 expression. p44/42MAPK and PI3K/Akt were also involved in stretch-induced SMC proliferation. Cerivastatin had no effects on stretch-induced p44/42MAPK and PI3K/Akt activation; however mevalonate induced pronounced RhoA activation, which inhibited the PI3K/Akt pathway and in turn reduced SMC proliferation (3).In contrast to statin, calcium antagonists inhibited PDGF induced cell growth but had no effects on responses to pulsatile stretch.Inhibition of S6K or tyrosine kinase by specific inhibitors prevented cell growth following either stimulus. Calcium antagonists inhibited PDGF induced cell growth but had no effects on responses to pulsatile stretch (4).

Conclusions and Relevance for 3R
This in vitro model could be used to test various drugs in human cells in response to classic peptide growth factors and mechanical stretch. The stimulatory effect of heparin calls into question the clinical use of this substance in patients following coronary bypass surgery (3). Several animal models have been established to study the effects of mechanical forces on venous graft disease. However, despite the importance of animal models, several concerns have been raised as to the suitability of these approaches for understanding human disease and human therapy due to species differences. The present system uses isolated human venous smooth muscle cells cultured under conditions mimicking

pulsatile forces observed in vivo. It will allow the discovery of new drugs interfering with cell growth in response to pulsatile stretch without the use of any animals. The results obtained so far will have great clinical impact for future treatment of venous bypass graft disease in patients.

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3R-Project 62-97

Development of an in vitro model system for cartilage invasion by synovial fibroblasts

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Keywords: *human*; *bone, cartilage*; *arthritis; inflammation*; *cell cultures: organ-specific*; *reduction*

Duration: 1 year **Project Completion:** 1999

Background and Aim
Animals are currently used to study certain aspects of the development, pathology and therapy of rheumatoid arthritis (RA). Among the most commonly used animal models of arthritis are the antigen-induced models (adjuvant arthritis in rats, type II collagen-induced arthritis or streptococcal cell wall-induced arthritis in mice) and animal strains that spontaneously develop arthritis, e.g. MRL-lpr/lpr mice. In humans, RA is characterized by the presence of hyperplastic synovial tissue that invades cartilage and bone. A very recent experimental approach in vivo is to transplant human synovial tissue into immunodeficient SCID mice. Synovial tissue from patients with RA has been transplanted directly into the knee joints, subcutaneously together with human articular cartilage, or under the renal capsule. This approach results in cartilage invasion that resembles the progressive-destructive aspect of human RA. The aim of this project is to establish an in vitro model to investigate destructive processes in RA, to study the interaction between fibroblasts, macrophages and chondrocytes, and to evaluate strategies to inhibit joint destruction in RA.

Method and Results
Human and bovine chondrocytes cultured in sponges pre-treated with native bovine embryonic extracellular matrix (B-ECM) produced a cartilaginous matrix. Sulfur-35 (^{35}S) was provided as a substrate for biosynthetic incorporation into proteoglycans. The 3-dimensional culture system was optimised for the number of chondrocytes (10^5 cells/sponge), the timing of ^{35}S incorporation (day 21 after chondrocyte isolation), and medium (20% FCS). RA synovial fibroblasts (10^5 RA-SF) were added and the destruction of the matrix mediated by these RA-SF was monitored by the release of ^{35}S. The system was modulated by the addition of cells from a human myelomonocytic cell line U937. RA-SF destroyed bovine cartilaginous matrix within 2 weeks (days 5-12) and human cartilaginous matrix within 3 weeks (days 10-18).). Compared with the effect of RA-SF alone (948 ± 180 cpm/week), the addition of myelocytic U937 cells, IL-1 beta or TNF-alpha to the incubation medium increased the destruction of human cartilaginous matrix by 71% and 91% (1618 ± 204 and 1802 ± 307 cpm/week). We found that RA-SF expressing the CD44v4 isoform have a high destructive potential.

Conclusions and Relevance for 3R
This model is envisioned to study distinct aspects of human destructive joint disease under in vitro conditions and to replace and/or supplement animal experiments in basic research and drug testing. Animal models are still required in cases in which the bioavailability of the drug (absorption-distribution) is of concern. Based on the fact that pro-inflammatory cytokines enhance destruction it is concluded that in particular anti-IL-1 directed therapies may prevent cartilage destruction in RA.

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3R-Project 63-97

In vitro study of pathogenesis of sepsis in a conditionally immortalized Kupffer cell line

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Keywords: *mice*; *liver*; *inflammation; septic shock models*; *cell cultures: organ-specific; cell cultures: transgenic*; *reduction*

Duration: 2 years **Project Completion:** 2000

Background and Aim
Hepatic macrophages (Kupffer cells) represent the major first-line defense in sepsis. Unfortunately, freshly isolated Kupffer cells survive and maintain their phenotype only for a short time in vitro. The goal of this project is to develop a Kupffer cell line suitable for culturing in vitro, so that interactions between these cells and bacteria or bacterial products in vitro may be studied.

Method and Results
1) Conditionally immortalized Kupffer cell lines from the H-2Kb-tsA58 transgenic mouse (Jat PS, PNAS, 1991) have been generated.
2) The inflammatory reaction and clearing function of these cells in response to gram negative bacterial products will be studied. Conditions under which there is an optimal balance between clearing and inflammatory response are searched. The modulation of cytokine liberation and clearing by in-vitro transfection of genes involved in both effector functions will be assessed.
Kupffer cells have been isolated from transgenic mice carrying a thermolabile SV40 large tumor antigen under the H2Kb promoter (kind gift of D. Kioussis, NIMR, London). The cells grow with Interferon-gamma at 33°C, at which temperature the promoter is turned on and the SV40T Ag is active. They differentiate at 39°C. These cells are now being characterised: cytokine and NO liberation is stimulated, surface receptors are assessed at the mRNA and protein level, and phagocytosis and uptake of bacterial components are being measured. Results will be compared with the functional characteristics of primary Kupffer cells isolated from normal mice (see also 3R project 73-00)

Conclusions and Relevance for 3R
If the Kupffer cells derived from the transgenic mice prove functionally normal, they will replace studies with primary Kupffer cells. Accordingly, animals will no longer be required for cell isolation.

3R-Project 64-97

Use of three-dimensional brain cell cultures as a model for ischemia-related research

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Keywords: *rat*; *brain*; *ischemia*; *cell cultures: co-cultures*; *cell cultures: organ-specific*; *refinement*

Duration: 2 years **Project Completion:** 2000

Background and Aim
Ischemic brain damage in human due to stroke or cardiac arrest is one of the most serious halth problems in our society. Adequate protective and therapeutic strategies require knowledge about the mechanisms involved. Most of the relevant research is carried out in animal models. The nature of these interventions and the lesions inflicted involve a high degree of distress. Models in vitro require a three-dimensional arrangement of the cells in order to reproduce the situation in a tissue. In this respect, aggregate cell cultures represent a highly promising in vitro system. These cultures prepared from fetal rat telencephalon and grown in a chemically defined medium reach a high degree of cellular differentiation and organization, ressembling in many aspects brain tissue in vivo. The present investigations were designed to test the suitability of this in vitro system for ischemia-related investigations.

Method and Results
Aggregate cell cultures containing either mixed rat brain cells or predominantly neurons derived from freshly isolated embryonic brain tissue can be obtained routinely by established procedures (1). Using biochemical and immunocytochemical criteria, we showed ischemia could be simulated in these cultures by a transient switch from rotatory to stationary culture conditions. This increased the medium-to-tissue glucose and oxygen gradients and caused selective neural cell death. The extent of the ischemia-induced damage correlated with the observed degree of glucose depletion (2). A similar pattern of adverse effects was obtained by transient glucose restriction. Further investigations showed that these deleterious effects were attenuated by antagonists of either NMDA receptors or L-type voltage-gated calcium channels, as well as by chelation of extracellular Ca^{2+}. These results are in accord with the view that the influx of extracellular Ca^{2+} is a critical event in ischemia-induced neuronal cell death (3, 4).
Work is now in progress to examine the implications of metabolic perturbations and glial reactivity in ischemia-induced neurodegeneration, and to evaluate potential strategies for neuroprotection.

Conclusions and Relevance for 3R
The results obtained so far indicate that aggregating brain cell cultures offer a useful in vitro model to investigate the mechanisms involved in ischemia. Promotion of this in vitro approach could lead to a reduction in the use of animal models for ischemia research in the future.

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3R-Project 65-98

Reconstructed human epidermis as model for predicting irritancy

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Keywords: *skin*; *barrier systems in vitro; irritation: eye and skin*; *cell cultures: reconstituted tissue*; *reduction; replacement*

Duration: 2 years **Project Completion:** 2001

Background and Aim
Reconstructed human skin equivalents are the model that most closely mimics normal human skin. They allow the topical application and skin irritancy testing of a great variety of products used in daily life. One of the major requirements for skin irritancy screening is the presence of a competent skin barrier. In native skin this barrier function is carried out by the uppermost layer of the skin, the stratum corneum (SC). In studies with skin equivalent cultures, it has become evident that this barrier function is impaired. In order to further improve this in vitro model, detailed information on lipid processing within the SC is required. The aim of the project is to define the changes that occur in lipids from the surface of the SC to the deeper layers. Based on this information, it might be possible to modify the culture conditions for the reconstructed epidermis in order to optimise the barrier function.

Method and Results
Up until now, lipid analysis of the SC has used extracts of the entire SC. Information on variation in the lipid composition in cross-sections through the SC is scarce. Tape stripping is a technique which removes corneocyte layers step by step with an adhesive film; it is the most frequently used method to investigate the skin penetration process. The use of this technique for lipid analysis has been hampered by the contamination of lipid extracts with compounds co-extracted by organic solvents from the tape. In the present study a suitable analytical method to determine the local SC lipid composition was established. This included optimising the solvents for the extraction and high performance thin layer chromatography analysis steps.

Conclusions and Relevance for 3R
The new method enables inter- and intra-individual differences in SC lipid profile in healthy and diseased human skin to be studied with regard to SC lipid organisation and to skin barrier function in vivo. It will provide information on differences in lipid profiles between native and reconstructed epidermis. This knowledge will be then used to further optimise the barrier function in the reconstructed epidermis, which will increase the reliability of the in vitro epidermis test for the detection of skin irritants. This in turn will lead to a reduction in the number of animals used for skin irritancy testing.

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3R-Project 66-99

The influence of enriched housing conditions on experimental results in laboratory mice

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Keywords: *mice*; *ethology: laboratory animals*; *ethology: enrichment*; *reduction*; *refinement*

Duration: 1 year **Project Completion:** 2001

Background and Aim
National laws, international guidelines and conventions on minimal housing conditions for laboratory animals require that the environments of animals meet their physiological and behavioural needs, as well as ensuring their health.
The environments of laboratory animals are standardised in order to reduce variation in the results, especially in regulatory studies performed during the development and registration of pharmaceutical products and chemicals. It has been questioned whether these standardised laboratory environments promote the well-being of the animals. In 'enriched' environments, the animals can perform more natural, species-specific types of behaviour. Enrichment provides the animal with a more stimulating and responsive environment such as cage mates, nesting material, hiding places.Little is known about the effects of environmental changes on the biological variation in experimental results. It is postulated that animals from enriched environments can cope better with novel situations, and thus might offer more relevant experimental results with less biological variation. This project will measure the biological variation in mice in response to routine tests when housed in either standard or enriched environments.

Method and Results
Groups of mice housed under standard and ‘enriched' environments will be subjected to two standard drug development tests:
1) The tetanus toxoid vaccine potency test (a serological method) from the National Institute of Public Health and the Environment (RIVM) and
2) The black and white box test for psychopharmaceuticals. This latter test consists of a black and white compartment. Mice are placed in one of the compartments and the latency and number of entries to the other compartment are measured. This test assesses the effects of drugs on anxiety levels in mice. It can also be used to investigate whether the different housing conditions influence anxiety levels. An anxiolytic drug (Diazepam) will be given to the mice to see whether the enriched housing conditions influence the behavioural effects of this drug. Enrichment will consist of tissues as nesting material, a next box, climbing grid, woodwool, tube (super enriched) or only tissues (enriched). Standard cages are provided with sawdust only.
In each case the biological variation in the results will be compared in the two groups of mice housed under different conditions. Data will also be compared with historical controls.
Expected results *(work in progress)*:Data from previous studies suggest that the effects of enrichment depend on the degree of enrichment and on the mousestrain studied. With regard to the variation in results, data from pilot studies suggest that this may depend on the parameter studied (e.g. physiological or behavioural).

Conclusions and Relevance for 3R
Housing in an enriched environment promotes the welfare of laboratory mice. If housing enrichment has no, or only minor, deleterious effects on the biological variation in results, there is no reason to deny laboratory mice a more natural environment. Moreover, if effects of enrichment lead to less variation in results, the number of animals used per experiment can be reduced. The results of this study will be brought to the attention of regulatory agencies with the aim of influencing future legislation on the housing of laboratory mice.

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3R-Project 67-99

Human monocyte-derived dendritic cells as in vitro indicators for contact allergic potential of chemicals

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Duration: 2 years **Project Completion:** 2003

Background and Aim
The only tests for contact allergic potential that are currently accepted by regulatory agencies are in vivo tests. The introduction of new methods (for example, the Murine Local Lymph Node Assay) has contributed to a reduction in both the number of animals used and in the stress experienced by the animals. The development and validation of in vitro methods using human material could lead to the replacement of animal tests entirely in the evaluation of the contact allergic potential of chemicals.
Human dendritic cells form an excellent basis for in vitro test systems since they play a pivotal role in the induction of contact allergy. In this project the reactions induced by contact allergens in human monocyte-derived dendritic cells (M-DC) will be investigated with the goal of developing a routine testing protocol.

Method and Results
The technology to culture dendritic cells from human blood monocytes has recently become available. Preliminary results from our laboratory indicate that human monocyte-derived dendritic cell technology has the potential to become a standard tool in contact allergy screening (Coutant et al., Toxicological Sciences, 1999). However, considerable work is still necessary to clarify the cellular, subcellular and biochemical events in M-DC following treatment with contact allergens. Within the ongoing project we refined the protocol for the generation of DC from human blood monocytes. CD86 up-regulation was used as a marker for M-DC activation. The M-DC were exposed to a dilution series of contact allergens or irritants in order to find the ranges necessary to produce a a concentration dependent up-regualtion of CD86. With our method we were able to distinguish between contact allergens and irritants in their mode of M-DC activation: contact allergens were able to activate the M-DC in the sub-toxic range, whereas irritants only activated M-DC at cytotoxic concentrations. For the screening method, both concentration ranges will be tested, and the measurement of activation markers and markers of cytotoxicity will be carried out in parallel. In addition, using genomic methods, we will search for a yet more sensitive marker to discriminate between contact allergens and irritants.

Conclusions and Relevance for 3R
Understanding the different effects of contact allergens and irritants on M-DC at the sub-cellular and molecular level is essential to provide the scientific basis for a standard in vitro testing protocol with human monocyte-derived dendritic cells. This project will deliver this background information. New markers that can discriminate even more sensitively between the mechanisms of M-DC activation by irritants and contact allergens may be found by new genomic methods. These will have to be validated using a large set of control substances.

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3R-Project 68-99

Genetic analysis of a 3D *in vitro* model of the rheumatic pannus tissue

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Duration: 1 year **Project Completion:** 2002

Background and Aim
Rheumatoid arthritis is a chronic degenerative disease of unknown etiology that affects about 1-2 % of the human population. Up until now no cure for the disease has been found; present treatments can only slow down the process of joint destruction. Crucial for the destructive joint processes is the de novo formation of pannus tissue. Due to the complexity of the disease, a wide variety of animal models has been developed to investigate the etiology and mechanisms in rheumatoid arthritis resulting in the development of new treatments of the disease.
Using single cell suspensions from synovial fluid cells of arthritis patients, we observed differentiation of three-dimensional tissues in vitro. This new model of pannus-like tissue (PLT) might be useful to study pannus tissue formation and differentiation. In the PLT cultures, we observed two cell types, fibroblast-like and macrophage-like cells, defined by their distinct morphology and major histocompatibility complex (MHC) by human leukocyte antigen (HLA) class II expression. We could discriminate several intermediate steps of differentiation which finally led to 3D villi-like structures. Secretion of interferon gamma, interleukin-10, and tumor necrosis factor alpha was measured in the culture supernatants.
Using methotrexate at various concentrations, the growth of PLT could be inhibited. We describe definite intermediate steps of differentiation. The present approach could be a suitable model for the in vitro study of pannus tissue formation.

Method and Results
Preparation of cells:
Synovial fluid cells (SFC) were obtained from patients with reactive and rheumatoid arthritis treated at local hospitals and rheumatology practices. In all instances, synovial tapping was therapeutically indicated and patients gave their informed consent. The synovial fluid samples were diluted 1:2 in phosphate-buffered solution (PBS), and mononuclear cells were depleted by Ficoll density gradient centrifugation. The pelleted cells were isolated, washed, and counted.

Tissue culture and cell lines:
Synovial fluid cells were taken into tissue culture at 106 cells/ml in standard tissue culture medium. Iscove's modified Dulbecco's or Roswell Park Memorial Institute 1640 medium were supplemented with antibiotics, glutamine, and 10% fetal calf serum. The cell lines were maintained in the same medium. K4 cells are SV40-transformed synovial fibroblast [16], U937, and MonoMac 6 are monocytic cell lines (ATCC, Rockwell, USA). Methotrexate was used at the indicated concentration.

Immunohistochemistry:
For immunohistochemistry, cells were grown on glass slides. Tissues were cut out of the culture using Pasteur glass pipettes and flash frozen in liquid nitrogen. Five-micrometer sections were used for staining. Cells/tissues were fixed in 100% methanol at -20°C for 60 min and air-dried. Endogenous peroxidase activity was minimized using 0.1% phenylhydrazin-hydrochloride with PBS. Then cells were blocked with PBS and 1% bovine serum albumin (BSA) for 60 min. Primary staining was done with the monoclonal antibody (mAB) L243 coupled to biotin for 20 min at room temperature. After washing in PBS and 0.05% Tween 20, cells/tissue were blocked with PBS and 1% BSA, and streptavidin-horseradish peroxidase oxidase was applied. Staining was done with amino-9-ethylcarbazol (Sigma A5754) in dimethyl formamide. For control, cells were incubated with antimouse mABs conjugated to biotin. Counterstaining was done with hematoxylin.

Cytokine enzyme-linked immunosorbent assays:
Sandwich enzyme-linked immunosorbent assays (ELISAs) were performed according to standard protocols. The following antibodies were used for the detection of cytokines: 9D7- and 12G8-biotin for IL-10, H34A- and H33-biotin for

tumor necrosis factor alpha (TNFalpha), and M700AE- and M701B-biotin for interferon gamma (IFN-gamma). Recombinant cytokines were used to generate standard curves. Unlabeled antibodies were used for coating and biotinylated antibodies for detection. All reagents were purchased from Endogen or Pharmingen (Heidelberg, Germany).

Conclusions and Relevance for 3R

The PLT developed from single cell suspensions derived from synovial fluids. The development occurred in several steps toward 3D structures which were reminiscent of the pannus tissue's villi. In cultures in which only the growth of fibroblast-like cells without attached macrophage-like cells was observed, no development towards PLT occurred. Though it is reported in the literature that synovial cells tend to grow without contact inhibition, in our system's limited association of macrophage-like cells with fibroblast-like cells, we observed no 3D growth. This phenotype was also found in the methotrexate-treated cultures. We therefore conclude that interactions of both cell types are necessary for the formation of PLT. Development and growth of cells was only observed in the initial phase up to 4 weeks, followed by growth retardation. At this point the project had to be interrupted because of the lack of synovial punctures, due to the introduction of significantly better therapies for patients suffering from Rheumatoid Arthritis (eg high dose methotrexate, anti TNF-alpha antibodies). The advanced therapies resulted in less punctures, a dramatically decreased volume of the punctures and bad conditions of the synovial fluid cells. However, using methotrexat, we could give the proof of principle that the in vitro pannus-like tissue model might be able to replace animal models.

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3R-Project 69-99

A human neural cell line as *in vitro* model system for the excitotoxic cascade during stroke

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Keywords: *human*; *brain*; *pharmacology*; *ischemia*; *cell cultures: organ-specific*; *reduction; replacement*; *pharmacological testing*

Duration: 2 years **Project Completion:** 2002

Background and Aim
Stroke is the third leading cause of death and an important cause of adult disability in industrialized countries. Most strokes are caused by an acute interruption of the brain's arterial blood supply, which leads to tissue ischaemia. Ischaemic neurons deprived of oxygen and glucose rapidly lose ATP and become depolarized. The resultant complex ischaemic cascade triggers an excessive release of the excitotoxic transmitter glutamate; this substance causes changes in intracellular calcium homeostasis and results in the production of free radicals. Apart from immediate damage, the ischaemic cascade results in a wave of delayed cell death. The latter process might be prevented by administering neuroprotective substances that are active at the various stages of the ischaemic cascade.
Currently, investigations on the cellular processes occurring in ischaemic nervous tissue and screening tests for neuroprotective compounds are carried out mainly in rodents. In order to minimize the use of experimental animals, the aim of this project is to establish a human nerve cell culture system as an alternative model.

Method and Results
NT2 cells are a clonal human teratocarcinoma cell line that yield terminally differentiated neurons after treatment with retinoic acid (Pleasure et al., 1992). We investigated a cell culture system in which the NT2 neurons are stimulated to differentiate in the presence of co-cultured glial cells, obtained from the same cell line. In addition, we have established a method for the rapid differentiation of NT2 neurons using an additional cell aggregation step. Using a variety of antibodies against neural markers, terminal differentiation to the functional neuronal phenotype can be shown. After several weeks in culture, terminally diffrentiated NT-2 neurons are subjected to anoxic and/or hypoglycemic conditions and the resultant changes in neuronal viability monitored. The influence of additional parameters on neuronal survival, such as type of culture medium, pH, glutamate concentration, and specific neuroactive drugs are presently being evaluated.
When the neurons have end-differentiated, ischaemic conditions will be simulated and the resultant changes in neuronal viability monitored. We will analyze, how pharmacological agents that rescue the ischaemic neurons from cell death, regulate cytosolic calcium levels and mitochondrial potential.

Conclusions and Relevance for 3R
This human cell culture system will allow ischaemic processes and the complex cellular mechanisms involved in ischaemic cell death to be investigated in vitro. Furthermore, it will be possible to use this model to screen potential neuroprotective substances for their ability to facilitate neuronal regeneration and repair. This model will help to reduce the number of experimental animals needed in pre-clinical drug testing and the development of neuroprotective treatment strategies.

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3R-Project 70-99

Sensory irritant screening using a stable cell line expressing the vanilloid receptor

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Keywords: *lung*; *sensory irritation*; *cell cultures: transgenic*; *reduction; replacement*; *toxicity testing: sensory irritants*

Duration: 2 years **Project Completion:** 2003

Background and Aim
Sensory irritation (stinging or burning sensations) caused by chemical agents is commonly measured in mice using the reflexive decrease in respiratory rate when the animals inhale the irritant. Studies in mice indicate that for many chemicals the sensory irritation is a process that is mediated by a membrane receptor protein, the so-called "sensory irritant receptor." This “sensory irritant receptor” is thought to be activated by a number of different sensory irritants, including capsaicin. A capsaicin receptor, the vanilloid receptor 1 (VR1), has recently been cloned. In this project, VR1 will be incorporated into a self-propagating cell line. The aim is to develop the first animal-free assay to assess the sensory aspect of an exposure to an irritant chemical.

Method and Results
The VR1 receptor was stably transfected into a cell line that does not constitutively express VR1. The VR1 receptor is a non-selective cation channel. Therefore activation of the receptor/ion channel can be monitored by measuring the increase in intracellular Ca^{2+}concentration. Changes in cytosolic calcium levels will be monitored with optical imaging (Fura-2). A number of sensory irritant chemicals will be tested using capsaicin as a positive control and capsazepine as a competitive antagonist. We will evaluate whether sensory irritation by known sensory irritants is entirely attributable to the activation of VR1 and whether the sensory irritants use the same binding sites as capsaicin. Finally, the sensitivity and specificity of the bioassay will be evaluated.

Conclusions and Relevance for 3R
Alternative methods already exist for the cytotoxic or tissue damaging activity of chemical irritants and for the ciliotoxic and ciliostatic potential of chemical additives. However, no in vitro method is yet available to detect the sensory irritant properties of chemicals. In the proposed cell culture model, the results will be scored with optical measurement systems amenable to a high level of automation. If the present project is successful, it may obviate the need to use animals for sensory irritant screening and contribute to the refinement of the existing mouse inhalation assay.

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3R-Project 71-00

Detection and characterisation of signs of pain in mice by the combined use of a telemetry model and behavioural observations

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Keywords: *mice*; *analgesia; ethology: laboratory animals*; *telemetry*; *refinement*

Duration: 3 years **Project Completion:** 2005

Background and Aim
Pain in animals is commonly assessed by observing behavioural aberrations. The diagnosis of pain can be confirmed by measuring physiological parameters (heart rate, respiration rate, bodily tension). An evaluation of pain is feasible in animals such as monkeys, dogs, cats and pigs, because these species show clear behavioural signs of pain.
In contrast, mice live in constant fear of falling prey to their enemies thus, the intention is probably to show as few signs of disease, suffering or weakness as possible. Accordingly, during animal experiments, or even when a person is simply present in the room, the mouse will hide almost all signs of slight or intermediate pain. Additionally, intrinsic analgetic systems that are well developed in mice for protecting them from intolerable fear and pain may influence their responses and make their pain less recognisable.
In consequence, there are no reliable indicators to detect low and intermediate pain in the mouse. Only when an animal is near death can we see that it is suffering pain. As a result, demands for adequate pain therapy are often ignored, or met with the anthropomorphic atti-tude "as long as the mouse shows no pain, it must feel no pain". Another consequence of the dilemma is that the success of a pain therapy is not visible and therefore doubts about the efficacy of many analgesic regimes came up - which were again counteracted by those, who claim, that every suspicious manipulation (as little as it might be) must be treated by a standardized pain therapy in mice even if this never would be done in other species or in man.

The aim of the project is to identify indicators for low or intermediate pain in mice using a combined analysis of physiological and behavioural parameters. This goal is becoming in-creasingly urgent, since, due to the increasing popularity with the advent of genetically modi-fied mice lines, this species is being used in increasingly complex biomedical models and procedures (e.g. the induction of stroke or ischemic heart disease, organ transplants, et al.).

Method and Results
Measurements of physiology and behaviour must be free of artefacts caused by external in-fluences such as the presence of the investigator. Therefore, the physiological data were collected by a telemetric system. For this, mice were implanted with transmitters able to measure ECG, body temperature and activity on a permanent basis. The transmitters are small enough not to be noticeable by the animal. This system allows data to be collected in the conscious mouse without the presence of the investigator in the room. Monitoring the animal over a number of days documents the time course of body core temperature, locomo-tion and heart rate/ECG. From this, the circadian rhythm of these parameters are drawn and heart rate variability and ECG wave forms can be analysed.

Aberrant circadian rhythms are indicative for disturbances in the animal’s welfare, whereas the elevation of heart rate and certain changes of heart rate variability and ECG wave forms are signs of pain, stress or an activation of the sympathetic nervous system. Data are com-pared with the daily food and water consumption and the body weight progression because these parameters are widely used clinical investigations for a rough assessment of the physi-cal condition as well as the aspects of the mouse`s welfare in biomedical experiments.

After instrumentation and recovery, mice underwent a defined surgical intervention to induce a stage of postoperative pain that is suggested to be of low to intermediate graduation. Pain was either untreated or treated with two different widely used analgetic regimes.
A few hours after the operation all animals, whether treated with analgetics or not, exhibited not overt signs of pain: their

fur and wounds were cleaned and the animals ate, drank and showed the spectrum of their normal behaviour in their home cage, such as walking around, climbing underneath the cage lid, digging or nest building activities.
The telemetrically measured locomotion activity counts are nearly unchanged indicating that the motorical behaviour is not depressed or even increased (e.g. restlessness), which con-firms the assumption of slight pain and not that animals suffered from severe or intolerable pain. Body core temperature showed only a marginal increase, suggesting, that post-surgical inflammation was of no influence.
Only untreated animals showed significant post-operative changes of the heart's actions (heart rate, heart rate variability, ECG wave forms) which suggests pain and sympathetic activation.
From the clinical parameters, the food intake was significantly diminished after surgery in untreated animals; body weight was only slightly reduced, also showing, that animals did not suffer from serious or long-lasting pain.
The analgetic regimes both were able to prevent all changes in cardial action. Post-operative food intake and body weight also remained constant when animals were under post-operative pain therapy.
Control experiments confirmed, that the anaesthetic and analgetic substances had no effect on the measured parameters per se.

The results show, that
i.) the model was able to identify and objectify signs of low to inter-mediate pain in mice, which could not be clearly detected otherwise in this species
ii.) analgetic regimes acted successfully in relief of low to intermediate post-surgical pain in mice.

Behavioural observations, data and score sheets are under investigation.

In the future, the model will be used for the assessment of pain in mice in frequently used, standardized biomedical experiments (e.g. antibody production), particularly for methods, in which the degree of pain and the benefit of pain therapy is a matter of diversity.
Simultaneously, the behavioural studies will go on with the aim to develop a score sheet for the detection of pain under routine laboratory conditions.

Conclusions and Relevance for 3R
The model will be a valuable tool for
i) objective classification of the degree of pain,
ii) pre-estimating the necessity of pain therapy in commonly used biomedical methods and
iii) the proof of the efficacy of various analgetic regimes used on mice.
The results will have a great impact on the refinement of animal experiments.

Referenc
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3R-Project 72-00

Development and applications of an *in vitro* cultivation model for *Neospora caninum* tissue cyst formation

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Keywords: *veterinary drugs; veterinary drugs*; *neosporosis*; *cell cultures: parasites*; *reduction; replacement*; *diagnostic approaches: parasites; diagnostic approaches: vaccines; veterinary drugs*

Duration: 3 years **Project Completion:** 2003

Background and Aim
Neospora caninum is an obligatory intracellular apicomplexan parasite which causes abortion predominantly in cattle and neuromuscular disorders in dogs (Hemphill and Gottstein, 2000). The parasite has a life cycle composed of three distinct stages, namely the asexual tachyzoite and bradyzoite forms, and the sexually produced sporozoites. Tachyzoites are virulent and transmitted from the mother to the fetus. Bradyocytes form tissue cysts that are orally infective. They can transform in their hosts into the tachyzoite stage. Tachyzoites can be cultivated in vitro in the presence of various cell lines (Hemphill, 1999). Knowledge on suitable culture conditions for the other life cycle stages, however, is rather limited. The aim of this project is to develop an *in vitro* cultivation system for generating *N. caninum* tissue cysts containing the respective bradyzoite stage. This will eliminate the need of animals for generating bradyocytes that can be used for e.g. primary drug screening experiments, the search for immunodominant antigens or studies on the basic cell biology of the parasite.

Method and Results
Cell culture derived *N. caninum* tachyzoites are grown for several cycles of infection within Vero cells. Parasites are isolated and monolayers of murine epidermal keratinocytes are infected. Infected cultures are then maintained in serum-free medium in the presence of 70 mM sodium nitroprusside for several days, and the conversion of the tachyzoites to bradyzoites is monitored by immunofluorescence, employing bradyzoite-specific antibodies. Quantitative Neospora-PCR revealed that nitroprusside treatment resulted in a distinct inhibition of parasite proliferation. After 8 days in culture, >60% of parasitophorous vacuoles had converted from the actively proliferating tachyzoite phase and were now expressing the bradyzoite antigen BAG1 and the cyst wall antigen CC2. Electron microscopical and biochemical analysis of these in vitro generated tissue cyst-like structures confirmed that stage conversion has taken place. This methodology is now applied to generate long term bradyzoite in vitro cultures, and the culture system has been modified for the use of Vero host cells. This allows for production and purification of larger numbers of *N. caninum* bradyzoites. Bradyzoite-infected monolayer cultures are currently evaluated for their use a a primary drug screening model. The efficacy of these drugs and possible bradyzoite-tachyzoite stage reconversion effects are monitored microscopically by immunocytochemistry, PCR and electron microscopy .

Conclusions and Relevance for 3R
An in vitro cultivation system for generating *N. caninum* tissue cysts harboring the bradyzoite stage of the parasite will reduce the number of laboratory animals currently used for this purpose. It will give researchers the opportunity of performing primary drug screening studies and other research on the general cell biology of *N. caninum* without the use of animals.

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3R-Project 73-00

Generation and functional characterization of a clonal murine periportal Kupffer cell line from H-2K^{b} -tsA58 mice

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Keywords: *mice; transgenic mice*; *kupffer cells; liver*; *phagocytosis*; *inflammation; septic shock models*; *cell cultures: organ-specific*; *reduction*

Duration: 1 year **Project Completion:** 2002

Background and Aim
Hepatic macrophages (Kupffer cells) are an important cell group because they offer the first line of defence in sepsis. These cells are difficult to study, however, because they survive and maintain their function only for a short time in vitro. Therefore, we established an immortal Kupffer cell line, which would allow the molecular analysis of Kupffer cell function, in particular the study of the interaction between Kupffer cells and bacteria, in vitro

Method and Results
Kupffer cells were isolated from H-2K^{b}-tsA58 transgenic mice. Four cell lines were cultured at 33ºC in a medium containing interferon-gamma and the supernatants from cell lines of hepatocyte and endothelial cell cultures. The line KC13 was cloned (KC13-2). It grew stably at 37ºC without interferon-gamma. Its phenotype and function were further studied.
In contrast to primary cells, the cells of the clone were uniform, survived detachment and could therefore be analyzed by cytofluorimetry. The cloned cells expressed many enzymes and markers that are typical of native Kupffer cells (non-specific esterase, peroxidase, MOMA-2, BM8, scavenger receptor A, CD14 and Toll-Like-Receptor 4 (TLR4), the antigen-presenting molecules CD40, CD80 and CD1d, and endocytosed Dextran-FITC. It lacked complement, Fc-receptors, F4/80 marker and the phagosomal coat protein TACO) (Figure 1). The clone exhibited CD14- and TLR4/MD2-independent, plasma-dependent lipopolysaccharide (LPS) binding, *E. coli* and *S. pneumoniae* phagocytosis and LPS- and IFN-gamma-induced NO production, but no TNF-alpha, , IL-6 or IL-10 release. The clone differed from peritoneal macrophages by the presence of CD1d and the absence of TACO expression and by the fact that LPS-induced functions were independent of CD14 and TLR4.

Conclusions and Relevance for 3R
Conclusions:
We succeeded for the first time in generating a stable, clonal Kupffer cell line.
The large size of the cells, their pattern of surface marker expression and their capacity to clear Gram-negative and -positive bacteria indicate that the clone was derived from the periportal large Kupffer cell subpopulation. Hepatocyte- and endothelial cell-derived factors were required to maintain the differentiated phenotype in the Kupffer cell clone, suggesting that a continuous stimulation is required to keep a differentiation state in this cell type. This property may offer the possibility of identifying which factors modulate gene and protein expression in Kupffer cells and to study interactions with other liver or blood cells.
Relevance:
The set of antigen presenting molecules in the clone allows the consequences of presenting antigens to MHC- and CD1-restricted T cells to be studied. *In vitro* studies on the consequences of antigen presentation (for example, T cell apoptosis) will also be possible. Moreover, the study of virus or parasite entry into Kupffer cells and the immunological response can be followed in the clone (Figure 2). The clone can be transfected and used to analyze signaling in Kupffer cells after stimulation with bacteria or bacterial products. In summary, the clone allows molecular studies of antiinfective and immune functions of Kupffer cells. It will reduce and replace studies with primary Kupffer cells obtained from mice.

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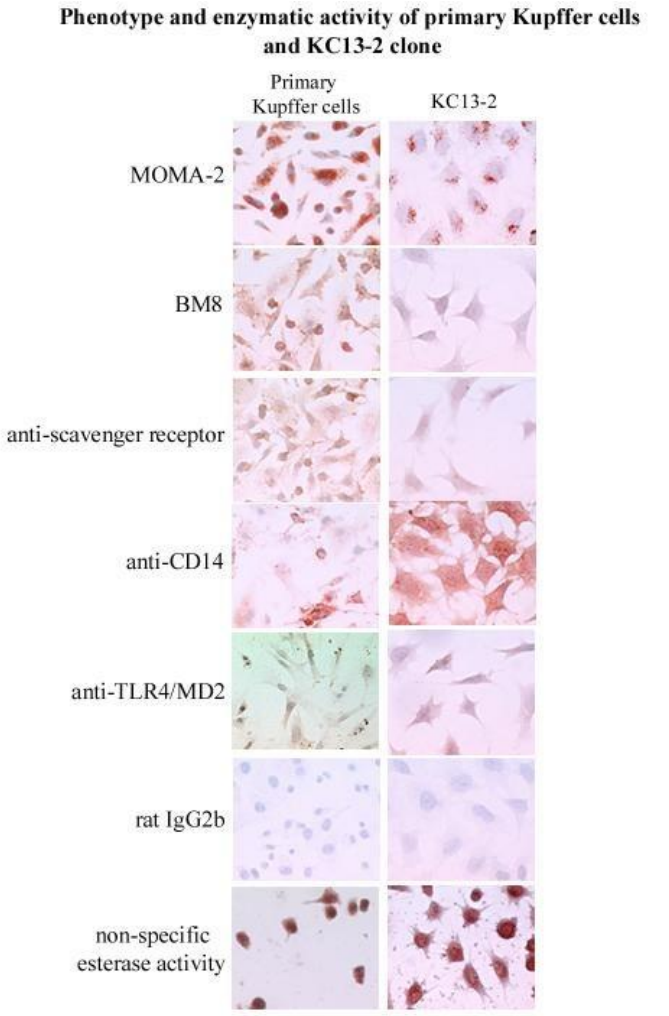

Figure 1

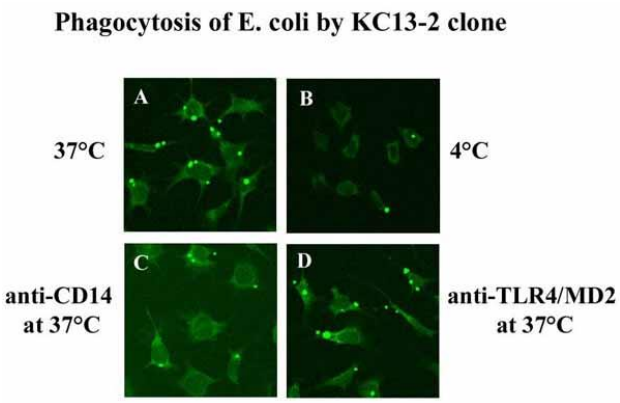

Figure 2

3R-Project 74-00

Validation of polymerase chain reaction assays as replacement of the Mouse Antibody Production (MAP) Test

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Keywords: *mice; viruses*; *molecular biology: pcr*; *reduction; replacement*; *antibody production test; diagnostic approaches: viruses*

Duration: 1 year **Project Completion:** 2002

Background and Aim
Rodent viruses may be transmitted through contaminated biological materials such as transplantable tumors, cell lines, etc. Such inadvertent transmission may cause endemic infections in the recipient colony, resulting in clinical disease and/or compromised research results. To avoid this, biological material must be screened for pathogens before it is introduced into rodents. This is currently done using the mouse antibody production (MAP) or the rat antibody production (RAP) test. In a previous project (3R Project 45-96, Homberger et al.), we established alternative assays using PCR technology to detect these viruses directly in the biological material. We were able to show that the complete spectrum of agents detectable by the traditional MAP/RAP test could be covered by these new assays. Since then, over 50 cell lines and tumour samples have been tested. Having demonstrated their potential, the aim of this study is to validate these PCR assays and compare them directly to the MAP/RAP tests (Kraft 1996, Rehbinder et al. 1996).

Method and Results
The validation was carried out using 1) routine samples submitted to our diagnostic lab for MAP-testing as well as 2) experimentally spiked samples. All virus strains (described in project 45-96) were cultured under their specific optimal growth conditions. The virus material was titrated to a concentration of between 10^3 and 10^{-3} infectious particles (IP/ml) and injected into mice (MAP-test) and collected for DNA/RNA-extraction (PCR) at all dilutions. The limits of detection (sensitivity) of the two techniques were compared.
For 6/16 viruses, the conventional PCR technique seems to be more sensitive and more specific in detecting murine viruses. In 12/14 viruses, the. RT-PCR (Real-Time PCR) is more sensitive than the MAP-test. In 2/14 cases all three detection methods have the same sensitivity. Furthermore, the PCR technology had a much shorter turnover time (two days) than the MAP test (one month to obtain seroconversion plus a few days for the serology results).

Conclusions and Relevance for 3R
In the past, the MAP-test was shown to be more sensitive than the direct isolation of virus particles grown in cell culture (de Souza & Smith 1989). Our study affirms that with PCR technology, the sensitivity of in vitro techniques can be sufficiently improved as to provide a viable alternative to the in vivo MAP-tests. The PCR-based technology was able to detect all viruses found in the context of the MAP-test, although the specific sensitivity pattern was somewhat different. The advantages of the PCR technique are its speed (2 days vs. 30 days for the MAP-test) and its contribution to the principles of 3R as a replacement technique. Currently, between 10 and 50 animals are used per MAP-test. The final results of this project will show whether the in vivo test can be completely replaced by the PCR technique. A final contribution to the principles of 3R: healthy, standardised, pathogen-free laboratory animals are a prerequisite in achieving reproducible animal experiments. With an effective in vitro method to monitor the pathogen free status of laboratory rodents, the number of animals used in in vivo experiments can also be reduced (Rehbinder et al. 1996).

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3R-Project 75-00

Internet laboratory for predicting harmful effects triggered by drugs and chemicals

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Keywords: *drug-design; pharmacology; qsar*; *in silico*; *reduction; replacement*; *toxicity testing: pharmaceuticals; toxicity testing: receptor mediated; toxicity testing: xenobiotics*

Duration: 1 year **Project Completion:** 2003

Background and Aim
We have created three-dimensional surrogatevirtual models of cellular receptors involved in chemically induced toxicity. The simulation uses a 4D/5D-QSAR concept developed at our laboratory (Vedani et al. 2000; Vedani and Dobler 2000). This model allows various chemical details and technical aspects of receptor – ligand interactions to be examined: multiple ligand presentation, induced fit, H-bond flip-flop and dynamic cavity shaping. As an example, we created a virtual Aryl hydrocarbon receptor (Ah receptor; this receptor binds dibenzodioxins, dibenzofurans, biphenyls, and polyaromatic hydrocarbons), and used a total of 121 binding compounds to calibrate the model and examine its behaviour. Our results yielded a cross-validated r^2 of 0.851 and a predictive r^2 of 0.792 (Fig.1). None of the compounds in either the training or the test set predicted false positive or false negative results (Vedani and Dobler 2001).
It is our objective to establish a virtual laboratory on the Internet to allow the harmful effects triggered by drugs, chemicals and their metabolites to be estimated in a virtual environment.

Method and Results
Free access to this virtual laboratory will allow any interested party to estimate the toxic potential of a given substance, even before the compound has been synthesised! This will be done by first modelling the three-dimensional structure of the substance and its possible metabolites in the computer, and then calculating the binding affinity of these compounds towards each receptor modelled in the database.
Testing a new compound for harmful effects involves the following steps: 1. Downloading the 2D/3D data of the substance on our server (or a local mirror) via an Internet protocol. 2. Automated generation of the structures of potential metabolites. 3. Identifying the most likely (i.e energetically favourable) 3D structural conformations for the parent compound and the metabolites. 4. Identifying likely positions and orientations of the parent compound and metabolites in interaction with every receptor in the database (using a Monte-Carlo search procedure). 5. Estimating the harmful potential of a substance by calculating its binding affinity and that of all metabolites towards each receptor.
Only those compounds and metabolites that pass through this virtual screening test with binding constants weaker than a threshold value (e.g. K> 50 mM) may be considered “safe” and cleared for further studies, including animal tests.
Presently, our database includes validated models for the Ah receptor (receptor-mediated toxicity), the 5HT-2A receptor (hallucinogenic activity), the cannabinoid receptor (psychotropic effects), the GABA-A receptor (receptor-mediated toxicity), and the steroid receptor (various undesired effects). The database will be continuously extended to include models of further receptors known or presumed to mediate toxicity or other harmful effects (Fig.2).
The following steps are necessary to add a new receptor to the database: 1. Selecting the training and test sets of ligands for which experimental binding data is available. 2. Generating all 3-D structures. 3. Conformational search. 4. Generating and validating the new receptor model. 5. Adding the model into the data base and external testing.
A noteworthy feature of this system is that the classified or proprietary data is used only for model construction but not for testing and validation, i.e. contributions from the pharmaceutical industry can be generated at their laboratories using a local mirror of the database.

Conclusions and Relevance for 3R
The proposed Internet laboratory contributes to two aspects of the 3R principle: Firstly, it allows potentially harmful substances, e.g. potential drug candidates, to be recognised in an early phase of development, before the compounds are synthesised or tested in preclinical trials including animal tests. In the case of industrial chemicals, e.g. chemicals that entered the EU market before 1981, the database will also contribute to the identification of harmful substances already

on the market. Secondly, the availability of the database should help to prevent redundancies in the testing of potential drugs or chemicals with identical or closely related biomedical targets.

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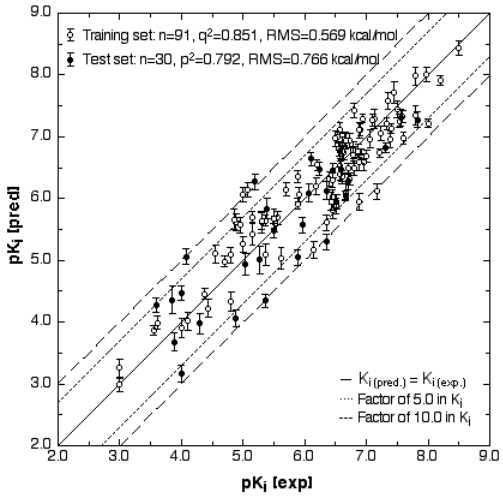

Figure 1

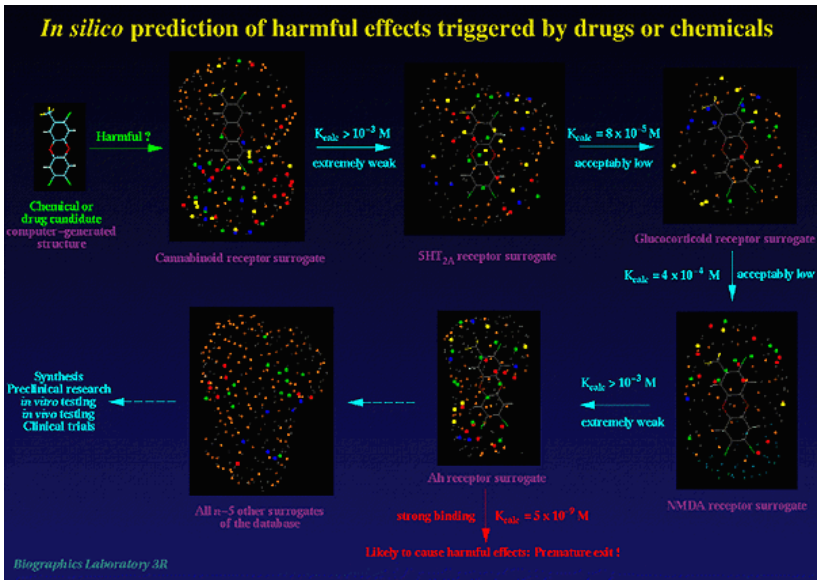

Figure 2

3R-Project 76-01

Development of a model of heart angiogenesis *in vitro*

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Keywords: *mice; rat*; *heart*; *angiogenesis*; *angiogenesis; ischemia*; *explants*; *reduction; replacement*

Duration: 2 years **Project Completion:** 2003

Background and Aim

Angiogenesis is a process of creating new arteries, veins and capillaries in order to increase the blood supply within an organ or tissue. It occurs for example in response to signals from an organ or tissue that is insufficient oxygenised. In the heart, angiogenesis occurs in response to acute or chronic blockages (occlusions) of coronary arteries. These occlusions induce a state of hypoxia known as myocardial ischemia.

Patients with coronary heart disease often have a better clinical outcome if angiogenesis can be stimulated and the collateral blood circulation within the heart can be improved. Because coronary heart disease is very common in humans, substantial research resources are currently being invested into studying the therapeutic stimulation of angiogenesis in the heart.

A growing number of experiments dealing with angiogenesis in the heart are performed *in vivo*. Current animal models of angiogenesis *in vivo* often use complete blockage by ligation or a gradual reduction of blood flow in a major coronary artery to induce hypoxia-driven angiogenesis. Many animals die due to myocardial infarction or cardiac rhythm disturbances during these sometimes cumbersome surgical procedures. Furthermore, myocardial ischemia is often quite a painful condition for the animals. Currently, no appropriate assay is available to assess angiogenesis in the heart *in vitro*. The aim of this study is to develop an *in vitro* assay of angiogenesis in the heart.

Method and Results

The present *in vitro* model uses small explants of hearts from laboratory animals killed for other purposes (mice, rats). The explants (~ 1 mm^3) are cultivated in fibrin gels for 10 days under different physiological conditions (e.g. normoxia and hypoxia) in standard 48-well cell culture plates. One single heart from an adult rat or mouse suffices to perform 24 different experiments in octuplicates. Evaluation of in vitro angiogenesis of the heart occurs after 10 day of cultivation. Angiogenesis is represented by sprout formation by endothelial cells growing out of the piece of heart in different shapes and forms. Sprout formation is analyzed morphometrically and cells forming the sprouts can be characterized. Characterization of outgrowing cells is performed by different methods: (i) by subcultivation of outgrowing cells with subsequent immunohistochemistry, (ii) by direct immunohistochemistry in the fibrin gel, (iii) by cryostat sections of the tissue explant embedded into the fibrin gel, or (iiii) by electron microscopy.

For validation of the *in vitro* assay we have investigated different physiological conditions (e.g. normoxia and hypoxia) and frequently used angiogenic stimuli (vascular endothelial growth factor: VEGF, basic fibroblast growth factor: bFGF and platelet-derived growth factor: PDGF). We found that hypoxia (3% O_2) is a prerequisite for angiogenesis *in vitro* to occur in adult heart and that the growth factors amplify and modify basal in vitro angiogenesis induced by hypoxia. Amongst the tested growth factor PDGF-BB displayed the strongest potential to induce in vitro sprouting, albeit in a disorganized form. VEGF, on the other hand, induces elongated unbranched capillary-like sprouts. By the use of pharmaceutical inhibitors we were able to investigate mechanisms leading to PDGF-BB-mediated heart angiogenesis. Thereby we found that PDGF-mediated *in vitro* heart angiogenesis under hypoxia is mediated via a mTOR dependent process.

In further validation of the assay, we also found that *in vitro* angiogenesis of the heart is more restricted with increasing age. In hearts of adolescent mice younger than 8 weeks, angiogenesis occurs faster and requires less nutrients in the microenvironment. Adolescent mice show *in vitro* angiogenesis under serum free and normoxic (21% O_2) culture conditions. In contrast, adult mice hearts (older than 12 weeks) require serum-enriched medium and hypoxic growth conditions (3% O_2). However, *in vitro* angiogenesis in adolescent and adult 12-week-old mice develops to similar degrees if adequate culture conditions are chosen. As age increases further (hearts of 20-week-old mice) the potential for sprout formation decreases compared to 12-week-old mice. Thus, angiogenesis appears to be more restricted with aging in our *in vitro* assay of the heart, very similar to corresponding observations *in vivo*.

Conclusions and Relevance for 3R

The heart angiogenesis *in vitro* model allows to assess angiogenesis in hearts of mice and rats. Both, induction and repression of angiogenesis can be investigated. The model allows rapid screening of all sorts of pharmacological compounds by using a small number of animals only. Thus, this model will reduce and partially replace the use of animals in the investigation of angiogenesis of the heart. The flexibility in the selection of experimental conditions and protocols will allow new compounds to be screened for their effects on angiogenesis in the heart.

Reference

Abstract:
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3R-Project 77-01

Reproducibility and external validity of findings from animal experiments: influence of housing conditions

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Keywords: *mice*; *ethology: laboratory animals*; *reduction; refinement*; *standardization*

Duration: 1 year **Project Completion:** 2004

Background and Aim
Increasingly precise molecular genetic and pharmacological tools are available to study the cellular mechanisms underlying complex brain functions in mice. Because the brain is ultimately responsible for the generation of adaptive behaviour, brain functions need to be defined in terms of behaviour. Thus, behavioural phenotyping following neural manipulation plays a crucial role in the study of brain functions. However, minor environmental differences between laboratories or between replicate studies within a single laboratory may cause fundamental differences in the behavioural expression of neural manipulations. Thus, behavioural phenotypes may be idiosyncratic to particular studies and, therefore, be of limited scientific validity. Growing evidence suggests that poor housing conditions might play a role here, since they seem to enhance sensitivity to environmental fluctuations that resist standardization (see also 3R-Project 66-99). This project consists of a systematic assessment in mice to determine (i) the reproducibility of genetic differences in behaviour and underlying gene expression in independent replicate experiments both within and between laboratories and (ii) whether improved housing conditions reduce the risk of obtaining idiosyncratic results.

Method and Results
Two transgenic lines of mice - one with mild, the other with moderate neurological and behavioural changes compared to wild type controls - will be bred in a central breeding facility. After weaning at 21 days of age, samples of both male and female transgenic animals and wild type littermates will be distributed to three independent test laboratories, where they will be housed in groups of four in either barren standard laboratory cages or in large enriched environments. When the animals reach adulthood, they will be exposed to four consecutive behavioural tests (Open Field, 24h Object Recognition Memory, Elevated Plus Maze, Morris Water Maze) followed by post mortem analysis of gene expression using microarray techniques. Three independent replicates of the whole procedure from breeding to testing will be conducted. This will allow us to analyse the effect of enriched housing conditions on the reproducibility of behavioural phenotypes and gene expression across independent replicate studies both within and between laboratories.

Conclusions and Relevance for 3R
Due to the ability to generate mice overexpressing or underexpressing single gene products at will, the number of mice used in research is currently exploding - this in sharp contrast to the general decline in animal use over the past 20 years. Mice are, therefore, a major target for the reduction of animal use and refinement of experiments according to the 3R principles. The present project is the first to systematically examine the robustness of results from experiments with genetically modified mice in the face of minor environmental differences both within and between laboratories. Moreover, it examines the impact of housing conditions on the robustness of results. This will generate significant new knowledge to improve the scientific validity of such experiments. It may also serve to promote the establishment of housing conditions which better satisfy the animals' behavioural needs. Thus, the present project serves the refinement of animal experiments in the best of meanings of the 3R concept.

Congratulations:
The 3R Foundation Switzerland congratulates Prof. H. Würbel for his work which received the Animal Welfare Award 2005 from the Bundesland Hessen Germany on Okt. 14, 2005 (Hessischer Tierschutzpreis 2005). The laudation contains the following:
“With his work *Laboratory animal welfare: Cage enrichment and mouse behaviour* Prof. Würbel contributes to

improvements of animal husbandry in general and to the discussion about enriched housing conditions for rodents during animal experimentations. His work is a step toward the realization of these enrichments in the future".

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3R-Project 78-01

Validation of a combined perfusion/loading chamber for ex-vivo bone metabolic studies and bone-biomaterial interactions

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Keywords: *sheep*; *bone, cartilage*; *perfusion chamber*; *reduction; replacement*; *validation*

Duration: 3 years **Project Completion:** 2005

Background and Aim
To date most *in vitro* work on trabecular bone is performed with either cell monolayers or organ cultures. However, both of these techniques have limitations: cell monolayers lack some types of bone and marrow cells as well as the native three dimensional structure of bone, while organ cultures can only be sustained for 3-5 days before necrosis sets in, due to oxygen diffusion limitations.
Numerous studies are also carried out *in vivo* especially using sheep models. These also have their limitations, including both ethics & costs. To overcome these drawbacks, an ex vivo system for the long term culture of trabecular bone, was developed, “ZETOS”, (D. Jones, Marburg, Germany and E. Smith, Madison, Wisconsin, 1997). ZETOS maintains the three dimensional structures of trabecular bone, and can keep all cell types (osteoblasts, osteoclasts, osteocytes and marrow cells) viable for at least 30 days (1). ZETOS has a unique loading system that allows physiological levels of strain in bone samples to be measured online. With this model, the process of bone ingrowth onto biomaterial surfaces can be studied *ex vivo*.

The aims of the project are
1) To calibrate the loading system, so that the displacement measured on the loading device can be correlated to the actual displacement that the bone receives.
2) To initiate the validation of the ZETOS system for ovine, bovine and human bone samples; Most *in vivo* tests for orthopaedics are carried out in sheep and this tissue can be used for comparison purposes. Since the system can use human bone it has the advantage of not being dependent on any shortcomings of animal models of osteoporosis and can use real human osteoporotic bone. Two advantages of bovine tissue are that firstly many antibodies are available against bovine proteins and secondly it is much easier (at least in Davos) to attain young bovine samples compared to sheep and human tissue.

Method and Results
Bone growth *ex vivo*: sheep femora, bovine metacarpals or human femoral heads were cut at the joint into 7 mm thick sections. Cancellous bone cores, 10 mm in diameter, were drilled from the sections and then cut parallel to the height of 5 mm. Throughout all cutting procedures the bone was irrigated with sterile saline to limit the amount of damage caused by heat and to stop the bone from drying out. Bone cores were washed in cell culture medium (containing tested foetal calf serum) and antibiotics for 30 minutes before being inserted into the chambers. The assembled system was allowed to settle for 36 hours at 37°C (Fig. 1).

The set up for the Zetos system consists of two parts. One part is a number of cross-flow circumfusion chambers, where trabecular bone cores are situated. These cores are circumfused by a constant flow of media, which is circulated in a closed system for 1-2 days. The second part is a computer controlled loading device, which is used to maintain the bone in a loaded environment resembling that of the *in vivo* situation. Without load, bone is believed to have a net increase in resorption, as is seen in patients after prolonged bed rest or astronauts in zero gravity. Inside the loading device there is a piezo stack that expands and contracts under high voltage, placing a force on the piston of the chambers. This force in turn causes a deformation (compression) of the bone cores. The expansion/contraction of the piezo is controlled by a sensor that acts in a feedback loop to control the amount of force applied to each chamber, thus in turn controlling the amount of deformation the bone cores receives. For 5 minutes daily, each chamber, with the bone core inside, is placed inside the loading device. The computer is programmed so that the piezo expands and contracts to give the bone compression of 20 and 5 micrometer in a physiological (walking, jumping) waveform at 1 Hz (once a second) for 300 cycles (5 min). By measuring the force required to deform the bone cores it is possible to calculate the apparent Young’s

modulus of each sample. This is a measure of its apparent stiffness, which is given in the unit Pascal. Previous work in Marburg showed that the chamber apparently maintained both the three dimensional structure of the bone as well as cell viability. Our hypothesis is that loaded bones should become stiffer, with a net increase of bone formation, due to the mechanical stimulation. Conversely, unloaded bone should reduce its stiffness, due to either a net increase in bone resorption and/or a net reduction in bone formation.

Achievements

To date we have cultures all three models (ovine, bovine and human) in the Zetos system. Several cell types have been viewed including, osteoblasts osteoclasts, osteocytes, lining cells, adipocytes, blood vessels, and haemopoietic cells (Figure 2). The three models have been investigated with regard to general histolomorphology, bone density with the use of radiographs, bone 3D structure with microcomputer tomography (mCT) and scanning electron microscopy (SEM) imaging (Figure 3). Molecular and histological techniques have been investigated to demonstrate cell viability and protein synthesis. The viability stain Cell Tracker Green and Ethidium homodimer 1 have been seen to be retained within cells through the fixation, dehydration and embedding procedure. This can therefore be applied in future Zetos experiments to demonstrate percentage of viable cells as the Cell Tracker Green enters viable cells and is cleaved by esterases in the cytoplasm and fluoresces in green, the ethidium homodimer binds to DNA of non viable cells and fluoresces in red. Immunohistochemistry techniques have also been developed to allow localisation of bone marker proteins such as alkaline phosphatase, osteonectin and RANKL. We are currently working on localising osteocalcin, osteopontin and procollagen type I for light microscopy and electron microscopy (Figure 4 & 5). These methods of viewing the bone specific proteins will be applied to bone core samples that have been cultured within the Zetos system for the validation of the system.

The Zetos loading has now been fully calibrated so that the displacement measured on the loading device can be correlated to the displacement that the bone receives. This was carried out with the aid of our collaborators in Marburg. A number of computer macros have been programmed for the Zeiss Axioplan imaging system allowing the number of cells, thickness of osteoid seems, bone apposition rate to be calculated.

Conclusions and Relevance for 3R

In 2003 we hope to show that the three-dimensional structure and viability of the ovine, bovine and human bone grown in this *ex vivo* system is comparable to that of the in vivo situation. We also intend to show that we have on-line protein production in the cells by using the above-mentioned immunohistological staining, and ^{3}H-glycine incorporation as markers.

After completion of the validation we would hope to study the effect of load and various chemical stimulators to force bone growth and force resorption. Other future work will be to evaluate standard biomaterial surfaces with this system and compare the results to the *in vivo* situation. This will all aid the goal of reducing the number of animals used in experiments to evaluate biomaterials and their surface coatings. By using human tissue we may be able to eliminate the need for an animal model to study osteoporosis in cancellous bone. In addition, the system may be useful for studying bone metabolism in pharmaceutical research and development, which would again reduce the numbers of animal experiments.

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Figure 1: System set up in the 37° C room once the bone preparation was complete. Media was circumfused through the bone chamber and bone core without loss of nutrients or the excess build up of wast, with media changed every 2 days.

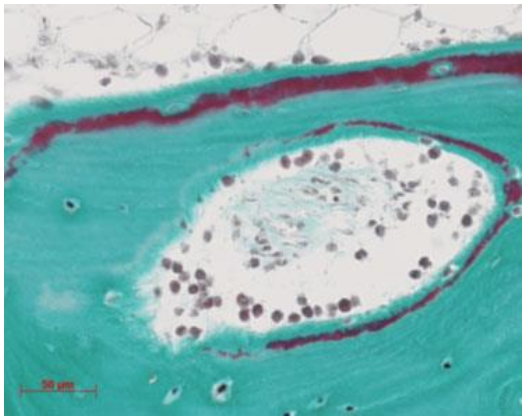

Figure 2: Histiological image of human bone cultured in the Zetos system for 30 days, stained with Masson Goldner stain, which shows the mineralised bone as green, the osteoid and cells in red.

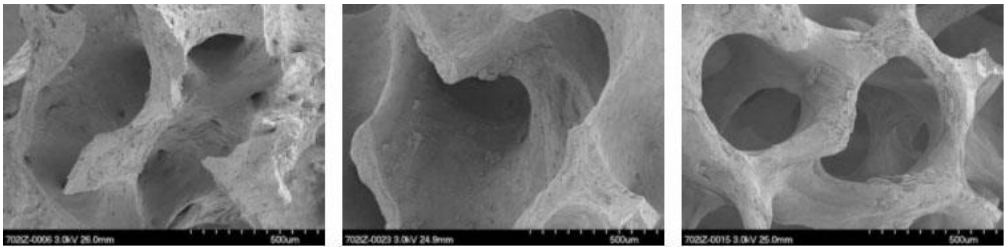

Figure 3: Scanning electron micrographs of ovine bovine and human tissue, respectively, to compare the difference in bone structure and density.

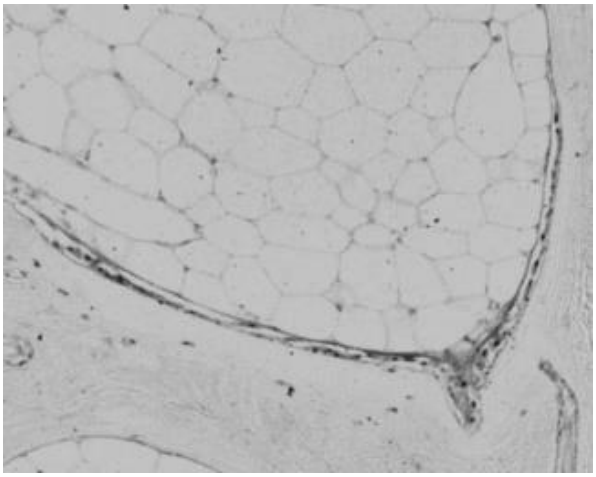

Figure 4: Histiological image of human bone labelled with a primary antibody for alkaline phosphatase.

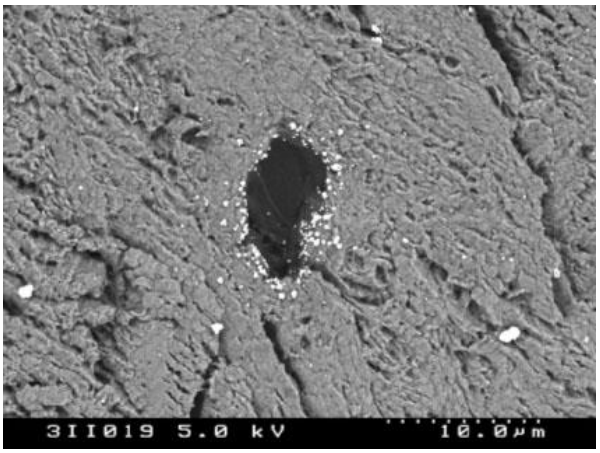

Figure 5: Scanning electron micrographs of a human bone sectioned labelled with a primary antibody directed against RANKL.

3R-Project 79-01

Screening methods for repellents and attachment deterrents for ticks in-vitro

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Keywords: *tick*; *veterinary drugs; veterinary drugs*; *reduction; replacement*; *diagnostic approaches: parasites; veterinary drugs*

Duration: 2 years **Project Completion:** 2003

Background and Aim
Because many insects have developed a resistance to pesticides, new strategies, such as the development of repellents and attachment deterrents, are required to protect people and animals from blood-sucking ectoparasites. The prevention of tick bites is a particularly important focus of such research. Ticks transmit Lyme disease and a range of other pathogens to humans and animals during their blood meal. The economic impact of ticks in livestock farming (free-ranging cattle and other domestic animals) is significant. Another current key area of work is the control of ticks on pets. Repellents and attachment deterrents are designed to disrupt the normal host-parasite relationship and inhibit the parasite's ability to suck blood from its host. Protection results in reduced loss of blood by hosts and reduced incidence of diseases transmitted. Our aim is to develop realistic *in-vitro* methods to screen repellents and attachment deterrents for ticks in order to reduce the need for mass screenings in live animals. We wish to bring our work on *in-vitro* assays to the attention of relevant practitioners at the start of the project in order to establish working contacts.

Method and Results
This project is based on our knowledge of the chemosensory pathways leading to host selection, attachment, feeding and mating in a range of tick species. Natural products that influence these behaviour patterns have been identified by *in-vitro* assays (http://www.unine.ch/zool/commun/articles.htm). The novelty of our approach is to measure the extent to which candidate repellents and attachment deterrents can inhibit *in-vitro* the effects of chemical stimuli inadvertently offered by the host. Only those products that can overcome the effects of the host's stimuli can be considered as effective repellents and attachment deterrents for protection of humans and animals.
We have three *in-vitro* test methods (Fig.1-3): i) a locomotion compensator that records the tick's movement towards test attractants from animals and the effects of repellents on this movement, ii) a test with an "artificial arm", and iii) artificial feeding membranes that simulate the skin, allowing attachment deterrents to be examined (see also 3R Project 10-88). The final phase of the project is reserved for communicating the findings at scientific and industrial meetings, and to transfer the *in-vitro* methods to the relevant end users.

Conclusions and Relevance for 3R
The proposed work will demonstrate that mass screenings for potential repellents and attachment deterrents for ticks can be carried out reliably*in-vitro*. By demonstrating the potential of the *in-vitro* assays for mass screening it is our aim that end users will be stimulated to switch from animal models to cheaper and more standardised *in-vitro* alternatives. To achieve the reduce/replace goal, an essential ingredient will be to work with practitioners from the outset by inviting them to submit potentially useful products of their own to demonstrate their efficacy in the *in-vitro* assays we are developing.

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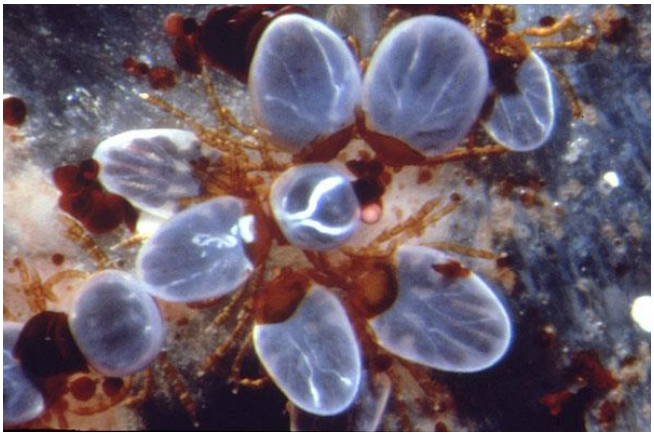

Figure 1: Mature larvae of the cattle tick Boophilus microplus feeding on blood through an artificial membrane. Most larvae are replete with blood. Note the brown faeces excreted by the ticks on the membrane. (Photo F. Kuhnert)

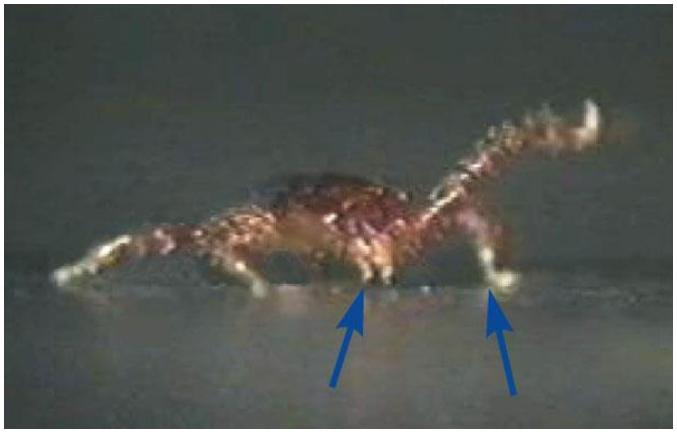

Figure 2: The larval stage of the cattle tick *Boophilus microplus* (total size 0.3 mm) has sensillae with chemoreceptors on the tips of its legs and mouth parts (blue arrows). The tick uses its sensillae to regularly probe the surfaces it walks on and in this way obtain information about the chemical nature of the substrate.

Figure 3: Larvae from the cattle tick *Boophilus microplus* aggregating in the quarter (upper left) of a Petri dish floor treated with steer hair extract.

3R-Project 80-01

Induction of a primary T cell mediated immune response against drugs and drug metabolites in vitro

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Keywords: *human*; *t-cell*; *immunology*; *allergy*; *cell cultures: organ-specific; cell cultures: primary*; *reduction; replacement*

Duration: 2 years **Project Completion:** 2005

Background and Aim
Side effects mediated by the immune system account for about 1/6 of all drug-related side effects. They are responsible for >60% of drug withdrawals, and thus represent a substantial health and economical problem. T-cells are involved in most allergic side effects to drugs. New data indicate that not only hapten like drugs, which covalently bind to peptides/proteins are immunogenic, but that also chemically inert drugs can under certain circumstances activate T cells via direct binding of drugs to the T-cell receptor for antigen (TCR) without previous hapten-carrier formation. The following questions were addressed:
a) can one generate an immune response in vitro to a chemically inert drug ?
b) what are the conditions of T cell activation by a drug and
c) how many T cells can react with a drug in sensitized individuals.

Method and Results
a) The data of this project demonstrate the successful induction of a primary T-cell immune response *in vitro* against a chemically inert drug using blood from healthy individuals, previously not exposed to the drug. Blood lymphocytes were stimulated by the chemically inert drug sulfamethoxazole and the protein-reactive drug-metabolite sulfamethoxazole-nitroso in the presence of IL-2. 3/10 reacted to the chemically inert compound sulfamethoxazole, while 10/10 reacted with the hapten sulfamethoxazole-nitroso. Drug reactive T-cells could be detected after 14-35d of cell culture by drug-specific proliferation or cytotoxicity, which was MHC-restricted. These cells were CD4, CD8 positive or CD4/CD8 double positive. The reactivity to the inert compound was confirmed by cloning sulfamethoxazole-specific T cells. These data confirm the ability of chemically inert drugs to stimulate certain T-cells by their TCR and may provide the opportunity to screen new drugs for their ability to interact with TCRs (Engler O. et al, 2004).
b) while previous attempts to induce a drug specific T cell response to sulfamethoxazole and other inert drugs failed if activated dendritic cells and drugs were used, the activation of T cells seemed to be the decisive difference enabling a response of T cells to inert drugs.
c) The above mentioned results raised the question how many cells are reactive with a drug in healthy and drug allergic individuals? Five patients with severe drug hypersensitivity reactions to amoxicillin, vancomycin, sulfamethoxazole and carbamazepine were analysed for the presence of circulating memory T-cells able to be stimulated by the drug. In addition 5 healthy controls without known drug hypersensitivity were analysed. Using ELISPOT with 4 cytokine determinations and CSFE staining for identifying drug reactive cells we found that between 1:250 - 1:3000 CD4 +T-cells of patients with well documented drug allergy reacted with the incriminated drug – which was equal or higher than the simultaneously detected reactivity of T-cells with tetanus toxoid. This reactivity could be found up to 12 years after the original reaction and persisted in spite of the strict avoidance of the drug. No detectable reactivity was found to unrelated drugs or in non allergic individuals (Beeler A et al, 2006).

Conclusions and Relevance for 3R
The stimulation of T-cells via TCR seems to be an important cause of unexpected drug hypersensitivity reactions and are usually not detected by animal experiments. The data show a possible in vitro method to detect such reactions. For future in vitro tests it is important that only pre-activation of T-cells (stimulated with IL-2) and a rather long cell culture duration is necessary to lead to a detectable response to this pharmacological stimulation of T-cells via TCR. In future, this system might be able to replace certain tests aimed to characterize the sensitizing potential of a drug that are currently conducted in animals (e.g. local or popliteal lymph node assays).
The analysis of precursor frequencies of drug reactive T-cells illustrates that non allergic donors do not harbour such cells

in a detectable amount. However, drug allergic reactions have lead to a massive expansion of such T-cells – which seem to persist for a long time. Tests to improve the detection of drug-specific T-cells may therefore rely initially on the characterization of such T-cells from drug allergic donors.

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3R-Project 81-02

In vitro model for the testing of endothelial cell activation and damage in whole blood

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Keywords: *human; pig*; *whole blood; endothelia*; *coagulation; complement system; transplantation*; *microcarrier*; *reduction; replacement*; *drug screening*

Duration: 2 years **Project Completion:** 2005

Background and Aim
Acute damage and activation of endothelial cells (EC), the inner lining of blood vessels, is encountered in sepsis or septic shock, in ischemia/reperfusion (I/R) injury, and in acute vascular rejection after allo- or xenotransplantation. There is increasing evidence that the complement system is a key player for the induction of EC damage. Ischemia-induced changes on the EC-membrane, or the binding of antibodies in the case of transplantation, lead to activation of the classical complement cascade. The main consequence of this complement-attack is activation of the EC, which means that heparan sulfate proteoglycans are released and a procoagulant cell surface exposed. This leads to activation of the coagulation cascade and, in turn, to more damage and activation of EC. One strategy to prevent EC damage is therefore inhibition of the complement and coagulation cascades. We have been developing soluble complement inhibitors and analyzing their potential role as "EC protectants". Up until recently, the interplay between EC, complement and coagulation in whole blood could only be studied in animal experiments because of the tendency of blood to coagulate spontaneously in vitro. However, a new technique in which EC are cultured on microcarrier beads now allows whole, non-anticoagulated blood to be incubated together with EC. Using this new EC culture system we have:
· establish an in vitro model for EC activation and damage that allows the complex interactions of non-anticoagulated, whole blood with EC, as normally observed in vivo, to be studied in vitro.
· (pre)validate the above model for the study of EC protection by using known "EC protectants", such as low molecular weight dextran sulfate (DXS)
In forthcoming studies we will screen and evaluate the potential of novel, soluble complement inhibitors / "EC protectants" mainly in the context of ischemia/reperfusion injury and transplantation.

Method and Results
1. Establishment of an in vitro model for EC activation and damage
EC damage in vitro has been explored in a pig-to-human xenotransplantation model. For this purpose, porcine aortic EC (PAEC) were cultivated on microcarrier beads to increase the surface-to-volume ratio and incubated with whole, non-anticoagulated human blood as an antibody and complement source. This system functions as a model for (hyper)acute vascular rejection in the setting of xenotransplantation. Immunohistochemistry and immunofluorescence analyses were used to measure activation of both the complement and the coagulation systems, as well as complement deposition on the EC-beads. EC survival was monitored and tests performed to measure the influence of EC damage on activation of the coagulation cascade (see Figure 1 and 2).

2. (Pre)validation of the above model to study EC protection
The known EC protectants dextran sulfate (DXS) was used in the EC activation and damage model and its effect on the different test parameters evaluated. This substance successfully prevented activation of the complement system, and therefore also the activation of EC.

3. Screening of novel, soluble complement inhibitors / "EC protectants"
Our group is currently developing fully synthetic, soluble complement inhibitors / EC protectants that have less systemic side effects on the coagulation system than DXS. These novel substances are currently being screened in both complement and coagulation assays with human plasma. Instead of immediate in vivo application in the hamster-to-rat transplantation model (the current procedure), the in vitro assay for EC protection with whole blood – as developed and validated under 1) and 2) – is being used in a second screening round.

Conclusions and Relevance for 3R

Currently, in most in vitro models in which whole blood or plasma is brought into contact with EC, anticoagulants are required to prevent clotting. This makes it difficult, if not impossible, to analyze in vitro the importance of the coagulation system in EC activation and damage. To date, the possibilities to analyze and screen substances in vitro that could potentially act as "EC protectants" are therefore limited. In consequence, animal models of e.g. I/R injury, transplantation, or shock have to be applied at early stages of scientific research and drug development. In the present project we strive to develop a model of EC activation and damage that can be used to study the interaction of EC with whole, non-anticoagulated blood in vitro. This model is now available and enable us to reduce the need of animal testing and replace part of the currently used in vivo experiments by in vitro screening.

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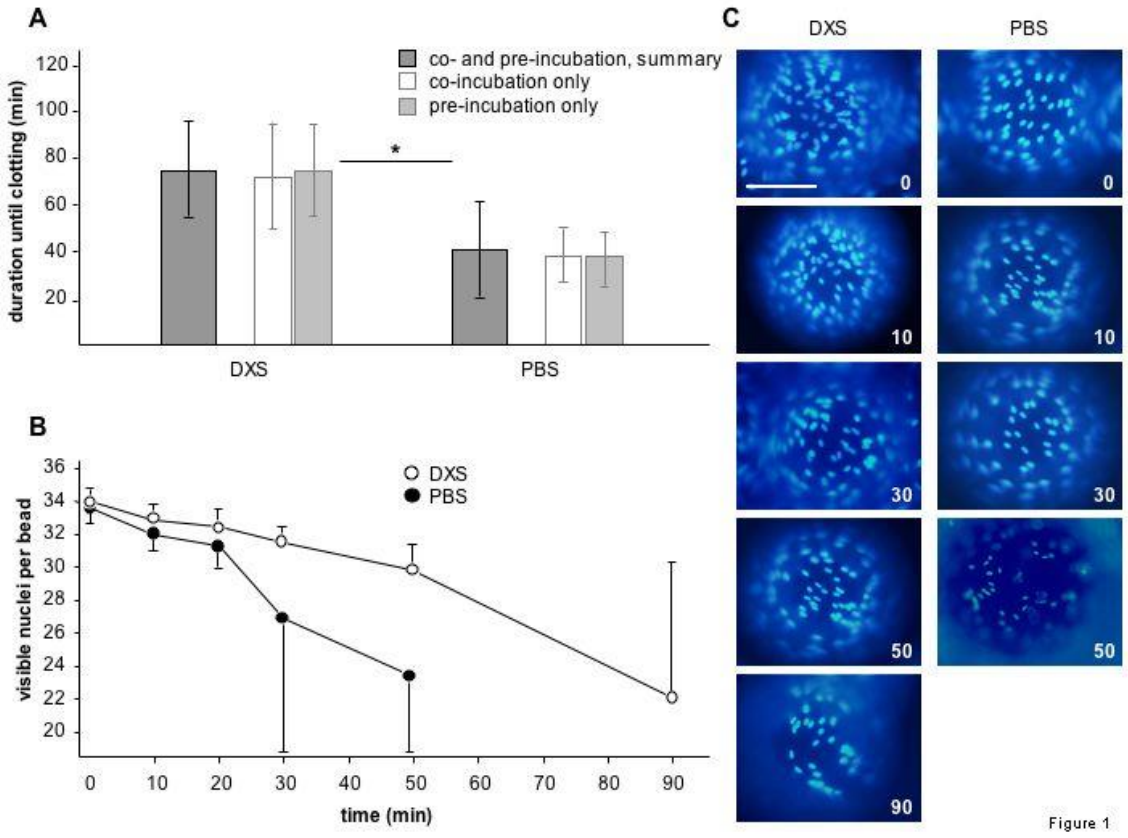

Figure 1: Shown is the duration of the experiments in minutes until occurrence of clotting (A) and the cell-count (porcine aortic endothelial cells) on the bead surface, counted as visible nuclei per bead surface (B). Representative images of single beads at baseline (0 min), after 10, 30, 50 and 90 min of incubation with human blood are shown(C). DAPI staining for nuclei. Scale bar represents 100 µm.

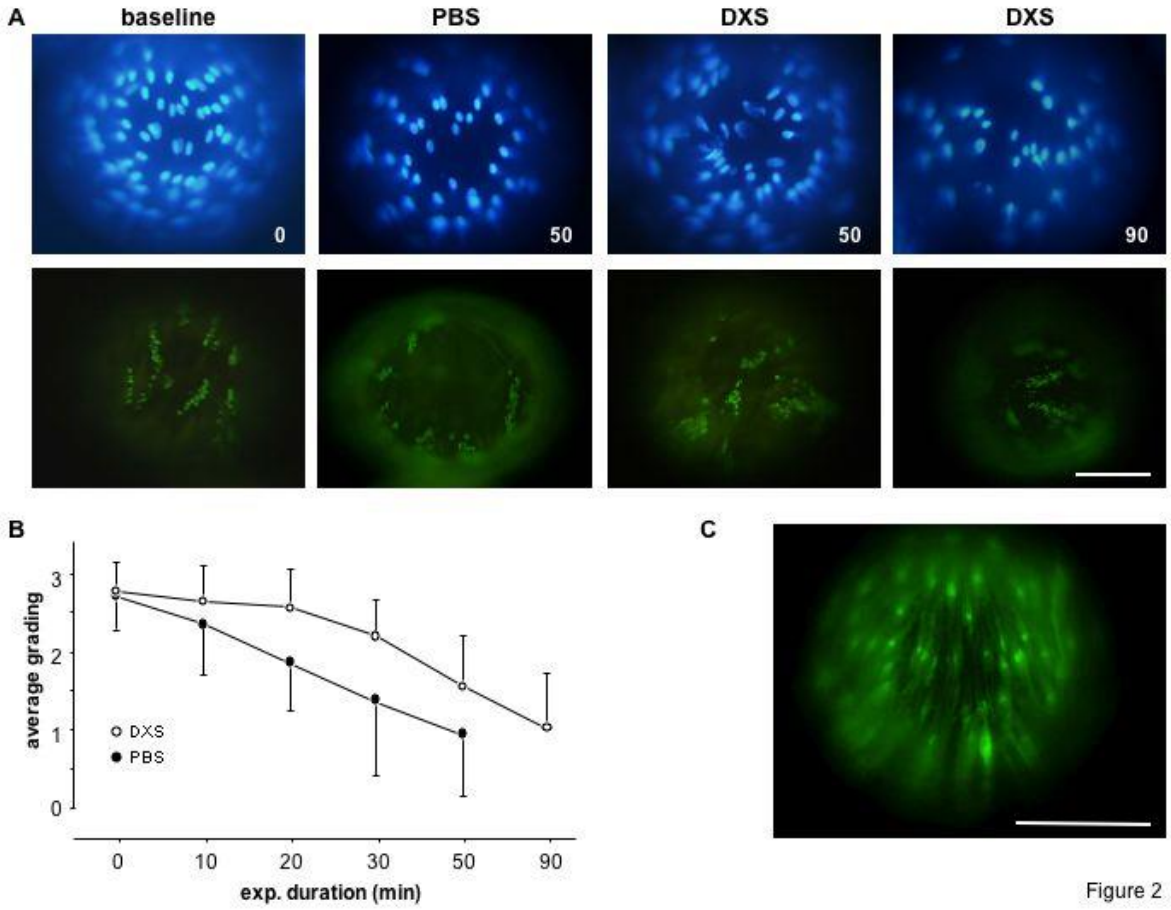

Figure 2: Immunofluorescence staining (A) and grading for expression of von Willebrand Factor with 0 = no positivity, 1 = minimal positivity, 2 = moderately strong positivity, 3 = strong positivity (B). Representative picture of a bead with fluorescein-labeled DXS bound to porcine aortic endothelial cells. Scale bars represent 100 micrometer.

3R-Project 82-02

Magnetic Resonance Imaging (MRI) for the non-invasive assessment of lung inflammation and pulmonary function in the rat

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Keywords: *rat*; *lung*; *asthma; inflammation*; *magnetic resonance imaging (mri); non-invasive*; *reduction; refinement*; *drug screening*

Duration: 2 years **Project Completion:** 2010

Background and Aim
Animal models and methods currently used to evaluate the efficacy of potential treatments for diseases of the airways are limited, generally invasive and terminal. The present project explores the flexibility of magnetic resonance imaging (MRI) to obtain anatomical and functional information of the lung, with the aim of developing a non-invasive approach to carry out drug screening in rat models of asthma and chronic obstructive pulmonary disease (COPD). Using MRI, the disease progression can be followed in a single animal. Thus, a significant reduction in the number of animals used for experimentation may be achieved, as well as minimal interference with their well-being and physiological status. Also, MRI has the potential to shorten the overall duration of the observation period after disease onset since the technique is able to detect changes induced by allergen before these are reflected in secreted parameters of inflammation.
Non-invasive MRI techniques shall be developed in order to
· assess pulmonary function (lung ventilation, perfusion)
· determine the pathophysiologic effects of chronic inflammation on the structure of lung tissues, e.g. airway remodeling and angiogenesis
· establish experimental protocols to measure the effects of drugs in both a) preventing and b) treating pulmonary inflammation in rat models of airway diseases

Method and Results
Procedures are described in detail in [1-3]. In brief:
1) Inflammation models: Two models of pulmonary inflammation will be studied by MRI: (i) Allergen-induced pulmonary inflammation in pre-sensitized rats, resulting from the intra-tracheal (i.t.) administration of ovalbumin (OVA). (ii) Endotoxin-induced pulmonary inflammation in non-sensitized rats, induced by instillation of lipopolysaccharide (LPS).
2) MRI: Measurements will be carried out with a spectrometer operating at 4.7 T. Animals will breath spontaneously during image acquisition.
3) Analysis of BAL fluid or Histology: These (terminal) methods will be used during the validation phase of the study to better characterize the signals detected by MRI and calibrate them against established methods of detecting lung damage. The lungs are lavaged and several inflammation parameters are assessed in the BAL, e.g. eosinophil and neutrophil numbers. Histology is carried out to assess e.g. perivascular edema and macrophage infiltration.

Conclusions and Relevance for 3R
The main advantage of using MRI as analysis tool in animal models of diseases is its non-invasive nature, which allows repeated measurements to be carried out in the same animal. This is of relevance in longitudinal studies since the inter-individual variance is eliminated and the number of animals to reach statistical significance is much smaller. In the case of rat models of asthma, we estimate we can reduce the number of animals used by approximately 80-90% as compared to conventional approaches [broncho-alveolar lavage (BAL) fluid analysis; bronchial biopsies; histology; weighing of lungs].

Since most of the studies are conducted on spontaneously breathing rats, the well-being of the animals during experimentation is improved, as invasive procedures like tracheotomy and/or intubation are avoided. Thus, repeated measurements can be carried out more easily, and the information obtained from time courses shall provide a better picture of disease development and treatment. A further aim is to replace current methods of ventilation assessment

involving the use of radioactive materials. Finally, we estimate that, by using MRI, the duration of the experimental period can be reduced in some of the applications as compared to conventional approaches.

Non-invasiveness is a major asset when studying chronic diseases. The flexibility of MRI makes it a widely applicable method. We are currently using MRI techniques to assess drug effects non-invasively in several models of diseases, e.g. arthritis, transplantation, neuro- degeneration and stroke. For more details, please see [4].

Outlook:

The established protocols will be useful in characterising new compounds intended to prevent/resolving inflammation in asthma and chronic obstructive pulmonary disease (COPD). Of special interest is the non-invasive detection of pathophysiologic changes associated with chronic inflammation in these models. Furthermore, we intend to develop procedures that allow lung function to be measured non-invasively, thus allowing questions concerning the functionality of the lung after therapy to be addressed. Also, it is expected that by using MRI as analysis tool the overall duration of the observation period after disease onset can be shortened since the technique can detect changes induced by allergens before these are reflected in secreted parameters of inflammation.

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3R-Project 83-02

The development of an in vitro intervertebral disc organ culture system

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Keywords: *bovine*; *intervertebral disc*; *mechanobiology*; *explants*; *reduction; replacement*

Duration: 2 years **Project Completion:** 2005

Background and Aim

Lower back pain is the most common health problem in individuals between the ages of 20 and 50 (1) with an estimated annual cost as high as $100 billion per year in the US alone. Despite the prevalence of low back pain, its etiology is largely unknown. There is mounting evidence, however, of a link between degenerated intervertebral discs (IVD) and clinical symptoms.

Several animals models are currently used to evaluate the effects of provoked disc injuries (mechanical and surgical) on disc composition and disc cell metabolism. These include: a) bipedal rats, in which rats forelimbs are removed, forcing the animals to walk on their hind legs and thus increasing mechanical stresses on their IVD (2); b) surgical treatments, such as anulus tears and external fixators (i.e. Ilizarov-type devices fixed to vertebrae and spanning one or more IVD) on sheep, dogs, pigs, rats, or mice to change the normal spine geometry and induce high stresses on certain areas (nucleus or anulus regions) of the instrumented (with fixator) or injured disc (for example: refs 3-8). To our knowledge, there is no suitable *in vitro* system to study the intervertebral disc.

The aim of this project is to develop a method for culturing intact intervertebral discs in vitro. In this method, discs will be explanted from bovine tails obtained from the local slaughterhouse, such that no animals are sacrificed specifically to fulfill the research aims.

Method and Results

Bovine coccygeal discs will be harvested from the tails of young cattle (6-8mos old) obtained from the local abattoir. It has already been established that such discs are a suitable model of the human lumbar disc (9). The discs will be cultured in a custom-built chamber similar to that used by Oshima, et al. (10). The chambers allow the discs to be submerged in culture medium with additional medium flowing over the top and bottom surfaces of the disc, and allow for loading of the discs by placing calibrated weights on the top of the chamber. To prevent swelling of the disc, the *in situ* swelling pressure of the disc will be balanced by weights resting on top of the chamber (mechanical compression).

In the first phase of this project, the appropriate culture conditions (magnitude of mechanical compression, media perfusion rate, length of culture) will be evaluated by assessing cell viability, metabolic activity, and matrix composition of the discs. Cell viability will be evaluated qualitatively using the calcein/ethidium live-dead cell assay (LIVE/DEAD^{R} Viability/Cytotoxicity Kit #L-3224, Molecular Probes) of fresh tissue samples. Metabolic activity will be assessed by ^{35}S-sulfate incorporation (into sulfated proteoglycans) and real-time PCR analyses of genes coding for matrix proteins and proteinases. Matrix composition will be evaluated in terms of water content (hydration) and proteoglycan content.

Once the appropriate baseline culture conditions are established, the utility of this system for mechanobiology studies will be assessed by measuring the cellular response to changing mechanical loads. Real-time PCR analysis will be used to evaluate the time-dependent response of the cells to changes in load magnitude.

Conclusions and Relevance for 3R

The goal for this project is to develop a system that maintains viable, metabolically active intervertebral discs *in vitro*. Specifically, our aim is to establish a system suitable for studying intervertebral disc mechanobiology. By conducting preliminary experiments*in vitro*, we will be able to focus animal studies around the most relevant questions. In the future, this system may also be useful for investigations into potential treatments of disc disease (e.g. pharmaceutical, physiotherapeutic, and tissue engineering treatments).

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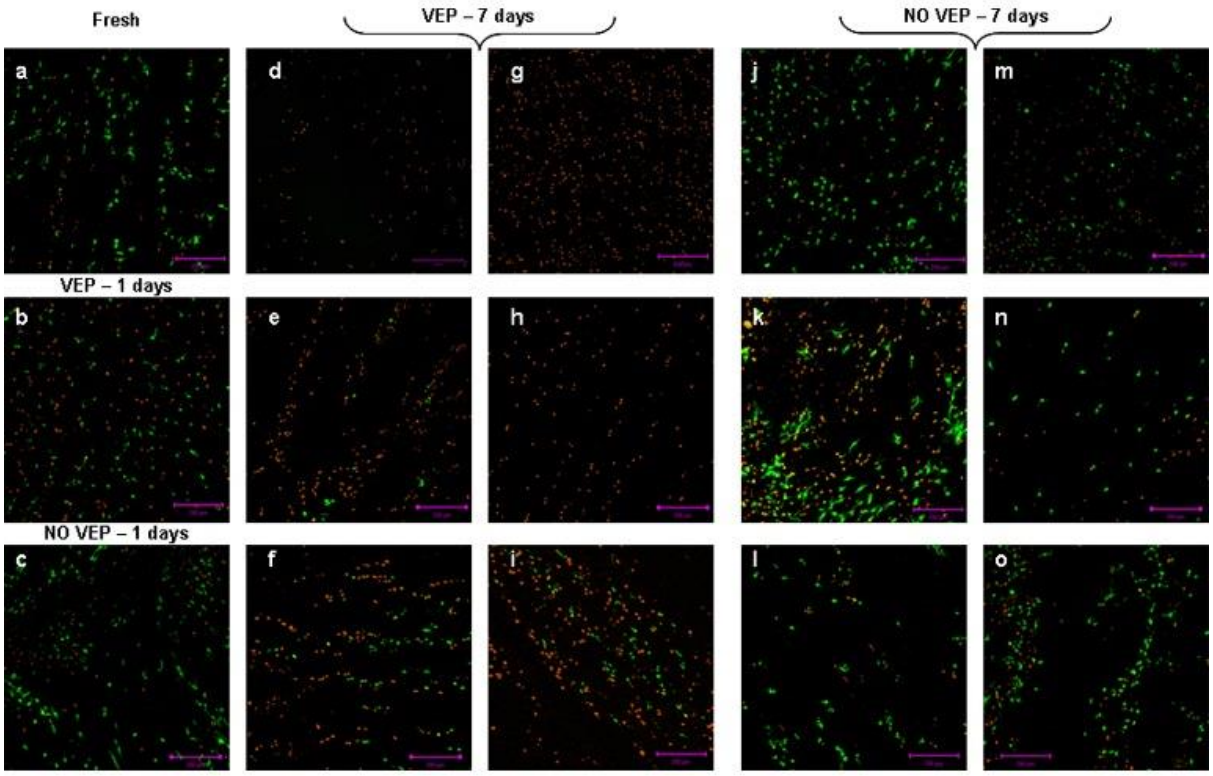

Figure 1: Nucleus cell viability staining of (a) fresh disc and discs cultured for one (b,c) or seven (d-o) days with (b, d-i) or without (c, j-o) vertebral endplate (VEP). Live cells fluoresce green, dead cells fluoresce red. Images represent cells in a 50mm slice of the tissue, starting at a minimum of 50mm from the surface.

3R-Project 84-02

Direct cloning of human antibodies from purified specific B-cells

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Keywords: *human*; *b-cell*; *cell cultures: hybridomas; molecular biology: pcr*; *reduction; replacement*; *diagnostic approaches: monoclonal antibodies*

Duration: 2 years **Project Completion:** 2010

Background and Aim
The classical method of producing diagnostic and therapeutic monoclonal antibodies (mAb's) is to fuse Ab-producing B-cells with cancerous mouse cells (hybridoma technology). The product of this cellular fusion serves as a source for the production of mAb's *in vitro* or *in vivo*. In either case large numbers of animals (i.e. syngeneic mice for in vivo production) or large amounts of animal products (fetal calf serum for in vitro production) are required. In addition, the syngeneic mice are, depending on the treatment and the type of adjuvants applied, often exposed to high physical stress. In addition, if these mAb' would be used in humans they would have to be engineered to contain only human DNA-sequences in their framework (humanization). We would like to replace these cumbersome and - in terms of animals sacrificed or fetal calf serum consumed - very intense procedures by a technology which starts directly with the immunological profile of human antigen-specific B-cells to produce pure human mAb's *in vitro* using recombinant technology based on bacterial or yeast cells. This method would not only replace the use of animals for production but also would provide a more direct and less expensive source of human mAb's.

Method and Results
in progress (present status)
Starting material is whole blood from healthy tetanus-vaccinated donors. Antibodies against the tetanus toxoid will be used as model mAb to establish the feasibility of the system, since they are well characterized at both the desoxyribonucleic acid (DNA) and protein level. The toxoid protein itself is readily available and sequences of hybridomas producing Ab's against Tetanus toxoid will be used for comparison of our recombinant Ab's..
In a first step lymphocytes are isolated using density centrifugation. B-cell enriched fractions will be generated by negative selection using magnetic bead separation technology. Antigen-specific B-cells will be isolated by tagging them with antigen (Ag) and by subsequent positive selection using a fluorescence activated cell sorter FACS sorter. Individual Ag-specific B-cells will serve as the starting material for single cell polymerase-chain reaction (PCR).
Ribonucleic acid (RNA) isolated from these cells will be first reverse transcribed and then amplified using primers specific for the variable regions of the Ag-binding sites of immunoglobulin G (IgG)-chains. Heavy and light chains will be amplified separately. In order to prevent any contamination –by unspecific DNA - which would compromise the sensitivity of single cell PCR - all laboratory procedures will be performed under an accredited quality-management-system in a laboratory designed to perform molecular diagnostic tests.
The resulting amplification products will be isolated and sequenced using standard methods. The sequences obtained will be compared to those published for the already known anti-Tetanus toxoid hybridomas. These sequences will then be cloned into specific expression vectors already containing the constant regions of IgG. The proteins will be expressed in either bacteria or yeast (Pichia pastoris). The choice of the corresponding expression vector will generate different IgG subclasses such as IgG1 or IgG4. The resulting IgG's will be examined for their specificity for Tetanus toxoid and will be compared to already characterized antisera to verify their correct expression.
To validate the procedure the next antigen expressed will be proteinase 3, a well characterized Ag and one of the most frequent autoantigens known. This antigen was chosen since it is notorious for its low antigenicity. If we are able to find B-cells directed against this antigen and clone their specificity using the procedure described above, it will be a good indication of the validity of our system (proof of principle).

Conclusions and Relevance for 3R
The general strategic directions of the project are according to 3R's aims refine and replace:
Refine:

Qualitative improvement of mAb production (light and heavy IgG-chains correspond to the situation *in vivo*);and production of these molecules in bacteria or yeast instead mice or cell culture (refine).
Replace:
Simplified production of human mAb's without the use of difficult-to-get or non-available hybridomas or humanization strategies. Replacement of the use of large numbers of test animals (replace).
Replacement of animal antisera (e.g. from horse) which are still in high use for therapy and diagnostics (i.e. anti-lymphocyte Ab, anti-snake venom Ab, anti-diphtheria Ab etc.) (replace).

3R-Project 85-03

Development of an in vitro culture model to generate *Neospora caninum* and *Toxoplasma gondii* oocysts and sporozoites

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Keywords: *parasites, ectoparasites: neospora caninum; parasites, ectoparasites: toxoplasma gondii*; *veterinary drugs; vaccination; veterinary drugs*; *neosporosis*; *cell cultures: parasites*; *reduction; replacement*; *diagnostic approaches: immuno; diagnostic approaches: parasites; diagnostic approaches: vaccines; veterinary drugs*

Duration: 2 years **Project Completion:** 2005

Background and Aim
Both *Neospora caninum* and *Toxoplasma gondii* are protozoan parasites of high human (Toxoplasma) and veterinary (Neospora) medical importance. Both parasites undergo a life cycle comprised of three distinct stages: Bradyzoites, tachyzoites and sporozoites. Bradyzoites represent a quiescent, slowly proliferating stage, in which parasites are enclosed in tissue cysts. Oral ingestion of tissue cysts containing *T. gondii* or *N. caninum* bradyzoites by a carnivorous host, namely cats (*T. gondii*) or dogs (*N. caninum*) leads to sexual differentiation of the parasites in their intestinal tissues. Parasite eggs become fertilized and form oocysts, which are then excreted in the faeces of the host animals. In the environment, these oocysts produce the third stage of these parasites, the sporozoites, through a process called sporulation. Sporulated oocysts are orally infective for both carnivores and herbivores, and are of prime epidemiological importance for both toxoplasmosis and neosporosis due to environmental contamination. Up to now, oocyst production for both species has always relied on extensive animal experimentation, employing either cats (*T. gondii*) or dogs (*N. caninum*).
During a previous 3R project, we established an in vitro tachyzoite-to-bradyzoite stage conversion technique to obtain cultures of *N. caninum* bradyzoites. The aim of this project is to employ in vitro cultured *N. caninum* and *T. gondii* bradyzoites to generate the sexual stages of *T. gondii* and *N. caninum* in intestinal cell cultures, for the production of oocysts in vitro.

Method and Results
A technique has been developed and refined, that allows to culture canine intestinal cells in vitro for up to 14 days. Cultures can be initiated from biopsies, but are more reliable when initiated from neonatal tissue. The cells carry to typical structural and morphological features of intestinal epithelial cells (polarized morphology, microvilli, desmosomes), and they react positively with antibodies recognizing a broad spectrum of keratin filaments, desmosomal markes and thight junction proteins. Only a small portion (<10%) in these cultures react with an antibody against vimentin, which stains contaminating cells of non-epithelial origin. This culture system has been successfulyl infected with N. caninum tachyzoites and bradyzoites, also obtained from in vitro culture (Vonlaufen et al., 2002; 2004). Further variation of culture conditions to induce sexual development is now in progress.
T. gondi and *N. caninum* bradyzoites have been cultured in vitro using our previously developed method. Bradyzoites were purified from these cultures (Vonlaufen et al., 2004), and a human intestinal cancer cell line (Caco2) has been infected. No stage conversion to enteroepithelial sexual stages could be detected, despite testing of a number of different cell culture conditions (changes in CO_2 and O_2 concentrations, including strictly anaerobic conditions, modulating the pH and temperature of the culture medium, and the addition of immunomodulators such as interferon-gamma or TNF-alpha, exogenous NO (from sodium nitroprusside) or antisera directed against bradyzoite and and tachyzoite antigens.
In addition, we demonstrated that sialic acid residues present on the host cell surface affect host cell invasion by *N. caninum* bradyzoites. Removal of sialic acid improves invasion efficiency, and treatment of parasites with a sialidase inhibitor also inhibits host cell iinteraction. This implies that parasites have evolved some means of removing sialic acid from the host cell surface, in order to gain entry. We have isolated a fetuin-binding protein fraction, which could be involved in this process, and we have shown that this fraction exhibits profound metalloproteinase activity.

Conclusions and Relevance for 3R

The development of an in vitro culture model to produce *T. gondi* and *N. caninum* oocysts would open the possibility of culturing all stages of the life cycle of these two parasites in vitro. This will enable studies on therapy and vaccination against toxoplasmosis/neosporosis to be performed without the use of animals. However, we have grossly underestimated the difficulties associated with the development of a reliable technique to isolate and culture intestinal cells of canine and feline origin. Further work is being carried out to achieve the final goals of this project.

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Congratulations:

The 3R Foundation Switzerland congratulates Prof. A. Hemphill for the awards he received for his work:
Dieter Lüttiken award for animal welfare on 2005. The laudation is as follows:
"The application not only focuses on the setup of a new method; it also concentrates on a strategic approach for fundamental and applied research. A spin-off to related areas can be expected. The study is highly relevant in terms of the 3Rs, in particular for Replacement."
Egon Naef Award 2006 for in vitro Research:
Honored for the development and application of *in vitro* cellular cultures for the study of interactions between parasite/host and for the discovery of treatment of the parasitic diseases in helminths and protozoa (*Neospora caninum, Girardia intestinalis*)

Figures

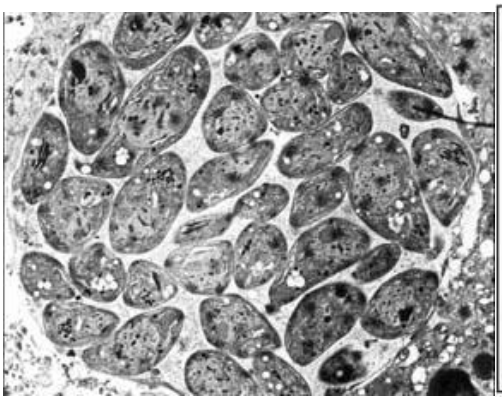

Figure 1

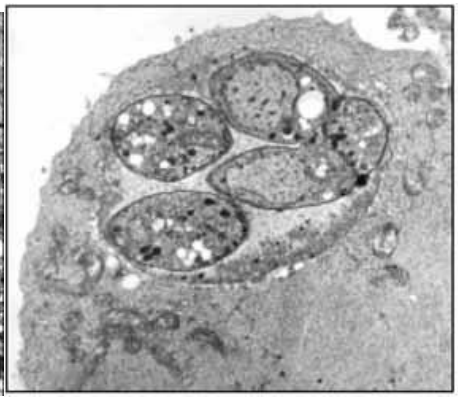

Figure 2

Figure 3

The three stages (Fig. 1-3) of the life cycle of Neospora caninum:

Tachyzoites occupying a parasitophorous vacuole.

Figure 2: Bradyzoites enclosed within a tissue cyst.

Figure 3: Sporozoites (within an oocyst).

3R-Project 86-03

Bone Formation in a loading chamber for ex-vivo bone culture

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Keywords: *human; sheep*; *bone, cartilage; osteocytes / osteoblasts*; *perfusion chamber*; *reduction; replacement*; *validation*

Duration: 1 year **Project Completion:** 2004

Background and Aim
The investigation of bone biology and changes within the bone, is normally undertaken by using either an animal model or cell culture systems. Animal models can not ensure the identical bone structure and biology to that within humans. The widely used two-dimensional cell culture lacks natural occurring cell integrity, three-dimensional relationships, phenotypic characteristics as well as natural load. In order to understand mechanical support and mineral homeostasis of bone, one must have both the cells and bone matrix combined in an isolated, 3D culture. A need exists for an ex vivo bone culture system, where a controlled biochemical and mechanical environment is created to be able to determine the influence of different parameters.Therefore a 3D loading culture system for human bone was developed as reported in project 78-01.

Method and Results
The method was modified from culturing of cancellous bone cores by Davies and colleagues, 2006 [1]. Cancellous bone explants were obtained from human femoral heads (Ethic Commission Graubünden approval (18/02)) were processed (Fig1) into cylindrical cores (5mm height, 9.5mm diameter) for the viability studies. The cores were inserted into the culture chambers, randomly assigned to groups and subsequently cultured for up to 14 days. Groups included, with or without TGFβ3 (15ng/ml) and with or without loading (300 cycles at 1 Hz, giving 4000 microstrain) and heat treated dead cores as a control. As fresh tissue controls bone cores were fixed with 70% ethanol immediately after excision (T0). The samples were then mechanically stimulated for 5 min each per day in the “complete jump” waveform which elicited a 4000µstrain at a frequency of 1Hz, and approximately 300 cycles. The maximum displacement of the tissue was 20 µm, and minimum 5 µm. Cores were cultured for either 7 or 14 days before undergoing viability assessment. Fresh tissue control cores (T0) were assessed for viability immediately after the washing sequence on the day of harvest from the femoral head to act as positive controls. Post culture cell viability was assessed by cutting the cores into 250µm thick sections and the LDH assay was performed [2]. All remaining cores were fixed in 70% ethanol, dehydrated through an ethanol series and embedded into Technovit 9100 New [3] for subsequent histological and immunohistochemical evaluation.

Histology of live cultured samples after 14 days in the Zetos system was comparable to fresh bone (T0) (Fig2). Non collagenous proteins such as bone sialoprotein and osteopontin were localised through immunohistochemical labelling of sections (Fig. 3). The LDH assay displayed a uniform purple/blue staining (LDH positive) over the entire section on a macroscopic scale of the fresh, live tissue. Many dark stained osteocytes were seen in the bone cores cultured for 14 days in the loaded Zetos culture after administration of TGFβ3. The dead sections however did not exhibit any dark, defined osteocytes and only the empty lacunae were visible (Fig. 4). The number of viable osteocytes observed in the fresh tissue (T0) was greater than after 7 and 14 days in Zetos culture (Figures 5-8). However, in all cases there appeared to be a positive effect of loading on the number of viable osteocytes present after 7 and 14 days in Zetos culture compared with the unloaded samples (Figures 5-8). In most cases the effect of loading plus TGFβ3 on viable osteocytes was even greater.

Conclusions and Relevance for 3R
The bone bioreactor system permits the culture of viable 3D human trabecular bone cores up to 14 days. The outcome of this work shows that this ex vivo loading bioreactor is able to maintain a high percentage (over 50%) of viable osteocytes throughout the bone cores after 14 days in ex vivo culture. Further to this, the combination of daily loading and TGFβ3 administration produced superior osteocyte viability at the core centres when compared to loading alone.
By allowing the bone cells to be cultured in their near to natural microenvironment the Zetos system overcomes the limitations observed with cell and organ culture. The bioreactor has potential in pre-testing the integration of human bone with biomaterials, studying basic bone biology including osteoporotic bone.

Acknowledgements
The authors would like to thank 3R #86/03 and ESA MAP project AO 99-122 for funding. Thanks to Dr. Thomas Perren (Davos Hospital), Dr.Heinz Bereiter (Chur Hospital) for supplying human tissue.

References

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Figure 1

Figure 1: Human femoral head being cut into 7 mm thick slices in order to bore cores, 10 mm in diameter, from the tissue that would be cultured ex vivo in the Zetos bioreactor.

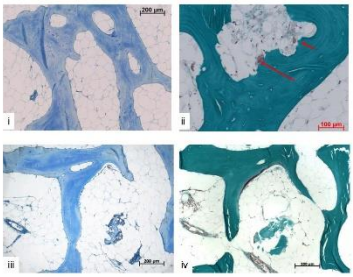

Figure 2

Figure 2: i) Toluidine blue stained human tissue (71 yr male), cultured loaded within the Zetos system for 14 days without the addition of TGFβ3. Not much new bone formation visible, most surfaces were quiescent. ii) Masson stained, tissue (81 yr male), cultured in the loaded system for 14 days without the addition of TGFβ3. Osteoclasts present in Howship's lacunae (red arrows) iii) Toluidine blue stained and iv) Masson stained human tissue (54 yr male), cultured loaded within the Zetos system for 14 days with the addition of TGFβ3, displaying normal morphology

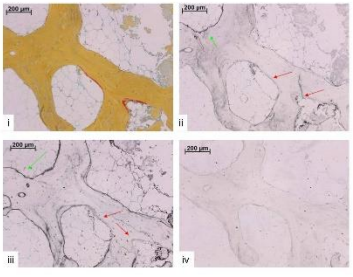

Figure 3

Figure 3: Human bone tissue (70 yr male), cultured in the Zetos system for 14 days. i) Movat stained section depicting osteoid seams in red and calcified tissue in yellow. ii) Bone sialoprotein labelled section (10 µg/ml polyclonal rabbit anti-human). Surface undergoing resorption - green arrow, osteoid seam - red arrows. iii) Osteopontin stained section (10 µg/ml polyclonal goat anti-rabbit). Surface undergoing resorption - green arrow, osteoid seam - red arrows. iv). Negative control – omission of primary antibody.

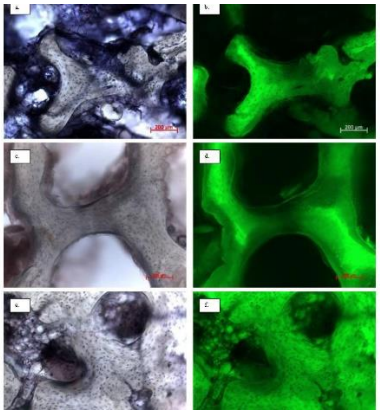

Figure 4

Figure 4 :High power images of human bone tissue (54 yr male). Mid-sections, 250 µm-thick stained for viability using LDH-assay. a. and b. Show an image taken from a T0 section, imaged in brightfield mode and the corresponding fluorescent image. The darkly stained osteocytes can be seen in the bone matrix in both images, and the viable marrow has stained dark purple in the brightfield image. c. and d. Show an image of a dead core taken after 14 days in loaded Zetos culture, imaged in brightfield mode and the corresponding fluorescent image. No darkly stained osteocytes can be seen in the bone matrix in either image, only the empty osteocyte lacunae. There is no dark purple-stained viable marrow in the brightfield image, only remnants of dead marrow. e. and f. Show an image of a core taken after 14 days in loaded Zetos culture after administration of TGFβ3, imaged in brightfield mode and the corresponding fluorescent image. Many darkly stained osteocytes can be seen in the bone matrix in both images. There is also dark purple-stained viable marrow in the brightfield image. Overview images only, not used for quantification.

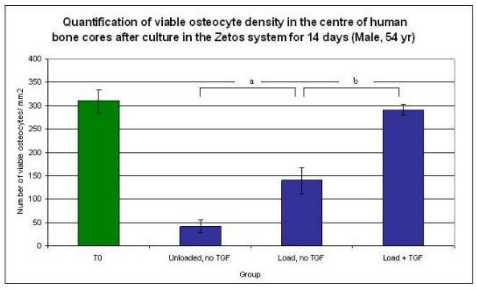

Figure 5

Figure 5: Graphical representation of the quantified osteocytes present in the central area of bone cores at T0 (fresh tissue) and after 14 days in Zetos culture (54 yr male), under different experimental conditions. Dark blue depicts 14 day time point. a Load (no TGF) group shows a significant difference from the unloaded (no TGF) group ($p<0.05$). b Load + TGF shows significant differences between Unloaded no TGF group ($p<0.01$) and Load no TGF group ($p<0.01$).

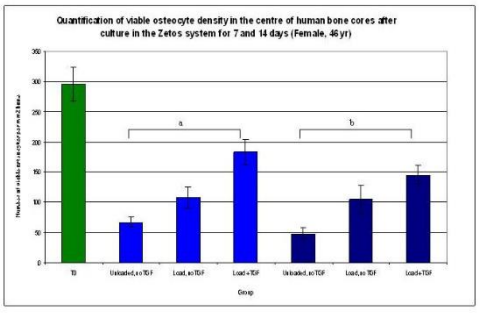

Figure 6

Figure 6:Graphical representation of the quantified osteocytes present in the central area of bone cores at T0 (fresh tissue) and after 7 and 14 days in Zetos culture (46 yr female), under different experimental conditions. . Light blue depicts 7 day timepoint, dark blue depicts 14 day time point. a indicates a significant difference of Load + TGF group when compared with Unloaded no TGF group at 7 days ($p\leq 0.011$). b indicates a significant difference of Load+TGF group when compared with Unloaded no TGF group at 14 days ($p\leq 0.001$).

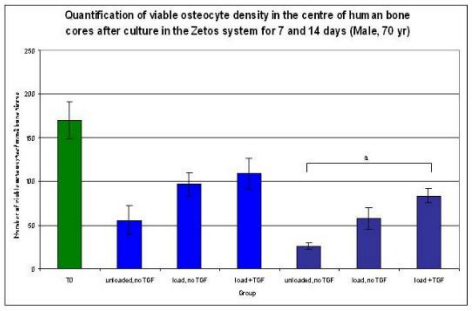

Figure 7

Figure 7: Graphical representation of the quantified osteocytes present in the central area of bone cores at T0 (fresh tissue) and after 7 and 14 days in Zetos culture (70 yr male), under different experimental conditions. Light blue depicts 7 day time point, dark blue depicts 14 day time point. a. indicates a significant difference between the Load + TGF group at 14 days and the unloaded no TGF group at 14 day ($p \leq 0.001$).

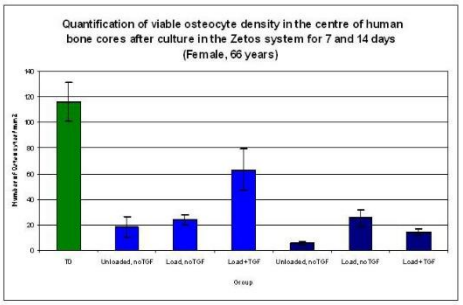

Figure 8

Figure 8:Graphical representation of the quantified osteocytes present in the central area of bone cores at T0 (fresh tissue) and after 7 and 14 days in Zetos culture (66 yr female), under different experimental conditions. Light blue depicts 7 day time point, dark blue depicts 14 day time point. No statistically significant differences in viable osteocyte density were found between treatment groups within each time point in this experiment.

3R-Project 87-03

Information on serum free cell lines, an interactive database

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Keywords: *cell cultures: serum free; cell cultures: serum free; data base*; *reduction; refinement; replacement*

Duration: 2 years **Project Completion:** 2006

Background and Aim
Serum is commonly used as a supplement to cell culture media. The most widely used animal serum is derived from fetal bovines (FBS). Serum provides a broad spectrum of macromolecules involved in growth, attachment, maintenance of metabolic competence and structure of the cells. However, sera are in general ill-defined components in cell culture media and accordingly reduce the reproducibility of the cell culture experiments (inter-laboratory comparisons). In addition, ethical concerns have been raised about the harvesting of serum and the need for bovine fetuses. In terms of the 3Rs, it is desirable to decrease the need for bovine fetuses in the future and to increases the reproducibility of cell culture type work. The production of a database in which available cell lines,adapted to serum-free and/or protein-free media are registered and available for public use, will be one approach in achieving this.

Method and Results
The interactive database SEFREC on-line since April 2006, includes available cell lines, which have been adapted to serum-free and/or protein-free media. In addition to information about the cell line, there will also be references to possible registered processes relating to the respective cells. With such information, the registration process could be simplified and completed more quickly. The principle of the interactive data base is to encourage change in relation to serum-free media. All information in the data base can be sourced by links to the origin data.
Data sources include specialized newspapers, congresses, workshops, information from the medium suppliers and from research groups. The collected data is compiled and digitized in a special data collection program. On conclusion of the official data input, the data is transferred, controlled and compiled in the data base.The links are activated to the respective sources of information and an input window is opened for new data. The data base is free of charge for registered persons.The selected information can be transferred in a word or excel sheet. An example of an input window see figure 1.
The data base SEFREC is available at http://www.sefrec.com . Industry and research groups are invited to place latest information about their products and keep the data base up to date. At the moment, 47 international members present more than 500 serum free medias and 32 serum free available cell lines. In the framework of a european network, this database might become the primary neutral source for informations about serum free cultures in life sciences.
Information about the data bank and maintenance please contact Claudio Strebel by Email: info@sefrec.com

Conclusions and Relevance for 3R
Become a member of SEFREC!
The free interactive data base, is a meaningful possibility for the systematic collection of information on cell lines and their serum-free media worldwide. Two targets could be achieved: Reducing the consumption of serum in cell biology media which increases the reproducibility of cell culture systems by using chemically defined media and to reduce the collection of serum from animals, which is often an animal welfare issue..

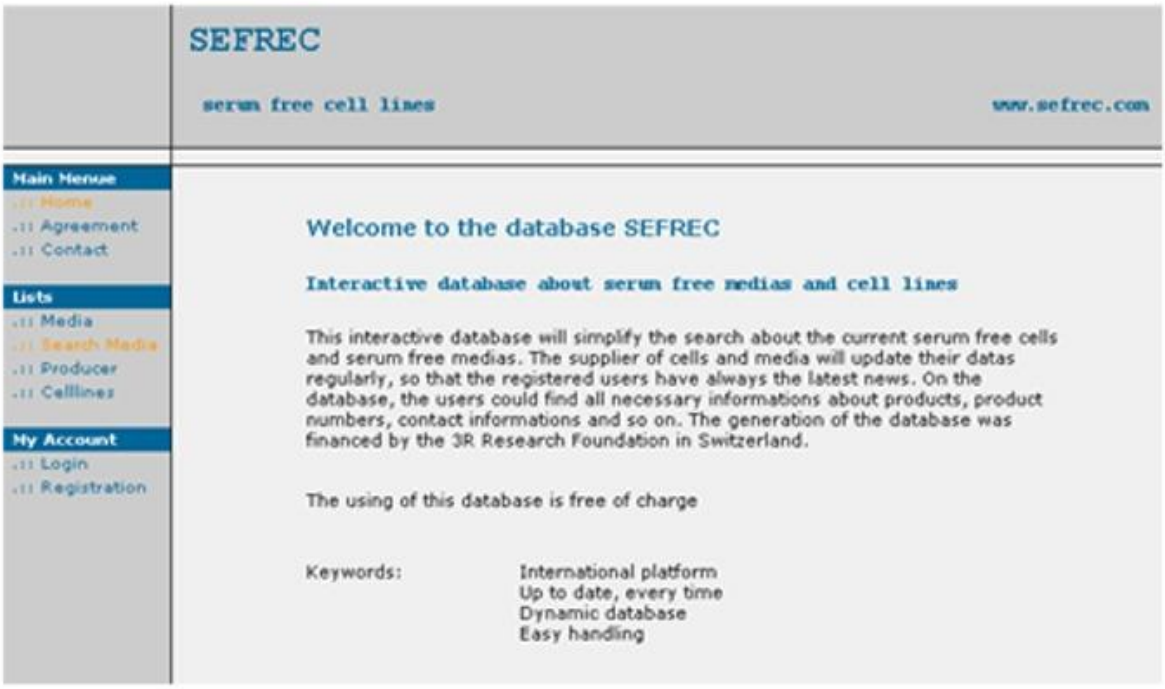

Figure 1: Home page of SEFREC

3R-Project 88-03

Assessing animal health and welfare and recognising pain and distress

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Keywords: *rodents*; *animal welfare*; *animal health; animal welfare; pain; distress; 3r education; refinement*

Duration: 1 year Project Completion: 2006

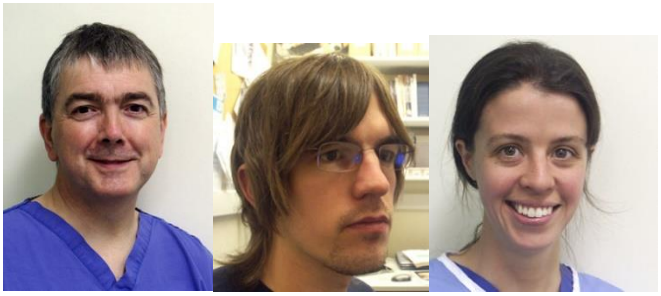
Background and Aim
We should always aim to minimise any pain or distress that may be experienced by laboratory animals. Considerable progress has been made concerning the application of the principles of reduction and replacement, but implementing refinements require that we improve our ability to recognise signs of pain and distress. If we cannot accurately assess pain or distress, then we cannot determine the significance of a refinement to a research protocol. We also need to be able to recognise, both signs of good health and welfare, and poor welfare, in a wide range of different circumstances, in order that appropriate actions can be taken.
A key issue is the training of new staff so that they can recognise these changes in the animals that they work with. Training courses for new research workers are now well established in many institutions, in many different countries, but delivering effective training requires good quality training material.
Many of those delivering training courses now use computer-based presentations, as this is a very flexible and effective means of providing stimulating and informative seminars. It also provides a very cost-effective means of distributing educational material. We have been developing a range of materials for this purpose (see www.digires.co.uk). We now intend to expand the material that is available and develop a web-based tutorial for use throughout Europe.

Method and Results
The illustrative material needed was obtained from a number of different research institutions and encompasses a wide range of different animal species. The material has been made available in several ways. A web-based tutorial has been produced (AHWLA=Assessing the Health and Welfare of Laboratory Animals), to teach research workers and others to recognise signs of health and good welfare and help them to become better able to identify signs of pain, distress and poor welfare in laboratory animals. The material in these tutorials has also been incorporated into teaching material on the 3Rs Foundation web site and was recognised by the authorities in Switzerland as a continuing education for one day (see Recognising post-operative pain in animals). All of the material on the web tutorials together with additional teaching resources is available for use by lecturers on training courses. The web-based tutorial is now freely available, and additional tutorials will be added over the coming years. Additional material has been distributed free of charge to course tutors in the UK and USA, and will continue to be available for a nominal charge (to cover CD duplication and distribution). The only restriction placed on the use of the material is that incorporation into commercial products requires prior permission and editing of the material for other electronic resources also requires approval. Permission would normally be given to all reasonable requests. When using the material, the support of the Swiss 3Rs Foundation should be acknowledged.

The project provides both still images and video material. Initially a general tutorial designed to assist new research workers in assessing the health and welfare a wide range of species was produced. An introductory tutorial on assessing post-operative pain in animals has now been added. We intend developing the web-site further, to include more detailed tutorials – for example methods of assessing welfare of trasgenic mice, and methods of pain assessment in rodents. We welcome enquiries about the work, and also invite colleagues to contribute material for incorporation on the web site and CD. Appropriate acknowledgement of the source would, of course, be given.

Conclusions and Relevance for 3R
Improving the ability of research workers and other staff involved with animal care to recognise pain and distress,will be a significant step in improving the welfare of large numbers of laboratory animals. This basic training underpins all other attempts at refinement (introduction of improved methods, use of more humane endpoints etc). We believe that providing these resources should be extremely timely, as many European countries are in the process of establishing training

courses for new research staff. In addition, those countries which already have training courses will benefit from the enhanced teaching and ease of use that can be provided with these new resources.



Paul Flecknell Jon Gledhill Claire Richardson

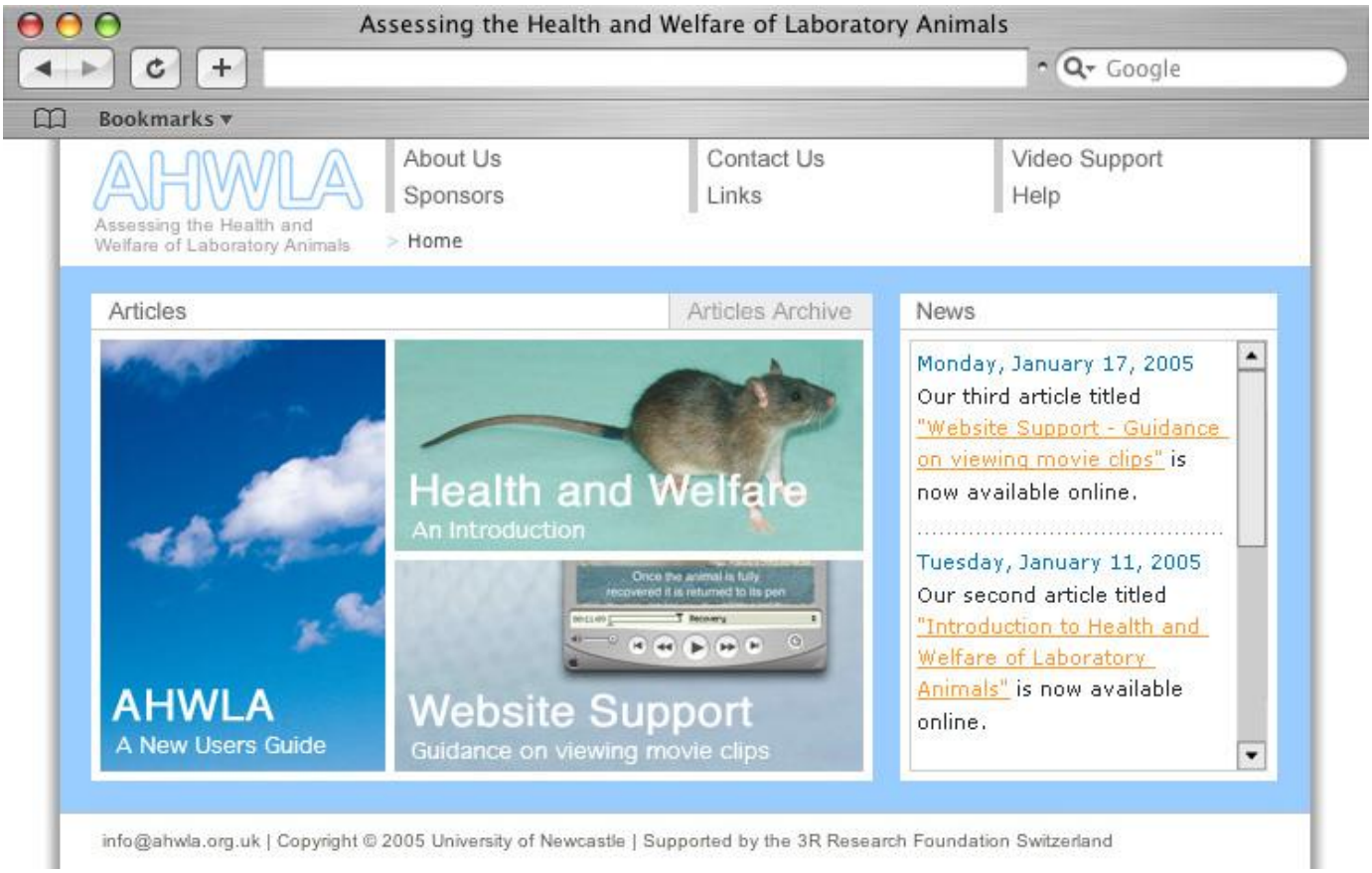


Figure 4: http://www.ahwla.org.uk

Web-tutorial 2005 about: Assessing animal health and welfare" and “Recognising post-operative pain in animals – an introduction”3R-

Project 89-03

In vitro replica of the inner surface of the lungs, for the study of particle-cell interaction

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Keywords: *human; pig*; *epithelia; epithelia; lung; macrophages*; *toxicology*; *cell cultures: 3d cultures; cell cultures: co-cultures; cell cultures: reconstituted tissue*; *reduction; replacement*; *toxicity testing: aerosols; toxicity testing: nano-ultrafine particles*

Duration: 3 years **Project Completion:** 2008

Background and Aim
Adverse health effects by inhaled and deposited particles are of great concern. In addition, therapeutic aerosols become increasingly important for the treatment of lung and other diseases. The interaction of particles with the inner surface of the lungs is fundamental for their biological effectiveness in the organism, though poorly understood and precluded from being studied in humans. A large number of animals are used to test the effects of aerosol particles on a living system by whole-body or nose-only exposures. Such experiments are time consuming and they are stressful for the animals. Furthermore, the increase of knowledge in the use of animal models is hampered for various reasons, such as the long time lapse between aerosol application and lung fixation, the lack of methods for direct studies, or artefacts produced by chemical lung fixation. Cell cultures which are in common use today are deficient in important structural components of the inner lung surface, such as macrophages, immune cells, the aqueous lining layer, and the surfactant film at the air-liquid interface. It is the aim of this project to establish a 3-dimensional (3D) primary cell culture system that reflects the differentiated inner surface of human conducting airways to study adverse health effects induced by aerosol particles.

Method and Results
The proposed primary cell culture system consists of the main structural components of the inner surface of the conducting airways which are known to acutely react upon inhaled and deposited harmful particles, i.e., differentiated (ciliated and serous) epithelial cells, macrophages and the liquid lung-lining layer with the surfactant film at the air-liquid interface.
1) Epithelial cell cultures: (i) Epithelial cells are isolated from human bronchi and thereafter expanded in petri dishes. For differentiation, cells are transferred to porous filter inserts in a two chamber system and grown at the air-liquid interface for at least 3 weeks. (ii) Fully differentiated epithelia obtained by microdissection from (porcine) trachea are cultured as explants on porous filter inserts at the air-liquid interface. (iii) Isolated porcine differentiated tracheal epithelial cells (passage 0) are directly seeded on porous filter inserts and cultured at the air-liquid interface.
2) Macrophages are recovered by bronchoalveolar lavage (BAL) from human or pig lungs. They are cultured as single cell cultures or added on top of epithelial cell cultures (co-cultures).
3) The production of a high-quality surfactant film by redifferentiated and by microdissected epithelial cell cultures is still questionable. Hence, the addition of an artificial surfactant film is recommended.
The integrity of the cell cultures and their state of differentiation are regularly monitored by morphological, physiological, and biochemical analyses.
The cell culture system described above is used in particle exposition studies to investigate possible health effects by environmental and manufactured nano-particles (e.g., Baltensperger et al., J Aerosol Med 21, 2008). Cell cultures are exposed in our novel exposure system for the efficient and controlled deposition of aerosol particles onto cell cultures (Lang et al., J Aerosol Med 20, 2007; Savi et al., submitted). Particle effects on cells are studied on-line during the exposure as well as off-line post aerosol exposure using laser optic and microscopic techniques including (immuno-) histochemistry, ELISA to assess (pro) inflammatory cytokine release (e.g., IL-6, IL-8, TNF-alpha) and cytotoxicity (LDH release). So far, the described cell cultures were used in studies with polystyrene particles (Lang et al., J Aerosol Med 20, 2007; Savi et al., submitted), as well as particles derived from secondary organic aerosols of different origins (Baltensperger et al., J Aerosol Med 21, 2008).

Conclusions and Relevance for 3R
The proposed fully differentiated 3D primary cell culture should reflect the organ-specific functions of the conducting

airways. This is an important prerequisite to replace animal models with in vitro ones. The suggested model allows studying particle effects at a cellular and molecular level. It may be used in different areas.
1) To assess possible health risks of newly produced nanoparticles and nanotubes.
2) To unravel the health risks by inhaled ultrafine particles generated by combustion processes, e.g. by diesel engines and wood burning stoves.
3) To investigate the health risks of (genetically-modified) microorganisms used in food processing industry.
4) To assess the biological effectiveness of new therapeutic aerosols to treat lung and systemic diseases.
Moreover, in the future, the proposed model system, which will at first represent the situation in the healthy lung, may be replaced by a replica of diseased lungs and, hence, reduce the need for experiments with diseased animal models. Since most animals in inhalation studies are used in the pharmaceutical industry, the introduction of the proposed 3D primary cell culture system in such companies has a large potential for 3R. A successful introduction of the proposed model in industry may reduce a substantial number of painful animal experiments, replace animal experiments by *in vitro* testing and refine *in vitro* model systems used today to study particle-lung interactions.

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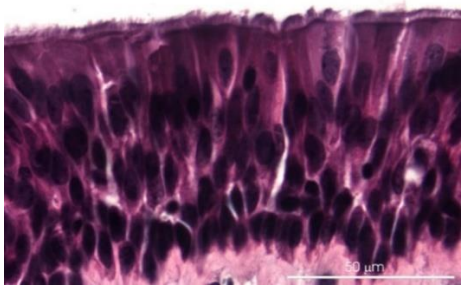

Figure 1:
Light micrograph of porcine tracheal explant with differentiated respiratory epithelium after 3 days in culture on filter inserts at the air-liquid interface. Paraffin section, hematoxilin-eosin staining.

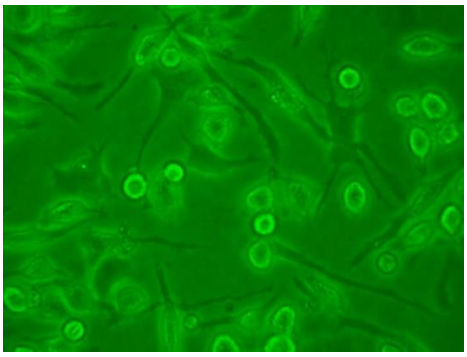

Figure 2
Porcine macrophages obtained from bronchoalveolar lavage in cell culture. Micrograph of living cells.

3R-Project 90-03

A non-mammalian system to study bacterial infections

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Keywords: *bacteria; protozoa: amoeba*; *infectious diseases*; *reduction; replacement*; *infectiosity*

Duration: 3 years **Project Completion:** 2007

Background and Aim
In the study of bacterial infectious diseases and in the development of new drugs, it is very often essential to test a bacteria's capability to cause a disease. To initiate such a situation, it is necessary to infect a host, typically a mouse, and allow the disease to progress. We are working on a new system where mice are replaced by a non-mammalian host: *Dictyostelium discoideum*, a soil amoeba. Very similar results are obtained in this system and in mammalian hosts. We now want to extend these results to validate this system as an alternative to mammalian models.

Method and Results
Dictyostelium discoideum is a powerful genetic system to analyze the complex relationship between bacteria and phagocytic cells. Our initial work was focused on the mechanisms controling phagocytosis of bacteria by amoebae (1). We have more recently focused on the interaction of Dictyostelium amoebae with pathogenic bacteria (Figure 1). We have shown that *Pseudomonas aeruginosa* makes use of a number of virulence factors to inhibit the growth of Dictyostelium amoebae (2). In particular virulence factors produced under the control of the quorum-sensing systems are crucial for Pseudomonas virulence against amoebae, while in another Pseudomonas strain, the type III secretion system was also important for virulence. Thus virulence factors characterized previously in mammalian systems, also play a role in Pseudomonas virulence against Dictyostelium amoebae.
More recent results allowed us to extend our initial findings to study many different bacterial pathogens, in particular *Klebsiella pneumoniae* (3). Based on our current results it is clear that, firstly this system can be adapted to study many different bacterial pathogens (4), secondly results obtained in this system are very similar to results obtained in mammalian hosts and thirdly the system is simple enough, to be easily used in non-specialized laboratories.

Conclusions and Relevance for 3R
As is summarized above, our project is to establish, extend and validate the Dictyostelium system as a relevant model in the study of bacterial infections. This would allow the replacement of animal experiments using mammalian hosts (typically mice or rats) with simple experiments, using Dictyostelium as a non-mammalian host. Experiments using animals to study infections usually require large numbers of animals. They inflict significant suffering on the infected animals and the conclusion of the experiments is often animal death. Thus it will be particularly useful to develop a credible alternative. Our project will allow the Dictyostelium system to be widely used by many research laboratories in studying bacterial diseases. This would make research in this field much easier and at the same time reduce significantly the need for animal experiments in mammalian species.

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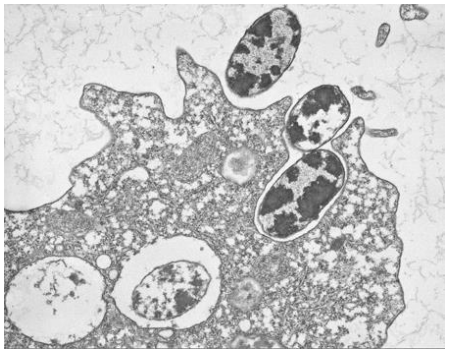

Figure 1: *A Dictyostelium amoeba eating bacteria*

Pathogenic bacteria often use the same mechanisms to defend themselves against unicellular amoebae and to infect mammalian animals. Consequently unicellular amoebae offer an alternative system to study pathogenic bacteria. This system could reduce significantly the need to use mammals to study pathogenic bacteria.

3R-Project 91-04

Establishment and validation of a Choroid Plexus Model to study drug transport

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Keywords: *pig*; *brain; choroid plexus; epithelia; epithelia*; *pharmacology*; *barrier systems in vitro*; *cell cultures: organ-specific; cell cultures: primary*; *reduction; replacement*

Duration: 2 years **Project Completion:** 2007

Background and Aim

Many CNS-drug candidates are ineffective in vivo, because they are not able to cross the barriers of the brain microvascular endothelial cells (blood-brain barrier) and/or the choroid plexus, which is localized in the cerebral ventricles and is formed by capillaries, surrounded by epithelial cells (Fig.1). There is an urgent need to characterize the mechanisms of transport across these barriers.
The aim of this study was the development of an in vitro choroid plexus (CP) cell culture model for studying transport of protein-mediated drug secretion from blood to cerebrospinal fluid (CSF) and vice versa.

Method and Results

Cells were isolated by mechanical and enzymatic treatment of freshly isolated porcine plexus tissue. Epithelial cell monolayers were grown and CSF secretion and transepithelial resistance were determined. The expression of f-actin as well as the choroid plexus marker protein transthyretin (TTR), were assessed. Permeability studies with marker compounds of different molecular weight were performed in order to assess monolayer integrity. The expression of the export proteins p-glycoprotein (Pgp, Abcb1) multidrug resistance protein1 (Mrp1, Abcc1) and Mrp4 (Abcc4) was studied by RT-PCR, Western-blot and immunofluorescence techniques and their functional activity was assessed by transport and uptake experiments.

Choroid plexus epithelial cells were isolated in high purity and grown to form confluent monolayers. Filter-grown monolayers displayed transendothelial resistance (TEER) values in the range of 100 to 150 Ohm x cm2. Morphologically, the cells showed the typical net work of f-actin and expressed TTR at a high rate (Fig. 2). The cultured cells were able to secrete CSF at a rate of 148.2 +/- 4.6 ul/cm2/h over 2-3 hours (Fig. 3). Permeation experiments with molecular size markers indicated the formation of intact tight junctions with transport rates being lowest after 10-14 days of culture. Transport experiments were performed using fluorescent substrates of Mrp1 and Mrp4 (Texas Red, Fluorescein-Methotrexate, Fluo-cAMP) and P-gp (Bodipy-Verapamil, Rhodamin-123). Whereas the Mrp-substrates exhibited directed transport across cell monolayers, hardly any active transport could be demonstrated for the p-gp substrates. The ABC-export protein Mrp1 as well as Mrp4 were expressed in the basolateral (blood-facing) membranes of cell monolayers and intact tissue (Fig. 4). P-glycoprotein showed only low expression within the apical (CSF directed) membrane but was located more in sub-apical cell compartments.

Conclusions and Relevance for 3R

It was demonstrated that CP epithelium can be isolated and cultured, with cells growing into intact monolayers, fully differentiating and with properties resembling the tissue in vivo. Thus, the established primary porcine CP model, allowing investigation of complex transport processes, can be used as a reliable tool for analysis of xenobiotic transport across the blood-cerebrospinal fluid barrier (BCSFB).

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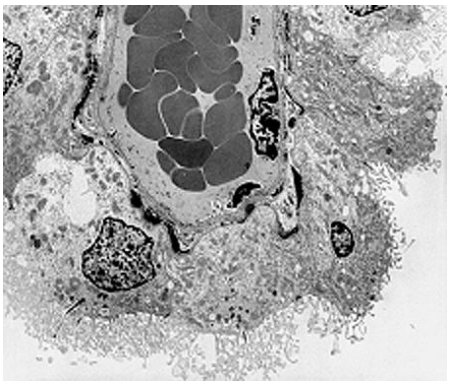

Figure 1: Elektron micrograph of a choroid plexus microvessel surrounded by epithelial cells. The dark cells in the center of the image are red blood cells in the luminal space of the capillary. Epithelial cells are covered by microvilli oriented to the cerebrospinal fluid.

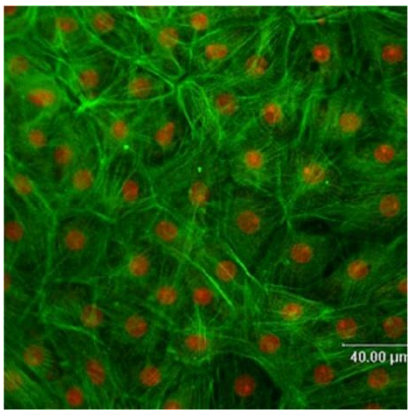

Figure 2: FITC-Phalloidin-stained porcine choroid plexus epithelial cells monolayer after 14 days cell culture (stained f-actin in green, propidium iodide-stained cell nuclei in red).

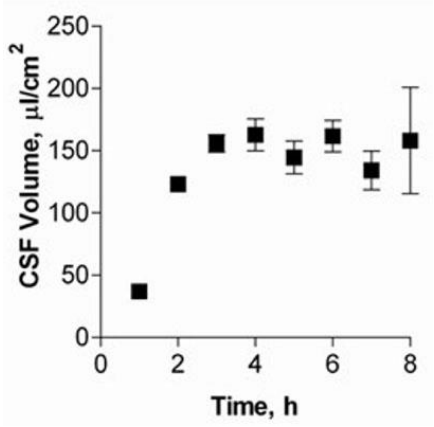

Figure 3:Choroid plexus epithelial cell secretion volumes after 14 DIC. Measurements were taken every hour up to 8 h (means ± SEM, n = 6).

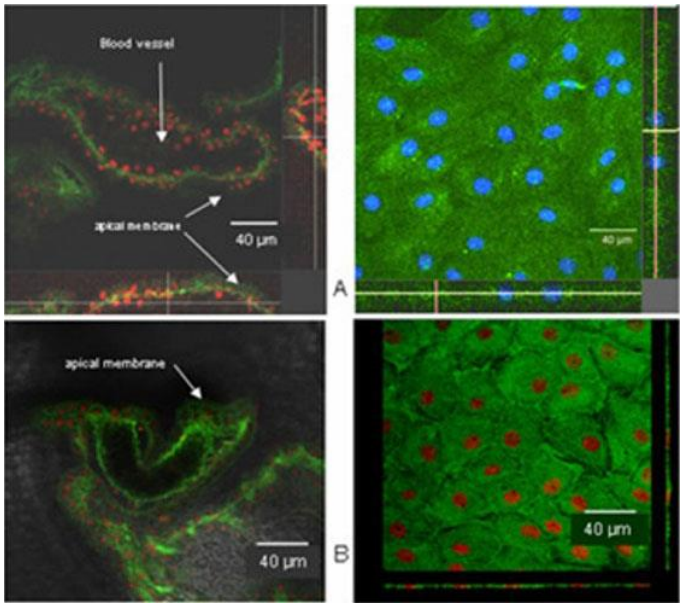

Figure 4: A) Confocal images of choroid plexus epithelial cell monolayers (right side) and freshly isolated intact tissue (left side). Pgp (immunostaining shown in green) is localized sub-apical (intracellular) rather than in apical membranes.
B) Immunostaining of Mrp1 in 14 day-old cell monolayers (right side) and freshly

3R-Project 92-04

Adjuvanticity of microbial-derived particles and synthetic analogs *in vitro*.

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Keywords: *human*; *dendritic cells; t-cell*; *cytokines, growth factors; vaccination*; *reduction; replacement*; *adjuvanticity*

Duration: 3 years **Project Completion:** 2009

Background and Aim
Methods currently used in toxicology for mandatory safety tests rely heavily on the dosing of animals, followed by the detection and pathological evaluation of manifested toxic effects. In the context of vaccinology, compounds of unknown functions are tested in vivo to demonstrate their immune enhancing properties without any clue of their toxicity. These procedures often cause animal pain, distress and even death before obtaining an indication of immune protection.
As new compounds become available, there is ethical, political and commercial pressure to reduce animal tests and ensures that tested animals undergo minimum pain and distress. In this context in vitro tests can represent a very valuable tool. New tests based on human lymphocyte cultures in vitro are particularly needed due to important differences between human and animals that render animal models poorly suited for identifying new safe immune enhancers for human vaccines.
The aim of this project was to develop an applicable system to identify human-compatible adjuvants in vitro.

Method and Results
Based on molecular profiles of human lymphocyte cultures and functional assays of T cell activation in the presence or absence of putative standard adjuvants, we have defined TNF/CXCL10 and IL-1 beta molecular signatures of adjuvanticity and pyrogenicity, respectively. We propose a new method of adjuvant screening. Proof-of-concept for this method was obtained by screening a small library of natural microbial compounds and synthetic analogues thereof, already tested in animal models.
Collectively, we propose a three-steps method of adjuvants screening (Fig.1), whereby potentially safe adjuvants are first identified in vitro for the capacity of inducing TNF and/or CXCL10 release by DC, and absence of IL-1 beta release by monocytes (Fig. 1, step 1). Selected compounds are then assessed for the capacity to enhance antigen-specific T cell activation in bulk cultures (Fig. 1, step 2) and limiting dilution assays (Fig. 1, step 3). Only compounds with high immune enhancer activity in vitro will be finally assessed in animal models before admission to clinical application.

Conclusions and Relevance for 3R
In the course of our investigation we have set the rational for the development of new applicable screening methods based on compounds pre-selection in vitro. The method we described models only few parameters of an immune response and presents a number of positive features. These include the simplicity and sensitivity of the biological responses assessed and the broad applicability of the assay to various compounds. Based on our experimental results we therefore propose to refine current methods of adjuvant screening by selecting candidate adjuvants prior to in vivo testing. This procedure will reduce the need of costly, demanding and potentially irrelevant animal studies.

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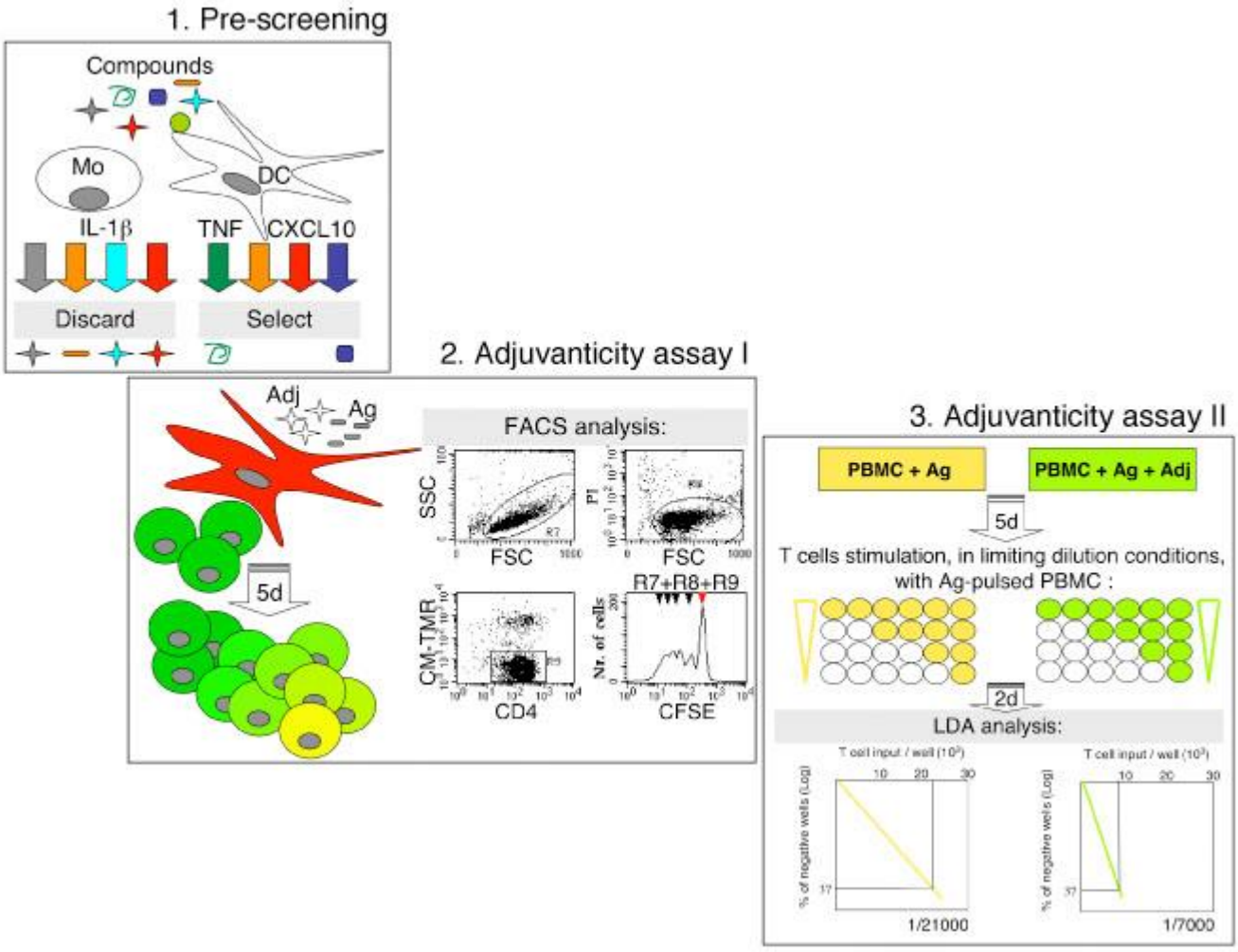
Figures

Screening of ajuvants for human vaccines.

Step 1: Pre-screening. Safe potential adjuvants are identified in vitro for the capacity of inducing TNF and/or CXCL10 release by human monocyte-derived dendritic cells (DC), and absence of IL-1 beta release by monocytes.

Step 2: Adjuvanticity Test I. Human monocyte-derived DC are labeled with the red fluorescent dye CM-TMR. Cells are co-cultured with autologous purified CD4+ T lymphocytes, labeled with the green fluorescent dye CFSE, in the presence or absence of antigen (Ag) and adjuvant (Adj). Ag-specific T cell proliferation after 5 days of culture is measured by the loss of green fluorescence in live CD4+ T lymphocytes (detected in R7 and R8, respectively) excluding red-labeled DC from the analysis (R9). In the CFSE staining histogram plot red arrow specifies the non-dividing parent generation. The assay can also be run in allogeneic settings, without antigen or in the presence of bacterial enterotoxins that expand specific T cell subsets.

Step 3: Adjuvanticity Test II. Human Peripheral Blood Mononuclear Cells (PBMC) are primed in vitro with Ag in the presence or absence of Adj, without exogenous cytokines. After 5 days, cells are seeded at limiting dilutions in a microtiter plate and re-stimulated with autologous Ag-pulsed PBMC. Two days later, T cell proliferation is measured by 3Hthymidine incorporation. The frequency of Ag-specific T cells is finally determined according to the Poisson' s distribution (LDA analysis).



3R-Project 93-04

Development of a novel multicellular 3-dimensional blood brain barrier in vitro model

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Keywords: *rat*; *astrocytes; brain; epithelia; endothelia; epithelia*; *cns, brain disorders; pharmacology*; *barrier systems in vitro*; *cell cultures: 3d cultures; cell cultures: co-cultures; cell cultures: organ-specific; cell cultures: primary; cell cultures: reconstituted tissue*; *reduction; replacement*

Duration: 3 years **Project Completion:** 2010

Background and Aim
The blood-brain barrier (BBB) plays a crucial role in preserving physiological brain homeostasis. Notably disruption or dysfunction of the BBB constitutes a well-described hallmark of many socially and economically important pathological states, thus understanding regulation of BBB is crucial to reduce/prevent injury/disease progression. Although different animal models are used to study various diseases characterised by loss of BBB integrity the cellular and molecular mechanisms involved are still only poorly understood. Since high complexity of the brain makes interpretation of *in vivo* data challenging BBB studies are frequently performed using highly simplified in vitro models. Despite this the translation of in vitro data to the *in vivo* situation remains inadequate. Remarkably, many models fail to address important features such as the 3D structure of blood vessels, the complex cellular interactions that occur *in vivo* or include the 3 specific cell types that comprise the BBB.
The aim of this project was to establish a cell culture system that contains the three major cell types that form the BBB *in vivo* : endothelial cells, astrocytes and pericytes. We have developed, characterised and validated an innovative *in vitro* system that is an accurate representation of the BBB *in vivo* and additionally allows dynamic movement and reorganization of cells in response to a changing environment - significantly refining current *in vitro* models. This model can potentially reduce and replace animal experimentation with *in vitro* testing and aid identification and refinement of new therapeutic targets and protective strategies. New innovations such as this therefore have potentially wide applications in basic science as well as medical and pharmaceutical industries.

Method and Results
The BBB cultures consist of endothelial cell, astrocytes and pericytes mixed in a ratio of 1:5:1 and suspended in a collagen matrix that solidifies at room temperature and is overlaid with media. The matrix allows cellular movement and enables each cell to display its specific unique morphology as occurs *in vivo*. During early development (up to 4 days) the culture manifests many endothelial structures such as cysts, intercystal connections and tube-like structures that appear analogous to those observed during vascular development in vertebrates such as blood islands, primary vascular plexus and angiogenic blood vessels (Fig 1). After 5 days our in vitro culture has stabilised and closely mimics the structure and interactions of cells comprising the BBB *in vivo* (see Figure 2). Validation of our model shows it correlates well to the in vivo situation in many important aspects. The blood vessels produced are hollow demonstrating the presence of a patent lumen. Furthermore astrocyte and/or pericyte interactions are required for adequate induction of well-established barrier properties such as proper localised adherens and tight junction complex formation as well as efficient vascular polarity. Challenging the cultures with stressors, such as exposure to hypoxia or mannitol that induce changes in barrier characteristics, convincingly reproduces the expected alterations in specific cellular interactions as well as protein localisation and expression. Thus the model is dynamic and accurately responsive to environmental influences.
We have used our model to differentiate the distinct roles of both astrocytes and pericytes at the BBB. Overall, our data suggest that during development pericytes play an important pro-angiogenic role whereas astrocytes are anti-angiogenic accelerating tube stabilization and maturation. In contrast during adverse conditions such as hypoxia or drug exposure our findings indicate that both astrocytes and pericytes significantly contribute to vascular stability, although to different degrees, possibly through synergistic signalling. We are now using our model to investigate the action of a number of compounds at the BBB. Furthermore we have performed cDNA array analysis at different stages of development to identify genes instrumental in induction, maintenance and break down of the BBB. A first manuscript with detailed description of the characterisation and validation of the model is currently being peer reviewed.

This versatile system will undoubtedly improve our understanding of the importance of cell-cell interactions and the mechanisms underlying BBB regulation during both physiological and pathological situations. We hope the future will see a wide use of this and related models in the BBB field.

Conclusions and Relevance for 3R

This system provides a unique opportunity to more easily study the BBB and reduces the need for difficult, invasive animal experiments. As such it has wide applications in basic research as well as medical and pharmaceutical industries. Furthermore, this model system will not only provide information on specific cellular interactions and signals that promote induction of BBB formation during development, but is readily manipulated and subjected to different insults to aid understanding of BBB breakdown. Thus utilisation of this model means research can be more focused and directed to the specific roles of individual cell types and barrier function as a whole with minimal use of animal experimentation.
In conclusion this work promotes a means for refinement of potential therapeutic tools and strategies prior to animal testing, and ultimately reduces animal experimentation, consumables and personnel costs.

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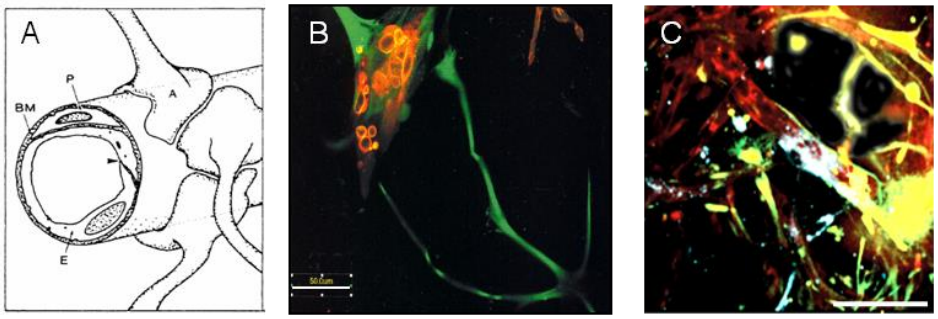

Figure 1: Brightfield and fluorescent images showing development of model over time reveals many endothelial structures (cysts, intercystal connections and vessel-like structures) analogous to those observed *in vivo* (eg blood islands, primary vascular plexus and angiogenic vessels).

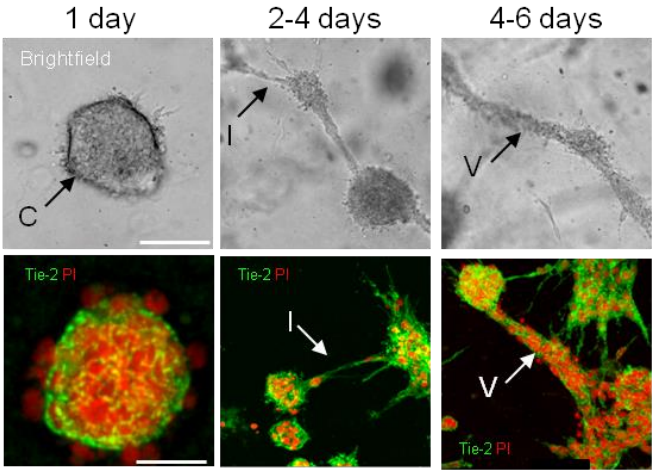

Figure 2: Diagrammatic representation of the BBB *in vivo* (A, from Abbot 1989). Cellular interactions in our *in vitro* model are analogous to the organisation *in vivo* (B and C). Red: endothelial cells; green/yellow: astrocytes; blue: pericytes.

3R-Project 94-04

Tumor targeted reporter gene expression to improve and refine traditional models of Tumor growth and metastasis

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Keywords: *mice; rat*; *tumour*; *tumourigenesis*; *reduction; refinement*; *toxicity testing: carcinogenicity*

Duration: 2 years **Project Completion:** 2007

Background and Aim
Background: Until today the use of animal tumor models is still the most informative approach to obtain pre-clinical data of potential anti-neoplastic agents. In most pre-clinical models, assessment of intraabdominal tumor location and size required sacrificing the animal. Furthermore, gene expression patterns between tumor cell implantation and tumor collection remained enigmatic.
- A large number of animals have to be sacrificed to evaluate tumor growth dynamics and kinetics of gene expression:
Over the past several years, methods for *in-vivo* analyses of tumor growth and gene expression have emerged. The most prominent approach, bioluminescent imaging (BLI) is an imaging method that allows the in-vivo analysis of cells expressing light-emitting enzymes like the luciferase (Luc) through the animal tissues. However, this non-invasive method to visualize tumor cells in-vivo required the cell-lines to be specifically engineered to emit detectable light. Likewise, in the few studies that used soluble reporter peptides like beta-Human Chorionic Gonadotropin (beta HCG) to monitor tumor growth in-vivo through serum level determination, tumor cells had to be stably transfected with the beta-HCG gene. This necessity for stable transfectants not only limits the testing of anti-tumor agents to a few tumor cell-lines, but it has also the disadvantage that the genetic engineering modifies genes of the maternal cell as well, thus altering the phenotype of the tumor cells in question.

- *In-vivo* transfection of tumor cells would eliminate the necessity for stably transfected cell-lines.
- Expression of reporter genes from a promoter specific to most tumors but not to normal cells would allow the systemic application of transfection vectors:
Re-activation of the human telomerase reverse transcriptase (hTERT) is a general principle of cancer cells, but not in normal somatic cells. We recently showed that tumor-specific transgene expression from the hTERT promoter enables the targeting of pro-apoptotic genes to cancer cells.

Aim: We want to test the possibility of tumor selective reporter gene (luciferase and beta-human chorionic gonadotropin) expression from the human telomerase reverse transcriptase (hTERT) promoter to detect early tumors, follow tumor growth and monitor telomerase activity of tumor cells as a surrogate marker for anti-tumor therapies

Method and Results
the project was terminated at an early stage
Bioluminescence imaging will be used to quantify and locate luciferase (reporter gene) expression after i/p luciferin injection. Serum level determination of beta-HCG will be performed with standard ELISA kits and by real-time PCR. Both reporter genes are expressed by the hTERT promoter, which is basically only activated in tumor cells. Plasmids have been already constructed and showed a satisfactory yield of transgene expression. Preliminary results indicated that the promoter is strong enough to allow detection of the reporter gene by BLI. Further methods will include: in-vitro: MTT-cell proliferation assay, Dual-luciferase assay, Effectene-transfection, Fluoro-Microscopy, Western-blot analysis; in-vivo: Immunohistochemistry, determination of liver transaminases, effect of telomerase-suppressive agents.

Expectations: We expect that both reporter systems will allow to quantify telomerase-active tumor cells after systemic vector application and that the effect of anti-tumor agents and of telomerase-suppressive agents can be monitored in the living animal.

Conclusions and Relevance for 3R
The development of novel anti-cancer strategies requires more sensitive and less invasive methods to detect and monitor minimal tumor formation in a broad variety of cancer models. This is especially true in pre-clinical efficacy testing of

molecular anti-tumor agents that display no direct cytotoxicity but rather demonstrate tumerostatic and anti-angiogenic effects. The search for surrogate markers for these agents of tumor-response is becaming increasingly important.
If, as we expect, tumor selective expression of either luciferase or beta HCG reporter genes can indeed be monitored *in-vivo*, it has the potential to be used in a very broad range of tumor models. This is due to the use of transfection vectors rather than stable transfected cell-lines, which will enable us to follow most established cell-lines as well as primary tumor cells derived directly from patients.

Relevance for 3R:
a) Reduction in animal experimentation
The bioluminescent reporter gene imaging technique (BLI) allows the non-invasive and repetitive image recording of the distribution of luciferase-expressing cells within the body. Hence, tumor-growth can be followed over time.
- It renders the sacrifice of animals in tumor-models that study the dynamics of tumor growth and the kinetics of gene expression patterns at different time-points unnecessary.

It enables the detection of tumors from early on, thus, avoiding treatment of non-tumor bearing animals. Often the implantation of tumor-cells in animals does not induce tumor formation in 100% of the animals. Because, so far, no method to detect tumor formation at an early stage existed, this incomplete tumor formation had to be countered by increasing the number of animals allocated to each treatment arm.
- *In-vivo* verification of early tumor formation results in a decrease of sample size per treatment group to reach statistical significant results

b) Refining experimental methods
Because telomerase activation is essential for unlimited cellular replication, 85% - 95% of the cancer cells display re-activation of this enzyme complex. The in-vivo monitoring of the hTERT activity in the tumor-cells can be recorded by using the hTERT promoter to enhance luciferase or beta HCG reporter gene expression This is especially useful in the context of novel anti-tumor agents that may act through downregulation of the proliferative capacity in malignant cells rather than through direct cytotoxicity. For these anti-tumor agents hTERT-activity can serve as a surrogate marker to test their efficacy.
- Monitoring of hTERT-activity as surrogate marker will be an increasingly valuable instrument to study efficacy of novel molecular anti-tumor agents.
- Repeated measuring of hTERT-activity will, additionally, allow to determine the kinetics of anti-tumor effects of these agents.

c) Expected achievement towards animal protection
Because both methods to follow tumor growth proposed in this study have been shown to be highly sensitive in previous experiments, the monitoring of tumor growth can start at a very early stage and conclusive results can be obtained at a much earlier stage of the disease.
- Early tumor monitoring allows to minimize tumor manifestation of the animals and to reduce the duration of treatment

3R-Project 95-05

Development of QSAR-Models for Classification and Prediction of Baseline Toxicity and of Uncoupling of Energy Transduction

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Keywords: *bacteria*; *qsar; toxicology*; *in silico*; *reduction; replacement*

Duration: 1 year **Project Completion:** 2007

Background and Aim
The pending implementation of the European chemicals regulation REACH (Registration,Evaluation and Authorisation of Chemicals) will have a significant impact on additional testing needs. One approach to potentially reduce the cost and number of animal tests required is the application of quantitative structure-activity relationships (QSAR) as a tool to prioritize further testing. However, in the field of ecotoxicology there are still large gaps for a full regulatory acceptance of QSARs, of which some are difficult to assess in terms of predictive power and often lack transparency.
Over the last years we developed an experimental in vitro test, the Kinspec system (bacterial test), which allows the determination of nonspecific toxic effects in membranes (Escher and Schwarzenbach, 2002). Kinspec covers amongst others the two nonspecific modes of action uncoupling (of oxidative and photo-phosphorylation) and baseline toxicity. One of the advantages of Kinspec is that it allowed new insights into the mechanisms of uncoupling (Escher et al., 1999). These insights lead to the development of a new QSAR model with a strong mechanistic basis and only three physico-chemical descriptors (Spycher et al., 2005).
The proposed project is based on the complementary use of experimental and theoretical work. In the initial phase the existing QSAR-model is used to design a diverse library of potential uncouplers. Subsequently, the compounds in this library will be measured with Kinspec and the results will be compared to the initial predictions of the QSAR model. In a final step a large database of compounds shall be prioritized for further testing. Therefore, this project can be seen as a pilot project for other specific modes of action in ecotoxicologically relevant endpoints to be tested under REACH.

Method and Results
In the Kinspec test system, toxic effects are quantified by changes in membrane potential of chromatophores extracted from the photosynthetic bacterium *Rhodobacter sphaeroides*. The effect concentrations, EC are derived from the concentration effect curves as described in (Escher and Schwarzenbach, 2002). The distinction of baseline toxicants and uncouplers is based on the calculation of toxic ratios (TR). Compounds with high TRs have a more specific mode of action than baseline toxicity alone. In case of the Kinspec test system, all compounds that accelerate the decay of the membrane potential at concentrations lower than those needed for baseline toxicity must imperatively be charge transporters and if they are additionally weak organic acids, it is clear that they are protonophoric uncouplers.
A set of 21 phenolic uncouplers was used to derive the present QSAR-model. The intrinsic activity model (based on concentrations in the membrane instead of aqueous concentrations) is based on three descriptors: free energies of solvation of the phenolic anion, ΔGsolv, an empirical parameter for dimer formation and the pKa as a measure for speciation. The two descriptors ΔGsolv and pKa are independent of (the) chemical class. The dimer formation descriptor is currently limited to phenolic compounds. thus, in a first step a way to generalize this descriptor will be developed. Then a first subset of Kinspec measurements of non phenolic compounds will be used to test if this generalization is successful. The focus of the QSAR-model is to achieve a high robustness, i.e. an ability to generalize to a large group of diverse chemicals. After these first steps the milestones illustrated in Figure 1 will be taken.
The possibility to test an already established model with completely new data is rare in QSAR-modeling and is a remarkable feature of this project. It will give an unbiased picture of the predictive power of the QSAR-model and its potential to screen large databases of thousands of compounds.

Conclusions and Relevance for 3R
From a QSAR perspective the proposed model has two features which make it suitable for regulatory use. The number of

descriptors is low and they have a physico-chemical meaning which makes the model transparent, a precondition to legitimize regulatory decisions based on QSAR predictions. The second feature is that it has the potential to be valid not only for one chemical class, but to all compounds acting according to the mode of action of uncoupling and this is still rarely the case in toxicity QSAR. Thus, the proposed project will help priority setting for testing within the framework of the future regulation of chemicals and therefore will save test animals.

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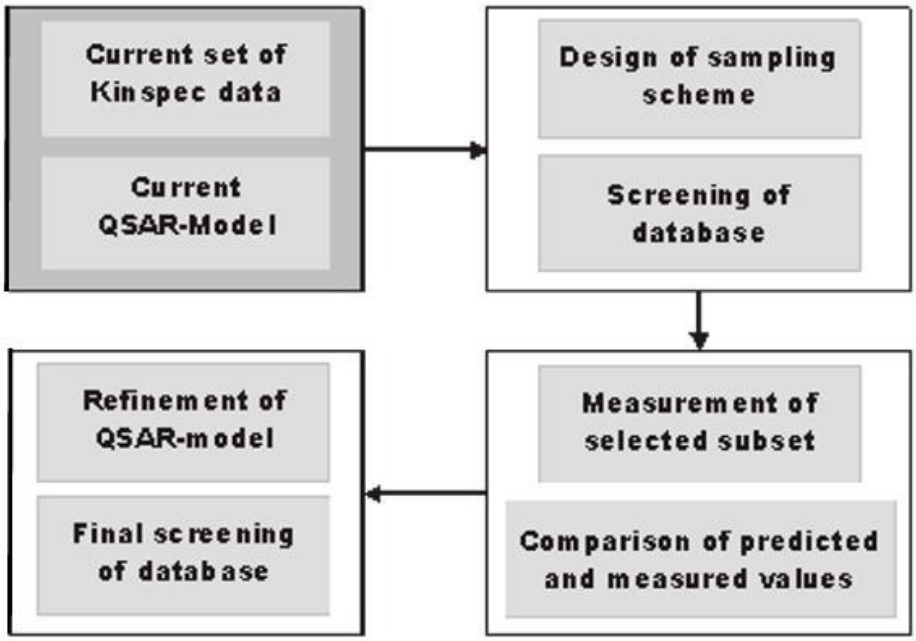

Figure 1: Main steps of research plan. The dark grey box indicates the status at the start of the project.

3R-Project 96-05

Assessment of pain and stress in mice by monitoring gene expression changes

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Keywords: *mice*; *brain*; *pain / stress*; *microarray; molecular biology: pcr*; *refinement*

Duration: 3 years **Project Completion:** 2009

Background and Aim
The overall aim of this project is to develop a rapid, reliable and objective method to assess pain and stress in mice on a molecular level. It is an extension of preliminary investigations using microarrays and complements ongoing studies at our Institute using telemetry and/or behavioural parameters to assess pain and stress in mice (see project 71-00). The problems proposed to be solved in the present study are, first of all, the identification of additional indicator genes and the validation of these genes using biopsy material from surgical models monitored by telemetry. A further step will be the development of a low-density microarray or a set of RT-PCR reactions with the relevant genes in order to monitor pain and/or stress. Finally, we would like to perform comparative molecular monitoring of surgical models and selected genetically modified mice (disease models from our breeding colonies) in order to validate the approach.
This molecular tool will be then used for the identification of different pain and stress levels in mice after experimental manipulations (e.g. surgery or pharmacological tests) and also for checking the housing conditions. In particular this tool is planned to be used to monitor potential pain levels in genetically modified mice

Method and Results
After careful research in available literature, we could identify about 300 genes related to pain, stress, and anxiety and selected 289 genes for spotting in triplicates on a low density microarray. We decided to spot 70 nucleotide long oligomers because this system allows a minimization of the secondary structure, high Tm's, and therefore a normalized hybridization temperature (Fig. 1).
We defined time points in postoperative mouse models for microarray analysis using a telemetric system. We are currently analyzing two different models, a first one where the animals are exposed to moderate pain and a second one with mild pain conditions. In order to identify the best suitable tissues we are currently analyzing expression pattern changes in total RNA pools from different tissues (e.g spinal cord, brain stem, hippocampus, cortex).
Preliminary hybridizations with triplicates were already performed with total RNAs isolated from whole brain. After a first comparison between the data obtained we could identify some genes whose expression was significantly changed in mice with pain.
At the moment, we are validating these genes with real time PCR.

Conclusions and Relevance for 3R
All experimental work with animals has to be monitored by a careful assessment and minimization of pain and stress. The same holds true for breeding of mutant animals. The microarray analysis will be used in parallel to behavioral observations in order to clearly define distress in genetically modified animals. By using post-mortem biopsy material, the microarray technology is much gentler in regards to animal welfare than other approaches like telemetry studies. Furthermore, this system will allow a clear dissociation between phenotype-linked data and artifacts due to the presence of pain/stress in the analyzed animals and therefore reduce the number of animals needed. This analysis tool will be very important for the improvement of anesthesia and analgesia in order to combine the best experimental conditions for both the animals and the needs of the experimentator, allowing a refinement of the animal experiment and at the same time a reduction of the number of animals. Last but not least we think that the objective assessment of pain/stress in mutant animals will play a crucial role in deciding which breeding strategy to choose in order to substantially minimize the number of affected mutant animals in breeding colonies.

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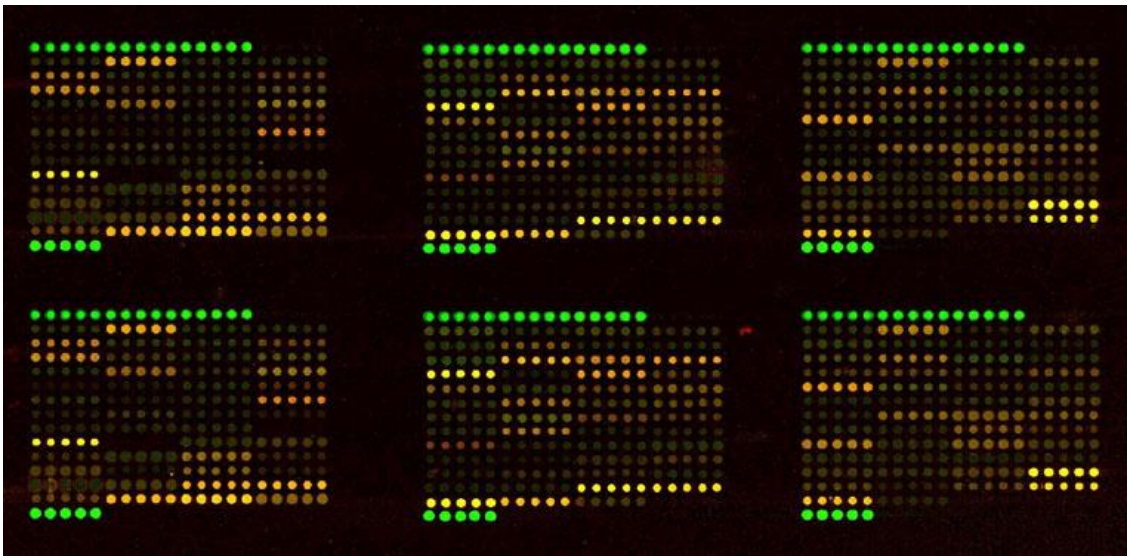

Figure 1A: Examples of signal obtained from total brain RNA reverse transcribed in presence of aminoallyl-modified dUTP and subsequently labeled with a cy-dye hydroxysuccinimide ester. Representative picture of an array-hybridization with aRNA labeled with aaUTP and hydroxysuccinimide ester of the cy-dyes (cy5= ctrl, cy3= brain of a mouse 5 days after telemetry transmitter implantation)

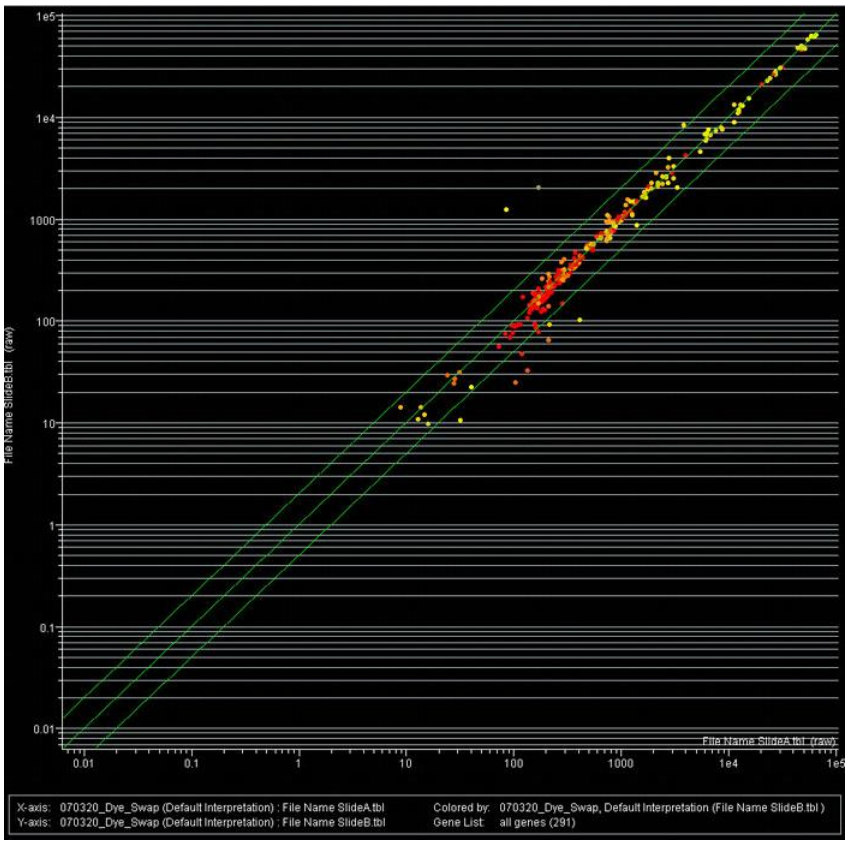

Figure 1B: Scatter plot analysis of a pain/stress microarray.

3R-Project 97-05

Development of a three-dimensional enteric cell culture model for in vitro studies of the intestinal eukaryotic parasites *Cryptosporidium spp.*

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Keywords: *parasites, ectoparasites: cryptosporidium spp.*; *intestine*; *cell cultures: 3d cultures; cell cultures: parasites; microcarrier*; *reduction; replacement*

Duration: 1 year **Project Completion:** 2010

Background and Aim
Although in vitro cultivation, be it axenic or in the presence of host cells, is feasible with species from all major groups of parasites, there are species of medical and/or veterinary importance which are refractory to such attempts. An example is *Cryptosporidium spp.* which are intracellular, intestinal parasites with a broad host range. Their transmission stages (oocysts) have to be produced in very young animals (neonatal mice, calves, lambs, piglets) in which such infections are difficult to control clinically, and death due to dehydration is observed regularly.
Continuous cultivation of the asexual stage of *Cryptosporidium spp.* in conventional 2-D in vitro systems is not possible due to the rapid death of the monolayer cells infected with *Cryptosporidium*. A novel option to overcome the limitations of 2-D cultures is to cultivate such parasites in three-dimensional (3-D) aggregates of intestinal epithelial cells mimicking their natural environment. Such 3-D cell aggregates can be produced under low-shearing conditions in the Rotary Cell Culture System (RCCS, Synthecon, Texas, USA), a technique which was developed by the National Aeronautics and Space Administration (NASA) in order to provide ground-based control experiments mimicking low gravity. The basis for this technique was the observation that cells in suspension tend to aggregate when exposed to microgravity in space. RCCS is an optimized form of suspension culture that - due to its low-shear and low-turbulence operation - minimizes mechanical cell damage and allows cells to aggregate, grow three-dimensionally and to differentiate. In a pioneering study, paper (Nickerson et al., Infect. Immun. 2001, 69, 7106), 3-D cell aggregates of an intestinal cell line (CCL-6) have been shown to display minimal loss of structural integrity and more rapid recovery of cell structure compared to 2-D cultures when infected with *Salmonella* in short time studies. We hypothesized that these particular features of 3-D aggregates should allow to sustain cryptosporidial infections and to support long-term growth of the parasites suitable for parasite propagation and studies on parasite-host cell interactions.

Method and Results
A number of intestinal epithelial cell lines, of human (CCL-6, CCL-241, CCL-244 [HCT-8], HTB-37 [Caco2], HTB-38, CRL-2102 [clone of Caco-2]) or bovine CCL-22 [MDBK] origin, were seeded, either singly of in mixtures, into Rotary Cell Culture System (RCCS-4) vessels containing carriers (Cytodex 3 microcarriers; Soft PGA Felt TE pieces). The cells were incubated in general for four but occasionally up to eight weeks. In order to promote cell differentiation, glucose concentration in the medium was gradually reduced and/or forskolin was added. Hence, multilayer cell aggregates were obtained with both carrier materials (Fig. 1) and, most interestingly in the case of the cell line HTB-38, also without attachment to a carrier surface (Fig 2). Microvilli were present at the surface but also in the intercellular space (Fig. 3), and tight junctions and desmosomes were prominent. Further, goblet cells were present, and the production of mucus could be demonstrated (Fig. 4).
Cell aggregates were exposed to varying inocula (between 10 to 10^7) of sporozoites obtained from oocysts of *C. parvum* (mainly from naturally infected calves but also from experimental animal infection), but also *C. hominis* and *C. serpentis* (isolated from specimens submitted to the diagnostic unit of our Institute) either directly in the RCCS-4 vessels or after transfer of the aggregates to 24-well tissue culture plates. Confocal microscopy revealed a successful infection process (Fig. 5). Characterization of parasite development and growth was done by reverse-transcription real-time PCRs of stage-specific genes, adopted from Jakobi and Petry (2006). None of the experiments revealed a propagation of the parasites beyond 72 h after incolulaton, which corresponds to the well-known situation with inoculations of two dimensional in vitro cell cultures. A recent publication confirmed our findings: Alcantara and colleagues (2008) using the very same cultivation

system (RCCS-4) with CCL-244 (HCT-8) cells, the most suitable cell line for 2D experiments with *Cryptosporidium*, grown on intestinal submucosa grafts as carriers, observed an increase of parasite growth until 48 h post inoculation (p.i.) by qPCR, a signal equal to the inoculum 72 h p.i.. and no signal at 96 h p.i.

Conclusions and Relevance for 3R

This start-up project revealed that a number of intestinal epithelial cell lines can be grown in the Rotary Cell Culture System over a long period of time to form 3-D cell aggregates, to provide an environment similar to that between brush border microvilli of epithelial cells in vivo but this condition did not suffice to propagate *Cryptosporidium* as hypothesized, presumably for reasons that might be associated with the very peculiar intracellular but extra-cytoplasmic localization of this parasite.

However, we reckon that this cultivation system might be of value to cultivate small pieces of calf intestine allowing for standardized and reproducible inoculation of parasites to analyze the transcriptomes of different developmental stages. Further, the 3-D cell aggregates produced in the RCCS-4 might comply with the requirements of a variety of other pathogens (virus, bacteria, protozoal and multicellular parasites) of medical and veterinary importance which infect epithelia of the gastrointestinal, but also the respiratory or urogenital tract, and such cell aggregates might serve as a relevant model to investigate a number of aspects.

Therefore, our work might contribute to bridge the gap between experiments done with 2-D conventional cell cultures with their inherent limitations and those performed in live animals. By providing a better model for what happens in vivo, these 3-D cultures will allow researchers to considerably reduce their use of experimental animals in their investigations on a variety of infectious agents.

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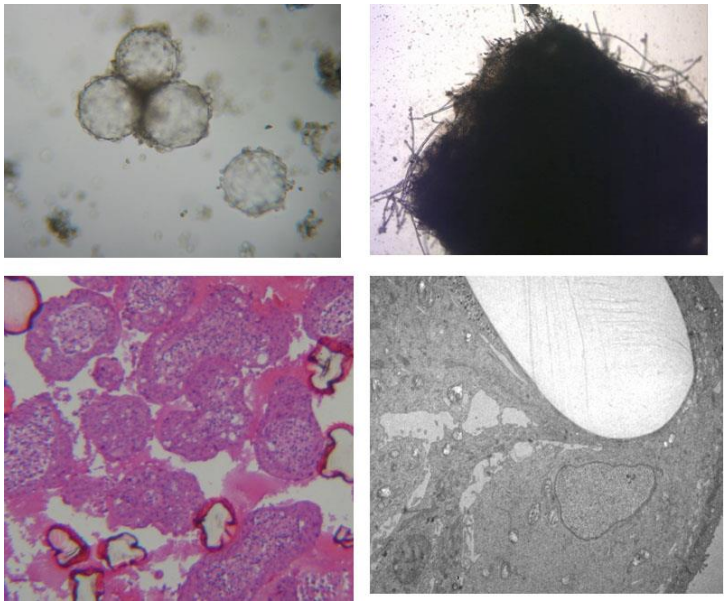

Figure 1: Cultivation of epithelial cells in ‚Rotary Cell Culture System' vessels with different carrier materials. Left column Cytodex 3 microcarrier beads (light microscopical image and histological overview, dark pink: cross-section of collapsed beads). Right column: PGA felt (light microscopical image and TEM with single light-colored PGA fiber) (TEM by A. Hemphill, Institute of Parasitology, University of Berne).

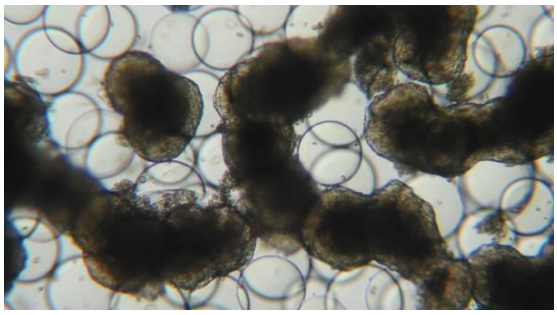

Figure 2: 3-D cell aggregates of the epithelial cell line HTB-30 grown in ‘Rotary Cell Culture System‘ vessels: Growth without attachment to carrier beads.

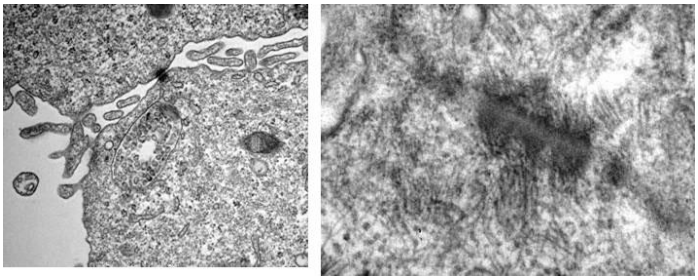

Figure 3: Formation of microvilli , tight junctions und desmosomes in cell aggregates from 3D-cultures (TEM by A. Hemphill, Institute of Parasitology, University of Berne).

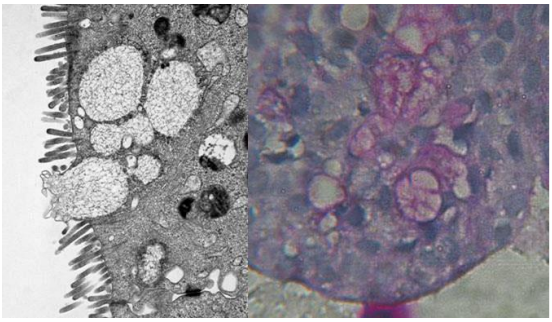

Figure 4: Formation of microvilli und goblet cells (TEM, left) und mucus (PAS stain, right) in cell aggregates from 3D-cultures (TEM by A. Hemphill, Institute of Parasitology, University of Berne).

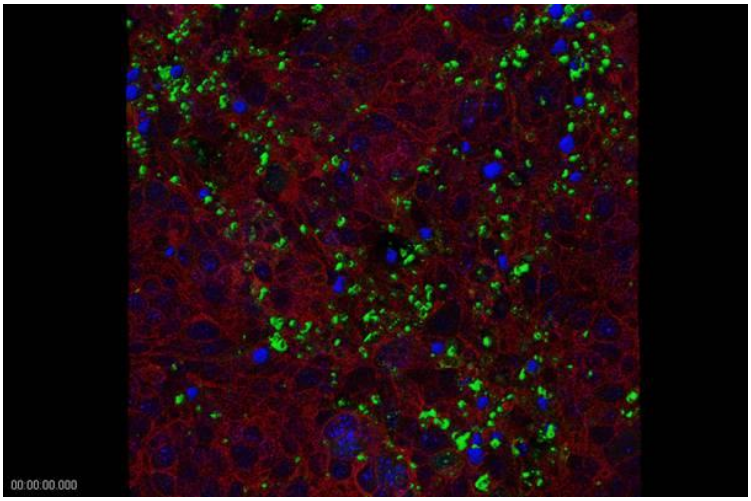

Figure 5: Intracellular stages of Cryptosporidium parvum (green) in HTB-38 cell aggregate two days after inoculation. Parasite staining with FITC-labelled HP-lectin (confocal microscopy; red: phalloidin staining of actin; blue: DAPI stain of nuclei) (Confocal image by A. Hehl, Institute of Parasitology, University of Zürich).

3R-Project 98-05

Establishment of a murine syngeneic co-culture system of intestinal epithelial cells with intraepithelial T lymphocyte subsets.

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Keywords: *human; mice*; *endothelia; intestine; t-cell*; *cell cultures: co-cultures; molecular biology: pcr*; *reduction; replacement*

Duration: 2 years **Project Completion:** 2008

Background and Aim
Investigations of the mutual cellular interactions between epithelial cells and intraepithelial lymphocytes (IEL) in the intestine are difficult to achieve due to the limited accessibility of this compartment to direct experimental interventions. Furthermore, relevant functional parameters, such as the influence of distinct T cell subsets on barrier function of the intestinal epithelium are difficult to determine in experimental animals. These aspects highlight the importance of establishing in vitro systems to obtain relevant information on the interplay of these cellular systems in maintaining local tissue homeostasis.
The establishment of suitable in vitro co-culture systems was so far hampered by the poor yield of IEL isolated from intestinal epithelium and particularly the short survival of some IEL subsets which may be < 12 hours in vitro under normal T cell culture conditions.
Hence, the specific aims of the present proposal are
1. to increase the yield of IEL isolated from the intestinal epithelium of mice and humans
2. to optimize culture systems for extended culture periods for all IEL subsets
3. to establish co-cultures of these IEL subsets with syngeneic intestinal epithelial cell lines and to determine whether and how antigen-specific activation of IEL affects the permeability of the intestinal epithelial cell layer and the pro- and anti-inflammatory properties of epithelial cells.

Method and Results
Protocols for the reproducible separation of intraepithelial lymphocytes (IEL) from contaminating epithelial cells by elutriation centrifugation will be established. With this technique we have already managed to substantially enhance the yield of IEL from human intestinal tissue samples (Table in Figure 1).
Currently, we are also optimising this method for the isolation of mouse IEL for subsequent co-culture with a syngeneic (H-2b) intestinal epithelial cell line (mICCl2, YAMC or MSIE). Culture conditions in vitro need to be established and optimised to allow for an extended co-culture of all IEL subsets with these epithelial cell lines. The mutual interactions and consequences of an IEL-epithelial cell culture will be monitored using transepithelial resistance measurements, gene expression profiles of all cell populations involved (e.g. using Realtime RT-PCR procedures), and ELISA- and Luminex® -based methods to determine secretion of cytokines.

Conclusions and Relevance for 3R
Reduction in animal experimentation:
The establishment of the envisaged syngeneic intestinal epithelial cell – intraepithelial lymphocyte co-culture system should allow to directly monitor time-dependent changes in these cell populations. Furthermore, it will allow direct experimental interventions such as antigen-specific activation of the IEL subsets (using MHC-restricted peptides), and mimicking an inflammatory situation by adding either recombinant pro-inflammatory cytokines or relevant inflammatory cells.

Refining experimental methods involving live animals:
The optimisation of the isolation protocols for intestinal IEL will not only lead to a more representative and thus more reproducible population of IEL, but may also substantially reduce the number of donor animals required for the isolation of IEL. Furthermore, the epithelial cell lines can be genetically modified (e.g. using siRNA) to experimentally assess the

direct functions of candidate genes and their products relevant for the IEL-epithelial cell interactions. In the future, this may make the generation and the use of genetically modified mice often obsolete for studying intestinal IEL functions and lymphocyte - epithelial cell interactions. Last but not least, with these co-culture systems we expect to obtain relevant information regarding the biology of intestinal IEL that will allow more precise analysis and interpretation of the pathophysiological role of IEL in humans.

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	Percoll (44/67%)	Elutriation Centrifugation
Total number of cells (IEC and IEL) after EDTA/DTT incubation ("starting material")	8×10^7	8×10^7
Total number of human small intestinal IEL in starting material	9×10^6 (11%)	9×10^6 (11%)
% T cells in IEL enriched fraction	25%	61%
Yield of CD3 T cells (in %)	2.3×10^6 (26%)	8.0×10^6 (89 %)

Figure 1: Representative example of a comparative isolation of human intraepithelial lymphocytes by Percoll density gradient: Equal numbers of cells released from the intestinal epithelium were subjected to either conventional Percoll gradient, or elutriation centrifugation, respectively.

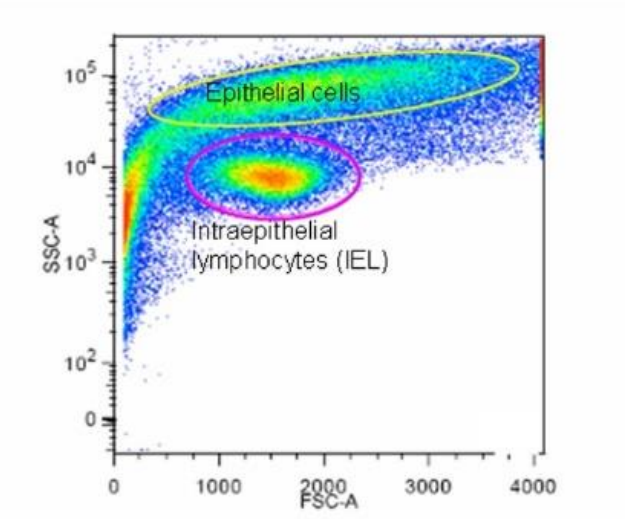

Figure 2: Scatter blot of the IEL-enriched fraction following elutriation centrifugation. In this preparation 88% of the cells are intraepithelial lymphocytes.

3R-Project 99-05

The NEMO network (Non-mammalian Experimental Models for the study of bacterial infections)

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Keywords: *bacteria; drosophila; protozoa: amoeba*; *infectious diseases*; *reduction; replacement*; *infectiosity*

Duration: 4 years **Project Completion:** 2010

Background and Aim
To test the ability of a bacteria to cause a disease it is usually necessary to infect a mammalian host and allow the disease to progress. These experiments inflict significant suffering to the animals. Our general aim is to stimulate the emergence of a community of scientists using alternative non-mammalian hosts for the study of bacterial infections. Our common belief is that many experiments currently carried out using mammalian hosts could be advantageously replaced by the use of alternative non-mammalian hosts.

The NEMO network of laboratories was created in February 2005, initially as an informal gathering of research groups involved in similar subjects. Our specific goals are:

1-To organize an annual meeting on the theme of Non-mammalian hosts for the study of bacterial infections, in order to stimulate exchanges among research groups.

2-To strengthen our research in this field through a series of collaborative projects, for which we hope to find financial support.

3-To publicize the use of alternative non-mammalian hosts in the scientific community.

The five research groups that originally created the NEMO network are:

Pr. Dr. P. Cosson, Centre Médical Universitaire, Geneva, Switzerland
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Project:
Extensive analysis of bacterial virulence in *Dictyostelium*

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Project:
Bacterial virulence and innate immune response: *Drosophila* as a model system.

Dr. G. Greub, Microbiology Institute, Faculty of Biology and Medicine, University of Lausanne, Switzerland
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Project:
Free-living amoebae as a tool to study intracellular pathogens.

Prof. Dr. Hubert Hilbi, Institute of Microbiology, ETH Zürich, Switzerland
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Project:
Amoebae: a cellular pathogenesis model for the legionnaires´disease agent *Legionella pneumophila*

Dr. Thierry Soldati, Department of Biochemistry, University of Geneva, Switzerland
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Project:
The *Amoeba Dictyostelium* as a model host for *Mycobacterium marinum* infection and persistence.

Method and Results

see publications cited in the Annual Reports

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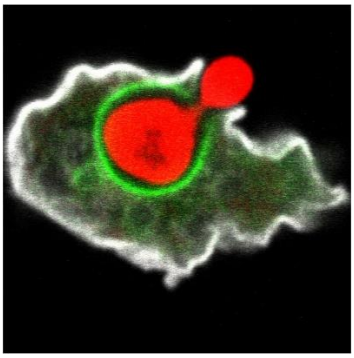

Figure 1: To study infectious diseases, it is not always necessary to infect animals. Researchers in the NEMO Network use more simple hosts such as amoebae, or drosophila flies. In this picture an amoeba (white) eating up a yeast cell (red).

3R-Project 100-06

Development of an in-vitro system for modeling bioaccumulation of neutral, ionizable, and metabolically active organic pollutants in fish

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Keywords: *fish*; *ecotoxicology*; *bioaccumulation*; *biochemical / analytical; in silico*; *reduction; replacement*; *bioaccumulation; drug screening*

Duration: 2 years **Project Completion:** 2008

Background and Aim

The recent implementation of the new European chemicals legislation (REACh) has produced the requirement of a lot of additional testing, including bioaccumulation assessment in fish and other aquatic species. In addition continued activities in the field of Persistent Bioaccumulative and Toxic (PBT) assessment and the introduction of new Persistent Organic Polutants (POPs) to the Stockholm convention confirms the need for robust and efficient methods to assess the bioaccumulation of chemicals. Bioconcentration testing is highly animal intensive. Thus alternative test methods have to be developed to reduce the number of test animals or to avoid the use of test animals by using alternative *in-vitro* test systems.
Bioaccumulation encompasses bioconcentration, i.e. the passive uptake (in fish via the gills), and biomagnification, i.e., the uptake via ingestion of contaminated food. Bioconcentration integrates the uptake, distribution and elimination of a substance due to water-borne exposure. In fish, bioaccumulation is typically dominated by bioconcentration due to the high surface area of the gill membranes. Metabolism decreases the Bioconcentraton Factor (BCF). Thus assessment models and in-vitro methods should account for metabolism. The *in-vitro* assay to be developed should therefore account for metabolic processes in fish.
The main goal of this project is the refinement of a new *in-vitro* method for evaluating bioconcentration kinetics in fish using the Parallel Artificial Membrane Permeability Assay (PAMPA) (Figure 1) to assess membrane permeation and membrane-water partitioning. With this information it should be possible to develop a prediction model to replace animal testing with the OECD 305 fish bioconcentration test. Low cost *in-vitro* tools are needed at the screening stage of assessment of bioaccumulation potential of new and existing chemicals because the number of chemical substances needs to be tested highly exceeds the capacity of in-vivo bioconcentration tests.
The second goal was to develop a prediction model to link the *in-vitro* results to *in-vivo* bioconcentration data in order to replace animal testing with the OECD 305 fish bioconcentration test.

Method and Results

The PAMPA system has been developed and widely used in pharmacokinetics research for assessing uptake of pharmaceuticals in the gastrointestinal tract and across other membrane barriers, such as epithelium cells. In a previous study we have used PAMPA for the first time to evaluate its potential for assessing passive absorption and elimination in small fish across fish gills. The initial work was very promising but the method still needed refinement to be applicable for hydrophobic pollutants of concern.
To overcome the difficulties associated with low aqueous solubility and high membrane affinity of highly hydrophobic chemicals, we measured the rate of permeation from the donor poly(dimethylsiloxane) (PDMS)

disk to the acceptor PDMS disk through aqueous and PDMS membrane boundary layers and term the modified PAMPA system “PDMS-PAMPA”. In a second step, we combined the PDMS-PAMPA with an *in-vitro* metabolism assay.
For highly hydrophobic chemicals, it is very difficult to evaluate how fast they cross the membrane by analyzing aqueous concentration due to their low aqueous solubility and high partition coefficient between membrane and water. Therefore, 1 mm thick PDMS disks were placed in the donor and the acceptor solution to serve as a passive dosing/sampling phase. Twenty organic chemicals of which uptake and elimination rate constants were reported in literature for small fish were chosen for validation of the modified PAMPA [1].
For highly hydrophobic chemicals, membrane diffusion is much faster than aqueous boundary layer (ABL) diffusion both *in-vivo* and *in-vitro*. We developed an *in-vivo* to *in-vitro* extrapolation model to use the proposed PAMPA system for prediction. There are three factors affecting the performance of the *in-vitro* to *invivo* prediction model, the ABL thickness, the partition coefficient to the membrane surrogate phase (PDMS in this study [2]), and the surface-to-weight ratio of fish. An advantage of the presented PDMS-PAMPA system lies in the fact that the prediction model to relate the *in-vitro* results to *in-vivo* is not just a best-fit model but a theoretical model based on the underlying mechanistic processes. Using this model we obtained a good correlation with measured *in-vivo* elimination rate constants in fish, with the exception of metabolizable compound. For those we presently work in supplementing the experimental device by a fish S9 model system to include biotransformation processes in the in-vitro assay.

Conclusions and Relevance for 3R

The measured permeability of the 20 test chemicals was proportional to the passive elimination rate constant in fish and was used to predict the “minimum” *in-vivo* elimination rate constant. The *in-vivo* data were very close to predicted values except for a few polar chemicals and metabolically active chemicals, such as pyrene and benzo[a]pyrene. Thus, PDMS-PAMPA can be an appropriate *in-vitro* system for nonmetabolizable chemicals.
If coupled with an *in-vitro* metabolism assay such as the fish S9 assay, which was also applied and refined in this project, a comprehensive *in-vitro* model is available for the prediction of bioconcentration potential of highly hydrophobic compounds.
Bioconcentration assessment in fish is highly animal and labor intensive. According to the OECD test guideline 305 „Bioconcentration: Flow-through Fish Test" (OECD, 1996) the test is performed during 28 days in two phases – uptake and depuration- with at least 9 sampling points using at least four fish each. Thus a minimum number of 40 fish is required for the determination of one BCF value for one fish species and one chemical. Considering that in the future more chemicals need to be assessed for their PBT properties (P = persistence, B = bioaccumulation, T = toxicity) due to the implementation of the new European Chemical’s legislation, there is an imperative need for alternative methods.

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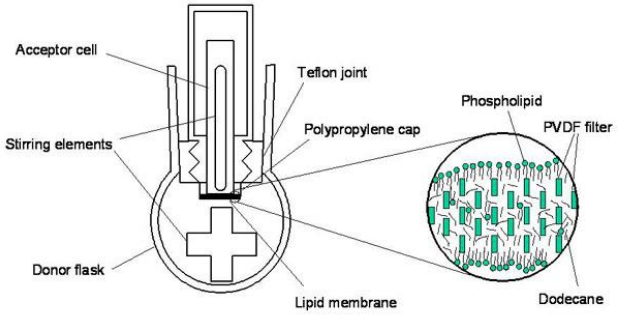

Figure 1: Experimental set-up of the PAMPA system

3R-Project 101-06

Organotypic CNS slice cultures as an in-vitro model for immune mediated tissue damage and repair in multiple sclerosis

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Duration: 3 years **Project Completion:** 2009

Background and Aim
Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system (CNS) affecting about one out of 700 adults in Europe. Histological data and results from animal experiments indicate that T cells, B cells, macrophages and antibodies contribute to the formation of immune-mediated lesions in the brain and spinal cord of MS patients. The presence of clonally expanded immunoglobulins in the cerebrospinal fluid (CSF) of MS patients is one of the most prominent hallmarks of the disease. However, neither the specificity of these immunoglobulins nor the triggers causing demyelination and axonal damage are known.

A large proportion of current MS research is currently performed in a model system called experimental autoimmune encephalomyelitis (EAE). For the induction of EAE, rodents or nonhuman primates are actively immunized with myelin antigens, thereby inducing an inflammation of the CNS. This inflammation leads to a progressive paralysis of the animals before they eventually are sacrificed. While CNS inflammation can also be induced by adoptively transferring MOG-reactive T-cells alone, antibodies are additionally required for the induction of demyelination in rats and nonhuman primates. Likewise, deposits of immunoglobulins and complement can be detected in MS lesions, supporting the role of antibodies in MS pathogenesis.

Although EAE has provided many valuable insights into the pathology of MS, it only partially reflects MS and it is difficult to discern the role of different arms of the immune system with respect to the induction of CNS damage. Special attention is currently being paid to mechanisms inducing axonal damage, which is thought to be the morphological correlate of permanent neurological impairment. Using organotypic CNS slice cultures, we have therefore established an experimental model system, in which the complex architecture of the CNS is maintained and which allows the detailed investigation of interactions between the CNS and components of the immune system, as well as mechanisms of CNS damage and repair.

Method and Results
Organotypic slice cultures provide an excellent tool to characterize the role of humoral and cellular immune effector mechanisms in MS separately. Using brain slices from transgenic mice expressing green fluorescent protein (GFP) in myelin (PLP-GFP), we established confocal live imaging of the immune – CNS interface.

In order to investigate antibody-mediated CNS tissue damage, we used an antibody specific for a myelin antigen (MOG, myelin oligodendrocyte glycoprotein) in the presence of complement (figure 1A, figure 2A). Addition of the antibody alone or of an isotype antibody with or without complement did not induce morphological changes (figure 2C). Time-lapse imaging showed that demyelination starts already after 4 hours of antibody/complement addition and is completed within 24 – 48h (figure 1A 43h). Interestingly, in spite of complete demyelination, axons remained intact (figure 2A'). After removal of the detrimental factors antibody/complement (figure 1A 43h), marked remyelination was observable (figure 1A 53h – 77h). We concluded that antibody-mediated demyelination alone is insufficient to induce axonal damage (Harrer et al., 2009).

Next, we investigated the effects of myelin-directed T cells. We demonstrated that an inflammatory CNS environment mimicked by IFNg can enable oligodendrocytes (ODCs) to process and present endogenous antigen via MHC class I molecules (figure 3). Consequently, autoaggressive, cytotoxic CD8+ T cells targeting myelinated structures strongly

invaded organotypic brain clices and directly transsected myelinated axons (figure 4). As a result, they not only caused patchy myelin dissintegration but alos widespread axonal loss due to “collateral bystander damage” (figure 5). Although the outlined scenario is most likely not the only relevant mechanism of CNS tissue damage in inflammatory CNS diseases, we are confident that our findings critically contribute to the understanding of CD8-mediated neuropathology in CNS inflammation, strengthen a pathogenic role of CD8+ T cells in MS, and advocate for the development of future immunotherapies aiming at the CD8-myelin/ODC interface (Sobottka et al., 2009).

Conclusions and Relevance for 3R

Reduce:
Using slice cultures of hippocampus and cerebellum, several experimental conditions can be tested with tissue obtained from a single mouse. In contrast, for the induction of EAE, several mice have to be included per experimental group. Using transgenic mice expressing GFP in myelin, experiments can be investigated by (confocal) live imaging.

Refine:
Induction of EAE leads to severe disability and suffering of mice. Organotypic slice cultures are performed after euthanasia, which substantially refines the experimental procedures. Bystander reactions are less likely to happen in an in-vitro setup than
in-vivo. Additionally, organotypic slice cultures reflect the three-dimensional in-vivo structure more precisely than conventional cell cultures.

Replace:
Organotypic slice cultures present an alternative tool to study the interactions between the CNS and specific components of the immune system. Therefore, many questions, which are now investigated in the animal model of MS, can alternatively be studied using this in-vitro system, thus replacing many in-vivo experiments.

Congratulations: The "Biogen Dompé Research Award for Multiple Sclerosis" was awarded to Dr. Sobottka and Dr. Goebels (2009) in recognition of the work "Collateral bystander damage by myelin-directed CD8+ T cells causes axonal loss".
See also http://www.forschungspreis.biogen-dompe.ch/front_content.php?idcat=202

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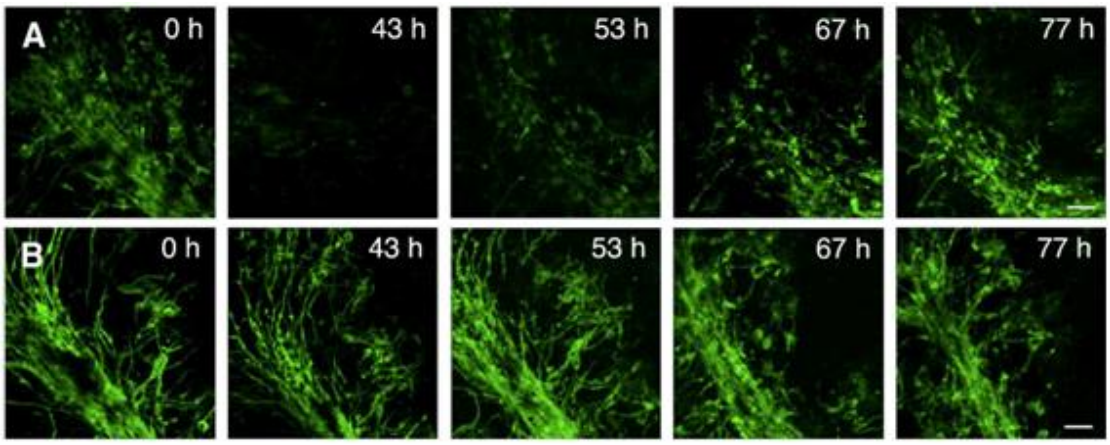

Figure 1: Confocal live imaging of remyelination. Micrographs of PLP-GFP OSC were taken before treatment (A 0 h, B 0 h), during demyelination with the anti-MOG antibody and complement (A 43 h) and during remyelination (A 53 h, 67 h, 77 h). Panel B show images of control OSC incubated in parallel with isotype control antibody and complement taken at the same time points as images in panel A. After removal of the anti-MOG antibody and complement, remyelination begins in the vicinity of scattered oligodendrocyte nuclei and gradually extends to form a new network of myelinated fibers (A 53 h, 67 h, 77 h). OSC treated with isotype control antibody and complement (panel B) show only minor morphological changes due to the experimental setup. Scale bar, 50 um (from Harrer et al., 2009).

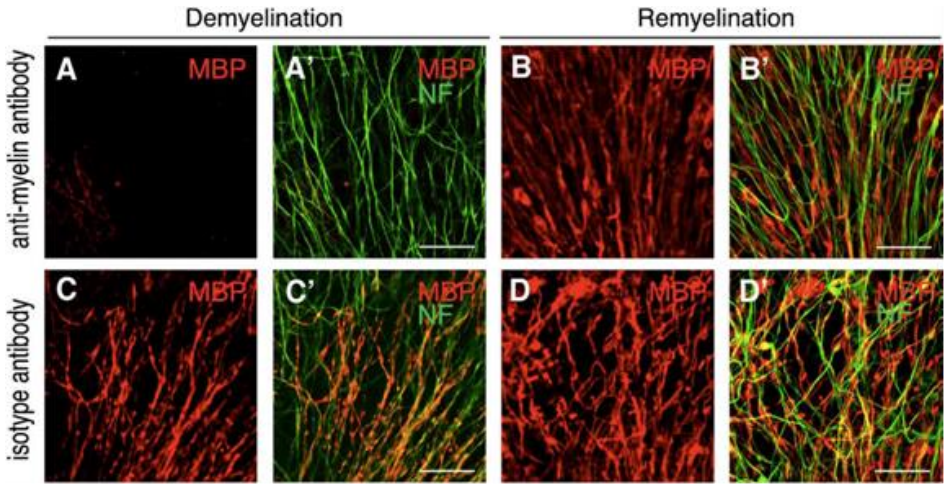

Figure 2: Remyelination is guided by morphologically intact axons. OSC were treated with anti-MOG antibody and complement (A, B) or with an isotype control antibody plus complement (C, D). After an incubation period of 48 h, OSC were either fixed (A, C) or further incubated for 10 days in fresh culture medium (B, D). Strongly demyelinated OSC (A) spontaneously remyelinated (B) at sites, where axons had remained morphologically intact (B"). The morphology of control OSC treated with an isotype control antibody and complement was not visibly damaged (C, D). Staining for MBP is shown in red, for Neurofilament200 (NF) in green. Scale bar, 50 uM (from Harrer et al., 2009).

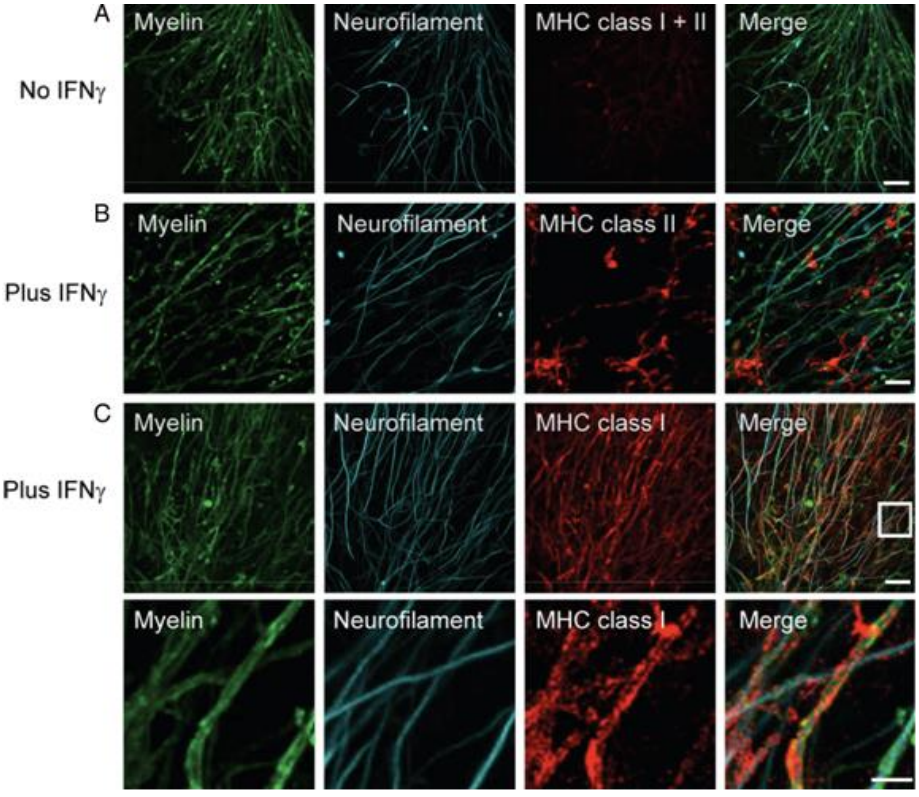

Figure 3:Incubation with IFNg induces MHC class I and II expression. Confocal images of costained MHC class I and II molecules (red) and NF (cyan) reveal only marginal MHC expression in untreated ODC-OVA x PLP-GFP brain slices (A). Yet, presence of IFNg (100 U/ml, 72 hours) induces MHC expression (B and C). Whereas MHC class II was most likely observed on microglia (B), MHC class I was mainly detected on oligodendrocytes (C). The lower panels in C reflect the boxed area in the upper merged panel. Scale bars equal 20 um in A, B, and upper panel in C and 7.5 um for lower panel in C (from Sobottka et al. 2009).

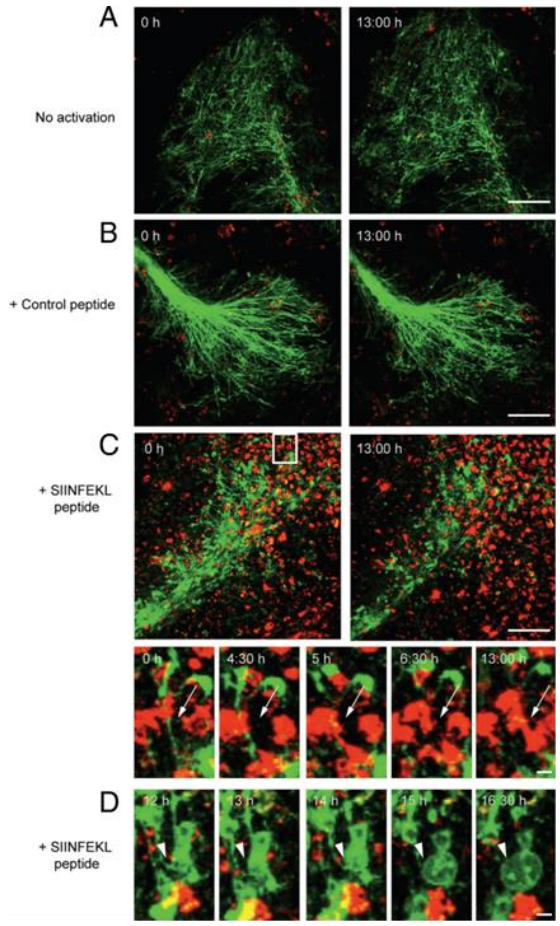

Figure 4: Direct transsection of myelinated axons by CD8+ OT-I T cells. Frames from confocal time-lapse sequences were captured and document only little invasion of nonactivated (A) or control peptide (B) prestimulated CD8+ OT-I T cells (red) into ODC-OVA x PLP-GFP brain slices. Myelin damage was not observed in either situation (A and B). Contrarily, SIINFEKL prestimulated CD8+ OT-I T cells entered brain slices to a great extent (C) and directly attacked myelin (green) as pointed out by arrows (subregion of C). Additionally, pronounced blebbing of oligodendrocytes after T cell attack was observed as pointed out by arrowheads (D). In the upper left corner, time elapsed after start of sequence is documented, scale bars equal 30 um in A, B, and C and 10 um for subregion of C and in D (from Sobottka et al. 2009).

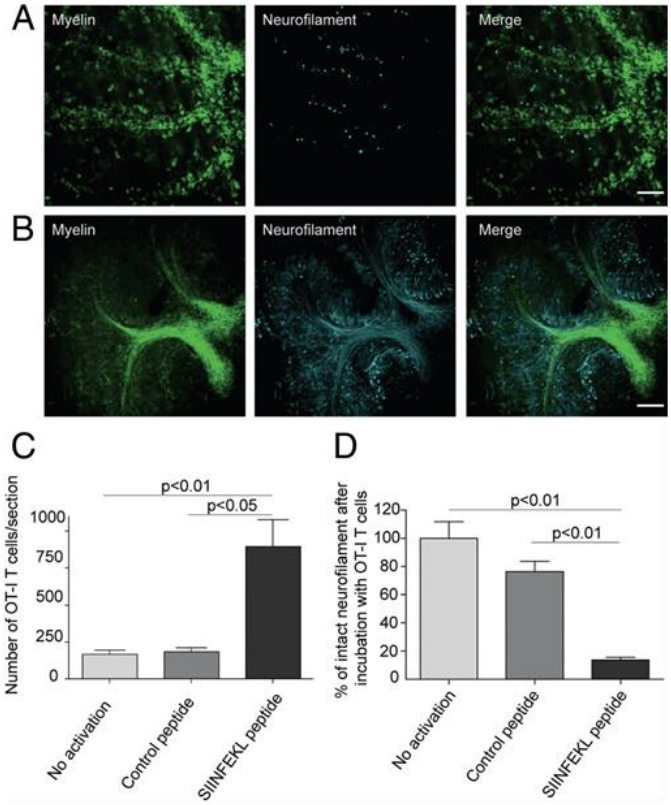

Figure 5: SIINFEKL-prestimulated cytotoxic OT-I T cells invade brain tissue and cause damage of myelinated axons. Spot analysis from confocal timelapse imaging revealed that SIINFEKL prestimulated CD8+ OT-I T cells invaded ODC-OVA x PLP-GFP slices to a significantly greater extent than nonactivated or control peptide-prestimulated CD8+ OT-I T cells (C). Extensive disruption and irregularities of both myelin (green) and axons (cyan) in ODC-OVA x PLP-GFP brain slices were observed after incubation with SIINFEKL-prestimulated CD8+ OT-I T cells (A) in contrast to well-maintained structures in slices incubated with nonactivated CD8+ OT-I T cells (B). Quantification of axonal damage confirms that neurofilament was significantly injured in ODC-OVA x PLP-GFP brain slices incubated with SIINFEKL-prestimulated CD8+ OT-I T cells when compared with controls (D). Results are expressed as percentage of intact neurofilament compared with ODC-OVA x PLP-GFP brain slices incubated with nonactivated CD8+ OT-I T cells. Values are +/- mean SEM, n = 3; for P value generation one-way analysis of variance followed by Bonferroni posttest was used to compare among groups (C and D). Scale bars equal 200 um in A and B (from Sobottka et al. 2009).

3R-Project 102-06

Isolated, autologous blood-perfused heart: Replacement of heterotopic heart transplantation

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Keywords: *rat*; *heart*; *transplantation*; *ischemia*; *reduction; replacement*

Duration: 2 years **Project Completion:** 2009

Background and Aim

The aim of this project is to develop a novel *ex vivo* model of rat heart perfused with autologous blood (see Fig 1). Responses of the isolated blood-perfused heart to reduction in oxygenation will be performed to characterise the model and define its potential to replace a common Tyrode-perfused Langendorff heart model and *in vivo* heterotopic heart transplantation model of ischemia-reperfusion injury.

Warm no-flow ischemia ultimately results in development of myocardial necrosis within 20-30 minutes after the interruption of blood circulation. In contrast, low-flow ischemia triggers a reversible reduction of contractile function in attempt to match ATP supply and consumption, a condition known as “hibernating myocardium”.

Mechanisms involved in a cross-talk between the myocardial contractile machinery, oxygen availability and metabolic pathways in hibernating heart are a matter of intensive investigation. According to our suggestion hypoxia is a major factor in tuning multiple responses which result in hibernation. Our new experimental model will allow precise control of blood oxygenation as well as blood flow rate thus mimicking hypoxia and low-flow ischemia conditions. Multiple parameters will be monitored in the heart tissue and in blood used for perfusion to evaluate its metabolic condition, redox state as well as the degree of myocardial stress and injury. The obtained results will then be compared with observations made on human patients with coronary artery disease resulting in acute or chronic low-flow myocardial ischemia.

Method and Results

Wistar male rats are anaesthetised with isofluorane, heparinised and 6-10 ml of blood are collected from the vena cava. The animals are decapitated and their heart harvested into ice-cold Tyrode buffer. After the circulation circuit is filled with blood the heart is mounted at the cannula and retrograde perfusion starts in a closed circuit with a hollow fiber oxygenator constructed by Johannes Vogel with blood oxygenated with a gas mixture containing O_2, 5% CO_2 and 75% N_2 (see Fig 1). After 20 min of equilibration at pO_2 20 kPa oxygen concentration, hypoxic perfusion is initiated by reducing the oxygen concentration in the gas phase of the oxygenator to 15, 10 or 5 %. During the perfusion heart rate is monitored using a piezoelectric pressure sensor.

After the perfusion protocol is completed the hearts are dismounted, and heart tissue harvested for the measurements of ion and water balance, metabolic and redox state markers.

Blood plasma samples are collected to measure (NO_{2^-}+ NO_{3^-}), BNP and TnT thus assessing mechanical stress and tissue damage. In a separate set of experiments, the use of glucose and fatty acids will be measured as a function of oxygen concentration in blood using autoradiography.

We are currently monitoring the changes in contractile rate, redox state as well as water and ion balance in the myocardial tissue of old and young animals exposed to hypoxic conditions. The preliminary data indicate that ageing makes the heart more vulnerable to the decrease in the blood oxygen pressure. Whereas decrease the hemoglobin oxygen saturation from 100 to 30 % triggers bradycardia and stunning in the aged heart, contractile rate of the young animal’s heart remains unaltered (Fig 2A and B). Hypoxia-induced decrease in contractile rate is the aged myocardium is followed by the oxidative stress, decrease in the hydrolytic activity of the Na/K ATPase and concomitant Na+ accumulation in the myocardial tissue. The observed changes in the heart function were not linked to the tissue ATP although basal ATP levels in the aged myocardium were significantly lower than those in the young animals.

Conclusions and Relevance for 3R

We have developed, optimised and validated the isolated autologous blood-perfused rat heart model. The heart sustains is spontaneous contractile activity for up to 3 h with the heart rate about 250-350 beats per minute and ECG parameters similar to those measured in an animal. Blood volume used for perfusion is known (5-7 ml) allowing precise adjustments of the concentration of any drug used. Temperature, blood perfusion rate and oxygenation state of blood are variables

that may be precisely controlled as well. Blood shear-induced trauma and haemolysis present the major limitation of the model reducing experimentation time to 2 hours when blood is not replaced. This experimental model was tested when investigating the effects of deoxygenation on the heart rate, electric activity, ion/water balance, glucose metabolism and redox state. The obtained data indicate the ability of the heart to sense the changes in oxygen availability and rapidly adjust its pumping function as well as ATP generation and expenditure to the reduction in oxygen supply in order to avoid acute myocardial degeneration.

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Figures

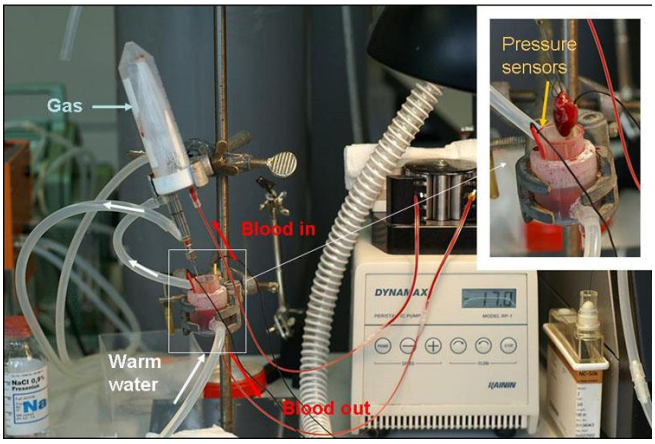

Figure 1: Experimental set-up

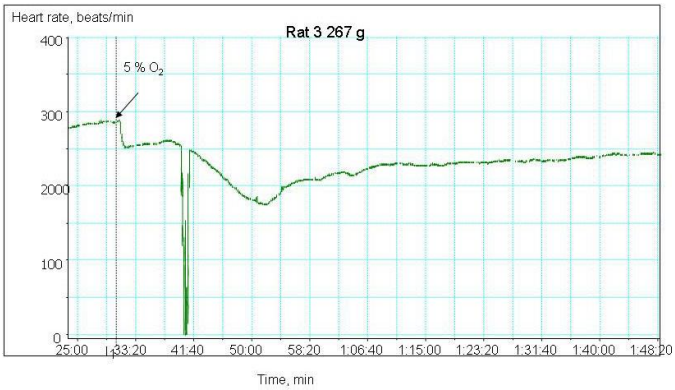

Figure 2A: Hypoxia-induced changes in heart rate in ex vivo perfused heart of a young animal

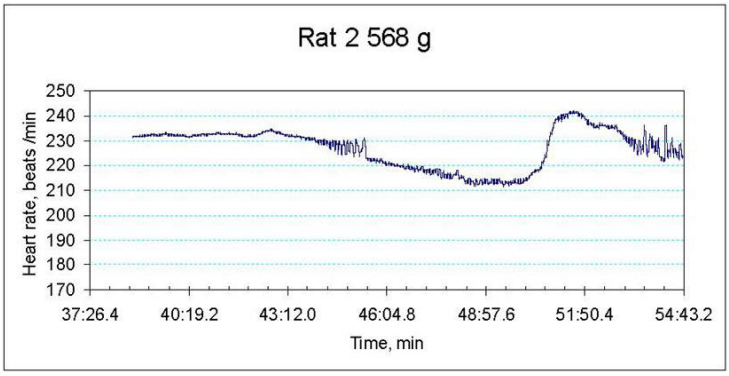

Figure 2B: Hypoxia-induced changes in heart rate in ex vivo perfused heart of an old animal

3R-Project 103-06

An in vitro Model of Central Nervous System Infection and Regeneration: Neuronal Stem Cells as Targets of Brain Damage & Regenerative Therapies in Bacterial Meningitis

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Keywords: *bacteria; rat*; *brain*; *transplantation*; *ischemia*; *cell cultures: co-cultures*; *reduction; replacement*
Duration: 4 years **Project Completion:** 2012

Background and Aim
Bacterial meningitis remains a devastating disease with high morbidity and mortality. Despite advances in treatment and care, the in-patient rate of mortality through bacterial meningitis is approx. 25% and, as a consequence of brain damage, clinically relevant neurological sequelae emerge in up to 50% of survivors. How infection leads to brain injury remains largely unexplained, but converging evidence suggests that the clinical outcome of bacterial meningitis is determined by the host's response to the infectious agent in the brain.
Owing to the multi-factorial pathogenesis of meningitis involving the interplay between the susceptible brain cell type, the bacterial pathogen and the host's inflammatory reaction, including potential therapeutic approaches supporting brain repair functions, the majority of the current research on meningitis is carried out *in vivo*. The degree of suffering according to the Swiss Federal Veterinary Office is considered intermediate to severe (e.g. categories 2-3) for many of these studies. In terms of animal use and welfare it would be highly desirable to replace part of this research by *in vitro* studies. We established *in vitro* models that reproduced important patho-physiological processes of damage and tissue regeneration in infectious diseases of the brain.
Brain injury caused by bacterial meningitis most frequently affects the cortex and the hippocampus (HC)[1, 2]. Cortical damage is associated with areas of focal ischemic necrosis. Hippocampal injury is documented in over 75% of patients who die from the disease and in corresponding animal models. This form of injury is characterized by apoptotic cell death of immature neurons, e.g. neuronal stem cells and/or their progeny in the dentate gyrus, often resulting in long lasting learning deficit. Hippocampal injury is limited to the dentate gyrus, a site of continuous formation of new neurons and therefore potentially well equipped for brain repair[3]. However, since the neurofunctional sequelae persist for decades after meningitis, the hippocampal repair potential seems to be ineffective or insufficient to compensate for the brain damage.
A potentially attractive treatment option for the support and/or reconstitution of neurogenesis after damage to the stem cell niche is the delivery of regenerative cells to the site of cell loss. Different sources of stem cells are currently being explored for potential use in repairing the brain. Importantly, these explorative studies aimed at assessing the potential of different stem cell populations are generally carried out by performing in vivo studies. A large proportion of the cell types evaluated are not suitable for transplantation. Based on this and as part of the 3R-funded project, an in vitro screening method was developed for identifying stem cells that can be used for regenerative therapy [4]. The results produced the scientific basis and methodological confidence for us and other groups working in this field to reduce and replace a substantial proportion of explorative animal studies [5].
We formulated the following aims for the project:
AIM 1: To reduce and replace studies in animals we developed an in vitro system for studying neuronal brain damage due to bacterial infection. HC-derived neuronal stem/progenitor cells were differentiated into defined developmental stages e.g. stem cells, immature neurons and mature neurons, and then challenged with different stimuli characteristic for bacterial meningitis, i.e. growth factor deprivation, TNF-α or bacterial components, to assess which developmental stage is most susceptible to bacterial meningitis.
AIM 2: To reduce and replace studies in animals we established a pre-screening system of organotypic hippocampal slice cultures (OHCs) challenged with live *Streptococcus pneumoniae* to evaluate different stem/progenitor cells for their repair potential in the injured HC and validated the *in vitro* findings in a consecutive *in vivo* project.

Method and Results

AIM 1

Method: The hippocampal-derived stem/progenitor cells were driven into neuronal differentiation during 21 days. After being challenged with the different stimuli, cell death was assayed by cleaved caspase-3, Annexin-V or apoptosis-inducing factor. The *in vitro* findings were validated *in vivo*in an infant rat model of pneumococcal meningitis. The capacity of hippocampal-derived cells to multiply and form neurospheres was compared between infant rats that survived pneumococcal meningitis and their uninfected litter-mates.

Results: Two types of apoptotic cell death were detectable. Caspase-3-dependent cell death after growth factor deprivation and TNF–α exposure (Fig. 1A) and a caspase-3-independent cell death (Fig. 1B) after the cells were challenged with bacterial components.

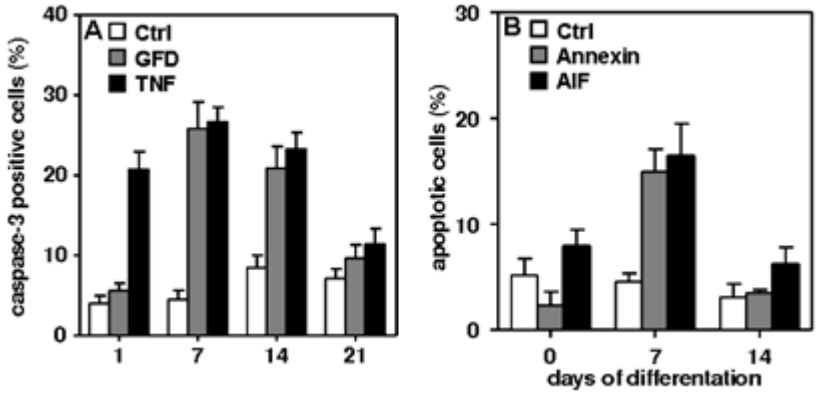

Figure 1: **(A)** Growth factor deprivation (GFD) and TNF induce caspase-3-dependent apoptosis. TNFinduced apoptosis early, at 1-14 d after differentiation. Apoptosis induced by GFD was most prominent at 7 and 14 d ($p < 0.05$).

(B) Bacterial components (BC) induce caspase-3-independent apoptosis. Hippocampal cells were exposed to BC for 2 h. Cell death induced by BC was compared to control cells using Annexin-V or apoptosis inducing factor (AIF) staining. Apoptosis induced by BC was most abundant 7 d after differentiation ($p < 0.05$). Overall, the likelihood of cell death after both challenges peaked at 7 d. P values were calculated by one-way ANOVA followed by Turkey's multiple comparison post hoc test. Data are presented as mean ± SEM of ≥ 3 independent experiments.

Cell death induced by bacterial components was characterized by the binding of Annexin-V and the relocalization of nuclear apoptosis-inducing factor. Immunocytochemical analysis revealed that immature neurons and stem/progenitor cells were most susceptible to apoptosis after the defined challenges (Fig. 2).

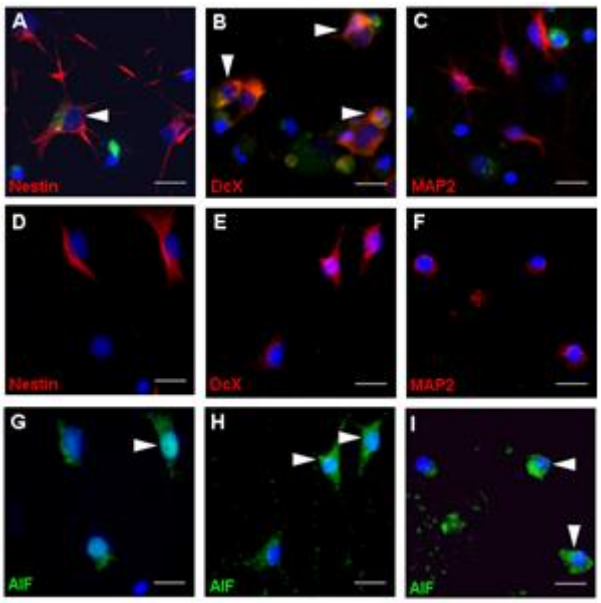

Figure 2: (A-C) TNF and growth factor deprivation (GFD) induced apoptosis in cells that stained positive for DcX and Nestin but not for MAP2. (D-I)After exposure to BC, AIF co-localized in mature neurons (MAP2; F and I), immature neurons (DcX; E and H) and stem/progenitor cells (Nestin; D and H). Scale bar, 50 µm.

In the *in vivo* system, significantly fewer colonies (formed by stem/progenitor cells) were obtained from the hippocampi of infected animals (Fig. 3), which is in line with the *in vitro* findings.

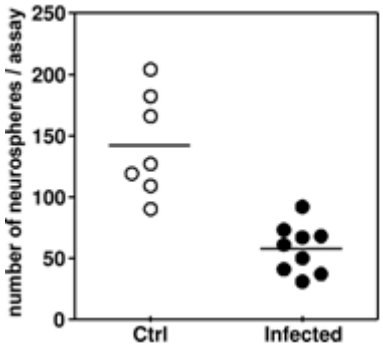

Figure 3: In an infant rat model of pneumococcal meningitis the ability of hippocampal cells from infected animals to form colonies was significantly reduced ($p < 0.05$), compared with that of cells isolated from hippocampi from mock-infected controls.

In summary, the *in vitro* model of differentiating hippocampal cells led to the observation that challenges characteristic for bacterial meningitis caused significantly more apoptosis in stem/progenitor cells and immature neurons compared with in mature neurons, a finding that may explain the persistence of neurofunctional deficit after bacterial meningitis.
The availability of this *in vitro* system allowed for a substantial reduction in the number of animals used, since it offered the possibility of screening pathogenic mechanisms and stimuli for their relevance prior to conducting large-scale studies *in vivo*[6].

AIM2:
Method: Green fluorescence protein (GFP)-expressing stem/progenitor cells from the fetal HC and ganglionic eminences (GE) were grafted into the DG of organotypic hippocampal slice cultures (OHCs) injured by challenge with live *Streptococcus pneumoniae*together with antibiotics to induce the release of bacterial components by killing and lysis of bacteria. The migration and differentiation of grafted cells were examined on cryosections of the OHCs using immunofluorescence and histomorphometry. Subsequently, hippocampal NPCs were evaluated in an infant rat model of pneumococcal meningitis. Application was done by stereotaxical transplantation of NPCs into the intact and injured hippocampal hilus region of the dentate gyrus within the OHCs. Survival and integration were monitored by immunofluorescence and histomorphometry at 3 defined points in time, i.e. 1, 2 and 4 weeks after transplantation [6, 7].
Results: In intact slices seven days after grafting, histomorphologic analysis revealed the migration of stem/progenitor cells from the site of injection into the molecular layer of the dentate gyrus, while in slices injured by challenge with live *Streptococcus pneumoniae* together with antibiotics the grafted cells migrated into the injured granular layer of the dentate gyrus (Fig. 4A/B) and differentiated into neurons (Fig. 4A/B insert).

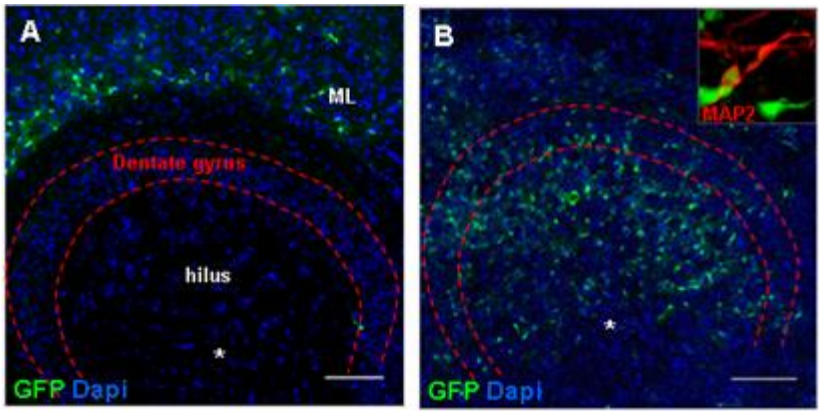

Figure 4: **(A)** In intact slices, hippocampal GFP-expressing stem and progenitor cells grafted into the hilus region of the HC migrated into the molecular layer of the dentate gyrus, while in pneumococci-injured slices grafted cells were found at the site of brain damage in the granular layer of the dentate gyrus. **(B)** Grafted stem and progenitor cells from the HC were characterized by a neuron-like morphological phenotype and expressed the neuronal microtubule-associated protein 2 (MAP2, insert). **(C/D**) In the infant rat model of pneumococcal meningitis, GFP-expressing NPCs migrated from the transplantation site to the site of damage in the granular layer of the dentate gyrus. Two weeks after transplantation (n = 3), the survivingcells differentiated into Doublecortin (DcX)-positive immature neurons **(C)**. After 4 weeks (n = 4), grafted cells differentiated into neurons (TUJ1, D). Scale bar A/B/C/D: 100µm.

In rats one week after cured bacterial meningitis, neuronal stem/progenitor cells from GFP-expressing rats grafted into the hilus region of the dentate gyrus migrated from the injection site to the injured granular layer of the hippocampal dentate gyrus (Fig. 4C/D). Migrated cells expressed markers of neuronal differentiation at 2 and 4 weeks after transplantation. Hippocampal injury induced by bacterial meningitis thus guides transplanted stem and progenitor cells to the site of brain damage. The availability of the OHC *in vitro* system leads to a substantial reduction in the number of animals used, since only approaches that prove successful in the *in vitro* system are considered for further evaluation *in vivo* [5-6].

Conclusions and Relevance for 3R

The experimental *in vitro* systems allow the following *in vivo* investigations to be reduced or replaced:
i) Testing of a pathogenic hypothesis by *in vitro* screening of potential bacteria-derived mediators (e.g. bacterial cell wall components) and potential host factors (e.g. host inflammatory mediators).
ii) Assessment of the intrinsic properties of the different stages of cell differentiation, which contribute to their selective vulnerability.
iii) Evaluation of therapeutic approaches that can counteract the selective vulnerability investigated under ii)
iv) Evaluation of therapeutic approaches that involve grafting stem/progenitor cells into brain tissue.
With the *in vitro* models we developed, studies for screening for pathogenic factors and therapeutic feasibility studies in animals can be substantially replaced by *in vitro* systems.
v) This model was successfully adapted to other pathologies also affecting the hippocampus. We recently used the model to develop an *in vitro* surrogate for the *in vivo* rat model of cardiac arrest/resuscitation that produces damage to the cornus ammoni 1 (CA1) region of the hippocampus. By submitting the organotypic slice cultures to oxygen/glucose deprivation we were able to induce the characteristic damage as observed in patients and animal models of cardiac arrest and resuscitation. Currently we are evaluating the model for regenerative therapy by neuronal progenitor cells (NPCs) grafting as an alternative to *in vivo* transplantation studies [7]

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3R-Project 104-06

Development of in vitro strategies to propagate and characterize hemotrophic mycoplasmas

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Keywords: *pig*; *whole blood; mycoplasma*; *vaccination*; *veterinary disease*; *cell cultures: co-cultures*; *reduction; replacement*

Duration: 3 years **Project Completion:** 2009

Background and Aim
Hemotrophic mycoplasmas (also named hemoplasmas) are the causative agents of infectious anemia in a wide range of domestic and wild animals (i.e. cat, swine, cattle, sheep, dog, lama, opossum). Current studies show that hemotrophic mycoplasmas are also apparent in primates (i.e. squirrel monkey, *'Candidatus* Mycoplasma kahanei'). The zoonotic relevance of hemoplasmas in humans is still unknown; the presence of human infections cannot be excluded. Hence, human infections with hemoplasmas are under investigation.
A major drawback for hemoplasma research is the unculturability of the agents. Thus, experimentally infected splenectomized animals are required for the propagation of hemoplasmas, e.g. as a source for microbiological, immunological and diagnostic analyses.
The aim of the research project is the establishment of an *in vitro* cultivation system for all hemotrophic mycoplasmas by using *Mycoplasma suis* as an appropriate prototype organism in order to replace the ethical questionable animal experiments.

Method and Results
Based on the recently established close phylogenetic relationship of hemoplasmas with the genus *Mycoplasma* we reason that hemoplasmas can be grown in pure culture by applying and diversifying proven culture techniques for mycoplasmas. Different culture approaches will be used to provide the microorganisms with the appropriate chemical and nutritional components of their natural environment, i.e. mammalian blood. Moreover, diffusion chamber methods will be applied in co-culture systems with eukaryotic cells and other fastidious mycoplasmal agents. Blood from experimentally infected pigs will be used as inoculum. The animals will be housed in the Clinic for Swines, Ludwig-Maximilians-University of Munich, Germany. The pigs need not to be infected within the scope of this submitted project but are a part of an approved experimental vaccination study. An *M. suis*-specific quantitative real-time PCR assay will be used to control the *M. suis* load of the inoculum and the growth and multiplication of *M. suis* in the different culture systems.

Conclusions and Relevance for 3R
The establishment of a hemoplasma *in vitro* cultivation system will replace all animal experiments which are currently necessary for the multiplication of these agents. New cultivation systems will open a wide range of possibilities:
- Clarification of pathogenetic phenomena in hemoplasma infections.
- Identification of virulence markers.
- Development and improvement of molecular and serological diagnostic assays.
- Realization of sequencing projects for hemoplasmas.
- Implementation of prophylactic measures since vaccine development and production is only possible from culture-derived hemoplasmas. Culture systems will allow attenuation and genetic manipulations of hemoplasma strains.

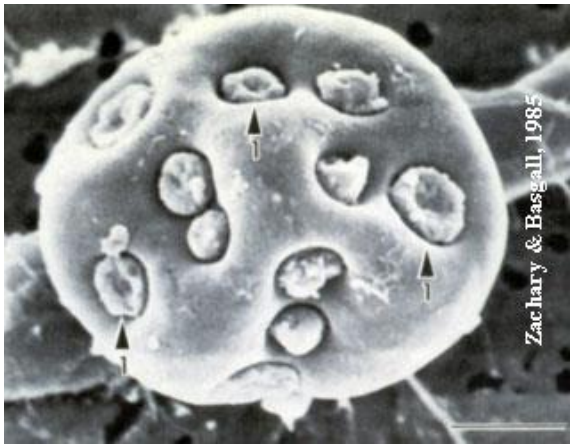

Figure 1: Electron microscopic picture of *M. suis* in close contact with the host cell (porcine erythrocyte; Zachary and Basgall, 1985).

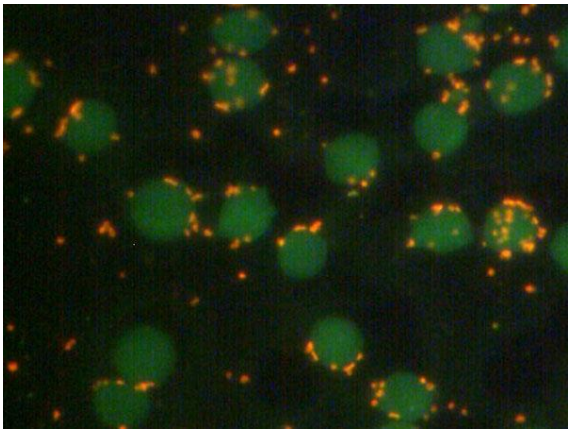

Figure 2: Acridin orange stained blood smear of an experimentally *M. suis* infected pig (erythrocytes: labelled green; *M. suis*: labelled orange; Hoelzle, 2007).

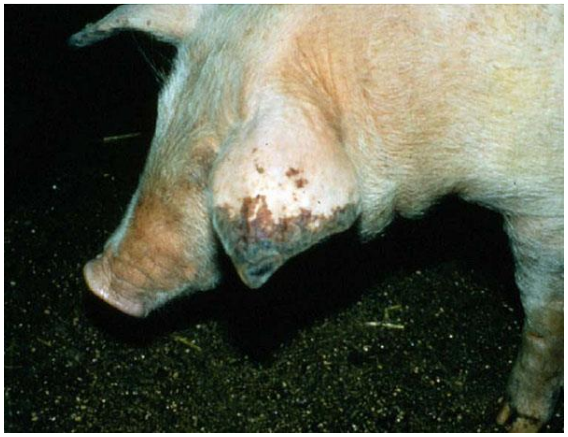

Figure 3: *M. suis* infected pig showing typical clinical symptoms (acrocyanosis; Hoelzle, 2007).

3R-Project 105-06

Establishment of an *in vitro* system for the prediction of the degree of virulence of classical swine fever virus isolates

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Keywords: *pig; viruses*; *veterinary drugs; veterinary drugs*; *infectious diseases*; *cell cultures: primary*; *reduction; replacement*; *veterinary drugs*

Duration: 3 years **Project Completion:** 2010

Background and Aim
The world organisation for animal health continuously registers numerous outbreaks of classical swine fever (CSF), a highly contagious disease of pigs caused by the CSF virus (CSFV) (http://www.oie.int/wahis/public.php?page=home). The economic consequences of such outbreaks are important, due essentially to the stamping-out policy and restrictions in the meat trade. A major problem is the persistence of CSFV in wild boar, the natural reservoir in which disease often remains unapparent. In domestic pigs the different CSFV isolates cover a continuous spectrum of virulence. Highly virulent strains are efficiently shed by infected pigs and spread particularly fast. On the other hand, low virulent strains - often diagnosed later - represent a particular problem for disease control. Hence, it is important to define the virulence of the CSFV isolates that are circulating in the wild boar reservoir and are posing a constant threat for reintroduction of CSF into the domestic pig population. Currently, the only possibility of classifying the virulence of a CSFV strain is by animal experimentation using pigs, which is ethically problematic. Previous work suggested that virulence is determined by multiple genetic elements. Simple comparison of genome sequences of strains differing in virulence does not yet permit prediction of the phenotype of a particular strain. As research in this area can currently only be performed with experiments involving pigs, the aim of the present study is to establish an *in-vitro* system enabling us to predict the virulence of a particular CSFV isolate. This is expected to drastically reduce the number of animal experiments in CSFV diagnostics and research.

Method and Results
In the past few years, we have made significant progress in understanding the pathogenesis of CSF and the interaction of CSFV with various cells of the porcine immune system (Balmelli et al., 2005; Guzylack-Piriou et al., 2006; McCullough, Ruggli, and Summerfield, 2008; Ruggli et al., 2009; Summerfield et al., 2006). The present project is aimed at exploiting these findings to establish in-vitro correlates of virulence. To this end, a set of CSFV strains of defined virulence are systematically analysed in terms of virus replication and of interaction with cells of the porcine innate immune system. A prerequisite for these analyses is a cell culture system that allows the propagation of virus isolated from diseased animals without selecting for cell culture adapted mutants. In common permanent cell lines, CSFV rapidly acquires mutations that confer enhanced binding to heparan sulphate (HS), which often results in virus attenuation. In the first part of this project, we have identified a suitable porcine endothelial cell line (Figure 1) for virus propagation and have demonstrated that these cells do not select for HS-adapted viruses. Using this culture system, we have produced stocks from 16 different CSFV isolates of defined virulence representing a broad range of highly-virulent to avirulent in-vivo phenotypes. Comparison of these CSFV strains at the levels of virus binding, entry and viral RNA replication did not reveal any correlate with virulence. The first promising results were obtained with the assessment of virus focus formation under a semi-solid medium overlay on porcine endothelial cells. On these cells, the highly virulent strains form significantly larger foci than the moderately or avirulent strains (Figure 2). Importantly, however, this system is applicable only to HS-independent isolates. Additional correlates with virulence were found by analysing the infection of blood-derived porcine macrophages and natural interferon producing cells (NIPC) by flow cytometry. Infection with highly virulent CSFV strains results in a higher percentage of infected cells compared to moderately-virulent and avirulent strains. Also the mean fluorescence intensity of the infected cells, which is proportional to the amount of viral antigen expressed, is higher with highly virulent than with avirulent strains. Whether the virus-mediated secretion of type I interferon (by NIPC) and of other cytokines also correlates with virulence is under current investigation.

Conclusions and Relevance for 3R
Taken together, the data mentioned above suggest that prediction of virulence cannot rely on a simple single assay.

Multiple factors, such as HS-binding, plaque size, interaction with NIPC and macrophages and other cells of the porcine immune system, have to be considered. The results obtained so far tend towards defining a multifactor system for the *in-vitro* prediction of CSFV virulence, consisting of a scoring system based on several virological and immunological criteria. Correlates between the *in-vivo* virulence phenotype and *in-vitro* characteristics of CSFV isolates will allow feasible and ethically acceptable CSFV diagnostic and research processes to be applied. An *in-vitro* system aimed at predicting the degree of virulence would significantly reduce the overall number of animals employed for experimental infections. The major refinement will be the gain of knowledge in terms of the molecular basis of the pathogenic characteristics of the virus, reducing to a minimum the number of animal experiments required in CSFV research. Although cell culture models will only enable us to predict the virulence of a particular virus isolate, in most cases this prediction will suffice to replace animal experiments. The knowledge created in this project will not only be useful in CSFV diagnostics but may also be applied in fundamental research aimed at determining the molecular basis of CSFV virulence.

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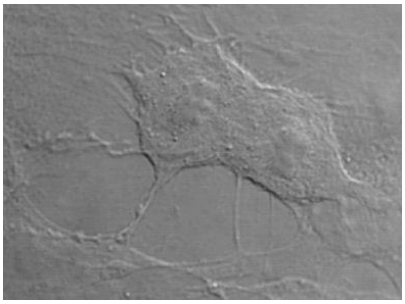

Figure 1: Porcine endothelial cell line used to propagate CSFV and to assess virus replication without selecting for heparan sulphate-adapted mutants. The cells are shown under native conditions.

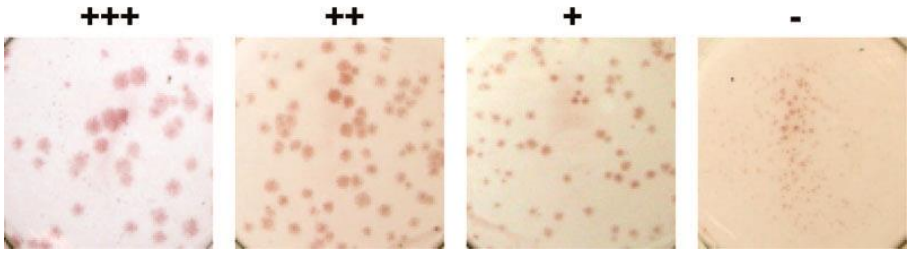

Figure 2: Focus formation of CSFV strains of different virulence on porcine endothelial cells. Cells were infected with highly-virulent (+++), moderately-virulent (++), low virulent (+), and avirulent CSFV. Focus formation under a semi-solid overlay medium was analysed 72 h post-infection by immunocytochemistry.

3R-Project 106-07

Standardization and Pre-validation of MucilAir: A novel *in vitro* cell model of the human airway epithelium for testing acute and chronic effects of chemical compounds

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Keywords: *human*; *epithelia; epithelia; lung*; *toxicology*; *cell cultures: 3d cultures*; *replacement*; *toxicity testing: aerosols; validation*

Duration: 1 year **Project Completion:** 2009

Background and Aim
Even though the alternative methods and models for addressing respiratory problems exist (Farmen et al., 2005; Matsui et al., 2005; Ostedgaard et al., 2005; Tarran et al., 2006; Geiser-Kamber, 2004; Kalberer et al., 2006), none of them have gone through the validation process. In this situation, it would be very difficult for industrial partners to use these alternative methods: Due to the legal constraints, the chemical industry, for example, cannot register products which are not tested with validated methods. As a result, animals will still be used in the future. With the implementation of REACH, more than 30'000 chemicals will have to be tested within the next 11 years. Thus, the standardization and validation of alternative methods is becoming a matter of urgency.

The compagny *Epithelix*, has developed and marketed a novel in vitro cell model of the human airway epithelium (*MucilAir*) which has some unique features:

It is of human origin and primary cell culture

It closely mimics the morphology and functions of the normal human airway epithelium

It has a unique shelf-life of one year

It is easy to handle and maintain

Adapted to high-throughput screening and testing

Serum free

These features of our model make it now possible to perform short-term and long-term toxicity tests using an in vitro cell model. The aim of this project is to collect enough experimental data on MucilAir so a dossier for pre-validation could be submitted to ECVAM.

Method and Results
According the ECVAM principles on test validity (Curren et al., 1995; Hartung et al., 2004), in order to validate an alternative method, a pre-validation step must be performed. This step includes several criteria:

1: Test definition:
- A definition of the scientific purpose of the test:
Test of the acute and chronic toxicity of the chemical compounds by inhalation.
- A description of the mechanistic basis of the test in view of the broader current scientific knowledge of the test endpoint: MTT, Resazurin test and TEER
- A definition of the protocol compliant with the Good Laboratory Practice.

2: Within-laboratory variability:
this addresses the variability over time and for different operators, but using the same laboratory set-up. Concretely, we propose to work out the following end-points:
a) Basal cytotoxicity tests: MTT and Resazurin test.
There are several traditional endpoints for evaluating the cytotoxic effects of chemical compounds, such as the MTT,

Resazurin test. The protocols for these two tests are well established.
b) Integrity of the epithelium: Trans-epithelial Electric Resistance (TEER): Cellular electrophysiological properties, such as trans-epithelial resistance, are excellent indicators of the integrity of the epithelium. Furthermore, the TEER measurement is a non-destructive method. Therefore, it can be used as an endpoint alone for long-term toxicity tests or combined with other endpoints (MTT, Resazurin).

The preliminary results of cytotoxicity tests:

A: Reproducibility:
In order to perform routine toxicity tests, measurable end-points have to be defined. We chose the MTT measurement as an endpoint evaluating the cyto-toxicity effects of the chemical compounds. Preliminary tests have been performed on MucilAir to establish a standard protocol, as well as to assess reproducibility. A protocol has been established. The results are reproducible when tested on 24 inserts, with a very small standard deviation (see Table in Figure 1).

B: Relevance - Cytotoxic Effect of Acrolein on MucilAir:
Next, we tested the cyto-toxic effects of Acrolein on MucilAir. The results obtained (Fig. 2) correlated very well with the published data (Haddad-Romet, 1993, Official Data of OCDE).

Conclusions and Relevance for 3R
Actually, there is no alternative method for testing the chronic toxicity chemicals. All tests have to be done on animals such as rats. We would like to offer a validated alternative method/model which will have profound implications in relation to 3R's animal protection principles.
First, unlike most of the airway epithelial cell models which can survive for only several days or weeks, our in vitro cell model has a shelf-life of one year. This unique feature makes it possible now to test the chronic effects of drugs or chemicals.
Secondly, the mission of Epithelix is not research per se; rather, Epithelix is actively applying and promoting the 3R principles by offering a ready-for-use in vitro cell model as a full package. Therefore, the impact on the animal experimentations is not limited to one research laboratory as it was often the case; instead, its impact is worldwide. If its full potential could be reached, then the life of a lot of animals might be saved.

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MTT Tests on Mucilair		
Number of tests	Mean OD value	Standard derivation
24	0.939	± 0.063

Figure 1: To test the reproducibility of the MTT test on MucilAir, 24 inserts of fully differentiated airway epithelium were submitted to MTT test, using a protocol that we optimized for MucilAir. As shown above, the MTT test using MucilAir is highly reproducible and reliable.

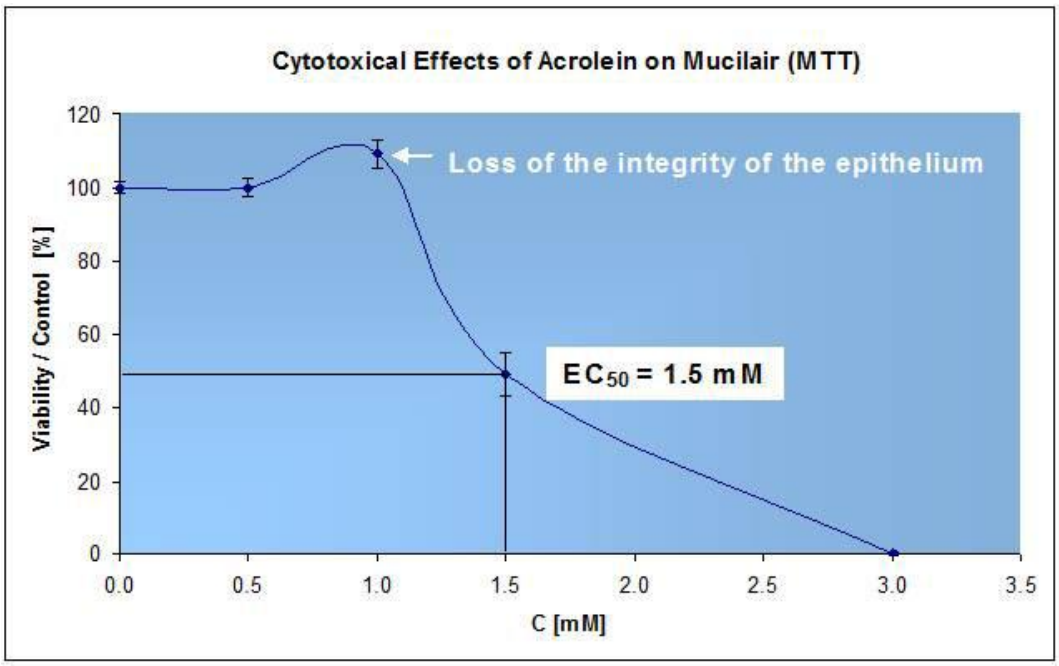

Figure 2: Cytotoxicity effects of Acrolein, a well-known toxic compound for the airway epithelium, were tested on MucilAir (incubation period of 24h). The “dose-effect” response is between 0.5 and 3.0 mM. The EC50 obtained (1.5 mM) was in good correlation with the published results (N=24).

3R-Project 107-07

Evaluation of an *in vitro* model to identify host parameters associated with virulence of *Toxoplasma gondii* strains

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Keywords: *human; parasites, ectoparasites: toxoplasma gondii*; *epithelia; epithelia; intestine*; *infectious diseases*; *replacement*; *infectiosity*

Duration: 2 years **Project Completion:** 2010

Background and Aim
The global prevalence of *T. gondii*, the causative agent of toxoplasmosis is estimated to be 30%. This intracellular protozoan parasite is one of the three common causes of congenital disease which can lead to severe malformation, mental retardation and spontaneous abortion. *T. gondii* is also the second most common cause of encephalitis in immunocompromised individuals.
Molecular epidemiological studies on a wide collection of human and animal isolates of *T. gondii* obtained from Europe and North America have revealed the predominance of three major clonal lineages classified as highly virulent Type I and less virulent Type II and Type III strains based on observation of mortality following inoculation of these parasites into laboratory mice.Recently, *T. gondii* organelles called rhoptries were shown to secrete kinases that dramatically influence host gene expression and are major parasite virulence factors (1) Our aim was to develop an in vitro cell culture based assay that would allow the early detection of *T. gondii* in clinical samples and would further allow an evaluation of its virulence towards the host. *T. gondii* infection acquired most commonly via the oral route, encounter small intestinal epithelial cells as early

targats. Human small intestinal epithelial cells were therefore tested as the prototype host cell.

Method and Results
Suitable cellular hosts in vitro
An immortalized small intestinal human epithelial cell line (HCT-8) was used as in vitro target for comparative studies of the three *T. gondii*strains which differ in their virulence. The cells were cultured to a monolayer (Fig. 1) and infected with an inoculum of the parasites (tachyzoites) (Figs. 2 and 3). Replication within HCT-8 cells, even at a very low multiplicity of infection, was observed of both the highly virulent Type I parasites and the low virulent Type II and Type III parasites. Type I parasites had a significantly higher rate of replication than Type II and III and this correlated with a deleterious effect on host cells, as indicated by the subsequent release of lactate dehydrogenase, a marker of host cell cytotoxicity.
Transcription factor and cytokine production
We compared the activation of NF-κB (a key transcription factor required for the activation of host genes), in HCT-8 cells by the three different *T. gondii*genotype strains. Type II parasites, that displayed intermediate replication, induced higher NF-κB activity in HCT-8 cells than Type I and III parasites, confirming previous observations in human macrophages (2). We further examined if the distinct levels of NF-κB activity could be correlated to downstream cytokine production. In concordance to the high NF-κB activity observed, Type II parasites secreted significantly higher levels of IL-8 and IL-6 than Type I and Type III strains from 8h until 48h post-infection.
Expression of human β-defensin 2
Human β−defensins (HBD) are effector molecules that play an important role in early intestinal innate immune defense by their dual function primarily as antimicrobial factors to defend against pathogens and secondarily as chemotactic factors to recruit cells for adaptive immune responses (3). Thus, the differences observed in the replication capacity between the three *T. gondii* types could reflect differences to stimulate IEC for the secretion of antimicrobial immune effectors such as β−defensins. To investigate this possibility, we examined the early expression of antimicrobial peptide genes HBD1, 2 and 3 in IEC upon infection by the three *T. gondii* genotype strains. A clear increase of HBD2 mRNA levels occurred after 3h of infection with Type II and Type III *T. gondii* strains but not with Type I strains. On the contrary, no significant induction of constitutively expressed HBD1 mRNA levels was observed following infection by each of the three v

genotypes. HBD3 gene expression on the other hand was down-regulated by all the three *T. gondii* genotype strains. Our findings support the notion that virulent *T. gondii* parasites (Type I), unlike the less virulence strains (Type II and III), do not activate HBD2 early after infection and use this as one of the mechanism to evade early host antimicrobial effect (4).

Conclusions and Relevance for 3R

A human cell cultures based assay able to rapidly detect the presence of *T. gondii* in clinical samples and in addition, able to provide information on the virulence of the infecting strain would be an ideal alternative to the current test in mice and be more predictive of virulence of the parasite in humans. Comparing the pattern of cellular response of human intestinal epithelial cells, the high virulence of strain I can be distinguished from the ones with low virulence (Type II and III). The virulence is described by the combined analysis of *T. gondii*growth, cytotoxicity of target cells and the early differential response of gene expression within 3-4 hours when there is no detectable replication of the parasite and therefore no cytopathic effect (Figure 4).

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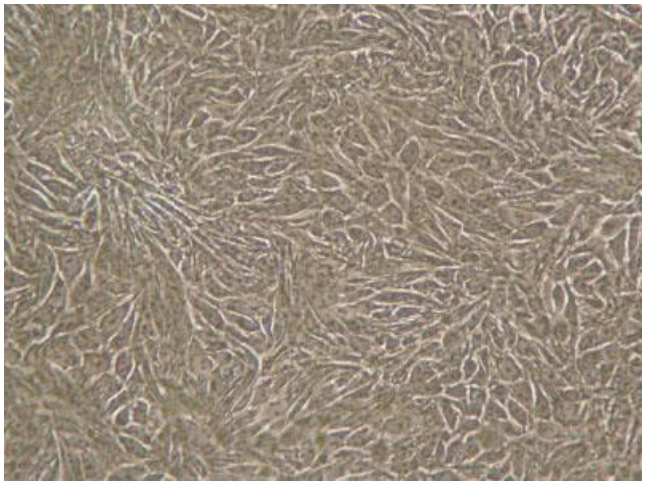

Figure 1: HCT-8 cell cultures under native conditions.

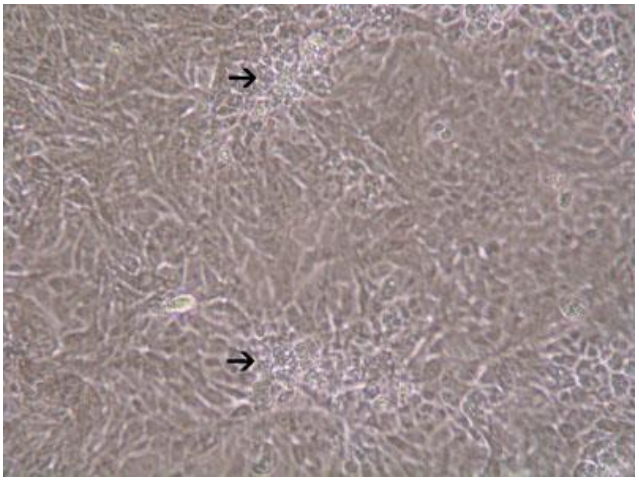

Figure 2: Plaque formation of HCT-8 cells infected with *T. gondii* (MOI=20)48hrs after infection.

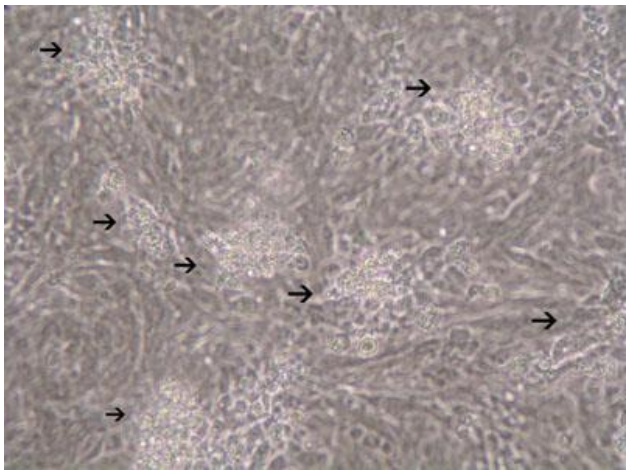

Figure 3: Increased number of plaques in HCT-8 cells infected with *T. gondii* (MOI=20) 72hrs after infection.

T. gondii virulence	*T. gondii* replication	Epithelial cell damage	Foci formation	HBD2 in epithelial cells
Type I (High)	+++	+++	+++	+
Type II (Intermediate)	++	++	++	+++
Type III (Low)	+	+	+	+++

Figure 4: Cellular host (IEC) correlates of T. gondii virulence

3R-Project 108-07

Cultured piscine hepatocytes as an *in-vitro* source of metabolic clearance data that is currently gleaned by *in-vivo* bioaccumulation testing in fish

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Duration: 2 years **Project Completion:** 2011

Background and Aim

Bioaccumulation is generally referred to as a process whereby the concentration of a chemical in an organism attains a level exceeding that in either the respiratory medium (in the case of fish: water), the diet, or both (Arnot and Gobas 2006). The bioaccumulation process embraces the absorption, the distribution, the metabolism and the excretion (ADME) of the substance. The bioaccumulation of a chemical is the result of its uptake via all routes of exposure, whereas the bioconcentration of a chemical is a consequence of the organism's exposure to it in the external aqueous medium, not of its uptake in the diet (Arnot and Gobas 2003). The extent to which a chemical bioaccumulates is expressed in such terms as the Bioconcentration Factor (BCF) - which is the ratio between the steady state chemical concentration in the organism and the chemical concentration in the respiratory medium - or the Bioaccumulation Factor (BAF), which takes into account also the concentration of the chemical in the food. In a regulatory context, it is usually the BCF that is implemented to identify bioaccumulative substances in fish. The standard procedure that is adopted for determining the BCF-values of chemicals in fish is an *in-vivo* test, which is conducted in accordance with the Guideline 305 of the OECD (Organisation for Economic Co-operation and Development). For the performance of one test alone, as many as 108 fish have to be sacrificed.

With the worldwide implementation of new chemical stipulations, the extent of bioconcentration testing for regulatory purposes will undergo a dramatic increase. In Europe, for instance, REACH requires an estimation of the bioconcentration potential in fish of chemicals with a log Kow-value ≥ 2.7 that are produced in weights exceeding100 tons per year. The implication of this new regulation is that the BCF-values of an estimated 3`000 to 5`000 substances might have to be determined, which will entail a massive increase in the number of fish that must be sacrificed for testing purposes. Hence, there is a need to develop alternative approaches to the testing of bioaccumulation *in vivo*.

In recent years, various non-animal-based strategies for the assessment of bioaccumulation have been discussed. A critical issue in the implementation of such non-animal-based predictions of bioaccumulation is the incorporation of information relating to chemical biotransformation (de Wolf *et al*. 2007, Nichols *et al*. 2007, Weisbrod *et al.* 2009). In principle, this information can be procured using either *in-silico* models, *in-chemico* assays, or metabolically-competent *in-vitro* systems of, for example, hepatic S9-fractions, isolated hepatocytes or microsomes. The aim of this project was to evaluate the potential of an *in-vitro* assay involving piscine hepatocytes to furnish data on chemical biotransformation rates.

Method and Results

Hepatocytes were isolated from the livers of rainbow trout using a two-step-perfusion technique. Fresh suspensions of the isolated cells were dosed with the test chemical and then incubated for 2 hours at physiological temperature (12-16°C). Samples were withdrawn from the incubation medium at regular intervals and processed for chemical analysis. The rate at which the test compound disappeared from the incubation medium yielded the intrinsic clearance rate (ml/h/10^6 cells) (Figure 1). Physiologically-based prediction models were implemented to derive the metabolic rate (k_m) of the test-chemical in the intact fish from its intrinsic clearance rate in the isolated piscine hepatocytes (Cowan-Ellsberry *et al.* 2007). This value was used to calculate either the BCF or the BAF.

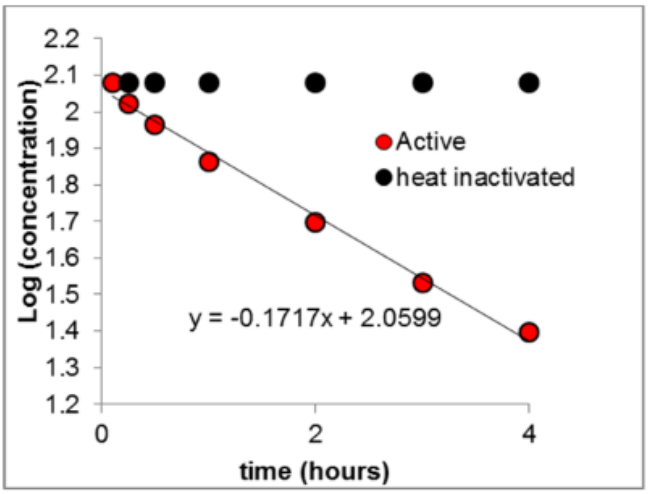

Figure 1: Determination of intrinsic clearance: The assay measures the temporal course of substrate depletion. The slope of the log-transformed substrate-depletion curve is used to calculate the elimination-rate constant (k_e). An estimate of the intrinsic-clearance rate [$Cl_{int,}$ (ml/h/10^6 cells)] is obtained when the elimination-rate constant is divided by the concentration of cells.

The project was successful in establishing a protocol of good intra-laboratory reproducibility for measuring the intrinsic hepatic clearance of lipophilic xenobiotics in freshly-isolated hepatocytes of the rainbow trout (Oncorhynchus mykiss). Seasonal fluctuations in the metabolic activity of trout have been identified as a major source of variability in the in-vitro assay. To overcome this limitation, existing methods for the cryopreservation of trout hepatocytes (Mingoia et al. 2011) have been adapted and further developed.

The project tested two extrapolation models for the prediction of the *in-vivo* metabolic rates of xenobiotics from the intrinsic clearance rates that were measured in the *in-vitro* hepatocyte assay. The models work well but would gain from an improved knowledge of physiological scaling factors.

Metabolic-rate values and BCF-predictions for three chemicals were generated and compared to *in-vivo* estimates. The *in-vitro* hepatocyte assay predicted well the *in-vivo* BCF-values, and, importantly, correctly classified the test-substances as “non-bioaccumulative compounds”. Although these findings confirmed the utility of the hepatocyte assay, the existing database is too small to support the drawing of general conclusions.

When aiming to reduce or replace *in-vivo* tests by *in-vitro* ones, the reproducibility of the latter has to be demonstrated. To test the intra- and inter-laboratory reproducibility of the hepatocyte assay, we participated in an international ring study, in which three laboratories determined the *in-vitro* intrinsic clearance rates of five reference compounds. The intra-laboratory variability (% coefficient of variance, CV) of the clearance rates for these five compounds ranged from 4.1 to 30%, whilst the inter-laboratory variability ranged from 27 to 61% (Fay *et al*. 2014). The inter-laboratory variability for the predicted bioconcentration factors, which were based on the *in-vitro* clearance values, ranged from 5.3 to 38 %. The results indicate that the *in-vitro* hepatocyte assay can be reliably used as part of a weight-of-evidence-based assessment of the bioaccumulation potential of xenobiotics.

Conclusions and Relevance for 3R

This project furnished proof-of-principle data that the piscine hepatocyte assay can contribute the information appertaining to biotransformation rate that is required for a non-animal-based bioaccumulation-testing strategy. The availability of such a strategy will be of paramount importance in helping to reduce *in-vivo* testing in fish, which, in view of the new chemical regulations that will be imposed worldwide, and which will call for BCF-information appertaining to thousands of chemicals, would otherwise increase explosively. The next step in the implementation of the hepatocyte assay in the regulatory praxis will be a formal OECD validation study involving large-scale, international-ring tests. This activity is being currently organized.

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3R-Project 109-08

Evaluation of lipid fractions for the substitution of serum in cell culture media

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Keywords: *rat*; *brain*; *cell cultures: aggregate*; *cell cultures: organ-specific*; *cell cultures: serum free*; *cell cultures: serum free*; *replacement*; *standardization*

Duration: 2 years **Project Completion:** 2010

Background and Aim
The widespread use of in vitro systems for biological research and industrial applications has greatly reduced the experimentation on living animals. However, most of the cell culture systems currently in use require fetal animal serum (typically 10% v/v fetal bovine serum) as media supplement, in conflict with the principles of the 3Rs. In the past 30 years, most of the components essential for cultured cells and tissues have been identified, including nutrients, vitamins, trace elements, hormones, and growth factors1-4. However, the nature of the indispensable component(s) contained in animal sera remained elusive. Recent findings suggested the involvement of serum lipoproteins. Therefore, it was our aim to isolate the lipoprotein fractions from serum and to examine their efficacy of replacing serum in cell culture media.

Method and Results
Lipoprotein (LP) fractions were isolated by density centrifugation from sera of different origin, and evaluated for possible stimulatory effects on cellular growth and maturation using serum-free aggregating brain cell cultures5-8 as the test system. The conventional LP tractions (VLDL/IDL, LDL, HDL) were prepared from (i) fetal bovine serum (FBS, GIBCO/Invitrogen, ref 10099-141, lot 6792117Y), (ii) newborn bovine serum (NBS, GIBCO/Invitrogen ref 16010-159, lot 6330496D), and (iii) human plasma (prepared at the CHUV from my own blood). Each liquid was first cooled in ice, then mixed with concentrated solutions of benzamidine hydrochloride (1 M; final concentration 1 mM) and PMSF (0.2 M; final concentration 1 mM), and the exact amount of solid NaBr required to give the lowest density (d = 0.019). Stepwise density gradient centrifugation each time for 20 h at 40'000 rpm with a Ti 40 swing-out rotor). After each centrifugation step, isolation of the respective lipoprotein fraction (aspiration of the yellowish supernatant using a 1-mL syringe) and addition of solid NaBr to give the next higher density. Thus, the fractions of VLDL/IDL (density 1.019), LDL (density 1.063), and HDL (density 1.21), as well as the remaining lipoprotein (LP)-free serum were isolated. Each of these fractions was dialyzed in Slyde-A-Lyser casets (Pierce, MWCO 10'000) against 2 L of NaCl (170 mM) solution containing EDTA (0.1 mM) and HEPES (0.5 mM), pH 7.3, and then tested in serum-free aggregating brain cell cultures for their possible beneficial effects, in comparison with untreated cultures and with cultures treated in parallel with either FBS or NBS. The dialyzed LP-free serum fraction was heat denaturized (as were also FBS and NBS). A dialyzed sample of heat denaturized FBS was also taken for comparisons. The criteria used to judge maturation-enhancing effects were the activities of cell type-specific enzymes, i.e., of 2',3'-cyclic nucleotide 3'-phosphohydrolase (CNPase, oligodendrocyte-specific); glutamine synthetase (GS, astrocyte-specific) as well as choline acetyltransferase (ChAT) and glutamate decarboxylase (GAD), both neuron-specific. The test fractions were tested in aggregating brain cell cultures, usually during the first 3 weeks in vitro. They were added after each medium replenishment, i.e., at days in vitro DIV 8, 11, 14, 16, 18, 20). Then the cultures were harvested at DIV 21. For each test point, quadruplicates of aggregating brain cell culture replica were taken.
In a first series of experiments, the effects of FBS and NBS were compared in aggregate cultures during early (DIV 7-21) and late (DIV 20-32) cellular maturation. The results showed (Table 1 and Table 2) that the two serum fractions had similar stimulatory effects on neuronal and glial maturation. During the early developmental period, both FBS and NBS stimulated neuronal as well as glial maturation. Interestingly, the commercially available LP-deficient FBS also stimulated glial maturation (oligodendrocyte maturation more than astrocyte maturation), but not neuronal maturation. In contrast, a commercial lipid preparation containing cholesterol and fatty acids was not stimulatory but rather detrimental to the cellular maturation (Table 1). During the late developmental period (Table 2), FBS still increased the activities of GS and ChAT but not of CNP nor GAD. NBS also stimulated GS and ChAT, although to a somewhat lower extent.
Further experiments were conducted to examine the effects of the lipoprotein fractions isolated from FBS and NBS.

Typical results from these experiments are presented in Table 3 concerning lipoprotein fractions isolated from FBS. Clearly, no stimulatory effect was obtained by the addition of any of the three lipoprotein preparations. The fact that most of the stimulatory activity was recovered in the lipoprotein-deficient serum fraction is in accord with the conclusion that the stimulatory activity of serum was not reproduced by lipoproteins. However, the finding that dialyzed serum was almost as active as non-dialyzed serum supported the view that the activity was due to macromolecular serum component(s) rather than low molecular weight factors such as hormones, growth factors, or nutritional additives. Experiments conducted with lipoprotein fractions prepared from human blood plasma produced similar negative results (Table 4), and again negative results were obtained from experiments in which lipoprotein fractions from NBS were directly compared with those from human blood plasma (data not shown). In the latter experiments, different lipoprotein media concentrations were taken, up to the 3-fold concentration used in previous experiments. An analogous test series was conducted with human lipoprotein fractions obtained from the group of Dr. A. Abderrahmani (DBCM, University of Lausanne), who prepares and uses routinely human lipoproteins for his actual research, and who provided us initially also with the detailed protocol for the preparation of lipoproteins by density centrifugation. The results from the testing of their lipoprotein fractions were negative as well (data not shown). In view of the finding that dialyzed serum retained much of the stimulatory activity, we finally tested additional blood proteins suspected to possess some of the wanted biological activity (based on either reports in the literature or on their relatively high plasma concentrations) such as fibronectin (0.5-5.0 µg/ml, Sigma no F4759), alpha1-antitrypsin (5-80 µg/ml, Sigma no A9024), fibrinogen (1-4 mg/ml, Sigma no F8630), and tissue-type plasminogen activator (Actilyse, 20-100 nM, Boehringer Ingelheim). In these experiments we used as endpoint only the CNP activity, which was specific for the stimulation in early cultures, and which was also completely recovered in dialyzed serum and in LP-deficient serum. However, the testing of these commercially available blood plasma proteins again produced only negative results (data not shown).

Conclusions and Relevance for 3R

The results obtained clearly show that lipoproteins alone are not able to replace the known beneficial effects of bovine serum in cell culture. This conclusion is based on experiments with aggregating brain cell cultures. Since this culture system contains different types of cells (stem cells, neurons, astrocytes, oligodendrocytes, microglia) and progressively runs through a sequence of histotypic stages of maturation (including glial cell maturation, segregation of "adult" stem cells, neurite formation, synaptogenesis, myelination), it appears to be the most versatile cell culture system to detect factors able to substitute bovine serum. Although the results are clearly negative concerning the lipoproteins as possible substitutes of bovine serum, one positive finding is that NBS was almost as stimulatory as FBS, and could therefore be used instead of the fetal serum (at least in the present culture system). The present data also indicate that the beneficial effects of serum reside in a macromolecular fraction that could be isolated and identified with great likelihood. Such an effort would certainly be worthwhile, because it would most probably be possible to obtain this component ultimately by animal-free synthetic means, such as proteins by recombinant technologies.

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Table 1: Effect of either lipid-deficient FBS or a commercial lipid supplement, in comparison to FBS and NBS, on neuronal and glial maturation in aggregating brain cell cultures

AGENTS added DIV 8-20	Total Protein and Total Enzymatic Activities determined at DIV 21				
	Total Protein [mg/sample]	CNP [U/sample]	GS [mU/sample]	GAD [mU/sample]	ChAT [μU/sample]
NONE	1.03 ± 0.12	2.66 ± 0.48	74 ± 15	1.58 ± 0.23	145 ± 11
FBS 5%	2.08 ± 0.15	5.91 ± 0.59	149 ± 14	2.19 ± 0.16	432 ± 40
FBS–LP 5%	1.62 ± 0.24	6.30 ± 0.71	110 ± 20	1.58 ± 0.33	192 ± 28
NBS 5%	2.26 ± 0.33	6.03 ± 0.57	178 ± 40	2.46 ± 0.20	302 ± 51
Ch+FA	0.84 ± 0.19	1.41 ± 0.44	52 ± 20	1.25 ± 0.24	134 ± 39

The data are presented as mean values ± SD of 4 replicate cultures.

FBS: Fetal Bovine Serum (GIBCO/Invitrogen ref no 10099-141, lot no 6792117Y)
NBS: Newborn Bovine Serum (GIBCO/Invitrogen ref no 16010-159, lot no 6330496D)
FBS and NBS were heat-denaturized (30 min, 56 °C) prior to use
FBS–LP: Lipoprotein-deficient FBS (Sigma ref no S5394) dialyzed against PBS, pH 7.3 and sterile filtered (0.2 μm filter)
Ch+FA: Lipid supplement containing cholesterol and fatty acids (GIBCO/Invitrogen 250x concentrated solution, ref no 12531-018, lot no 317757), diluted 250x in the medium.

Table 2: Effect of late addition of FBS and NBS on neuronal and glial maturation in aggregating brain cell cultures

AGENTS added DIV 20-32	Total Protein and Total Enzymatic Activities determined at DIV 21				
	Total Protein [mg/sample]	CNP [U/sample]	GS [mU/sample]	GAD [mU/sample]	ChAT [μU/sample]
NONE	1.37 ± 0.19	1.60 ± 0.23	72 ± 10	2.28 ± 0.41	296 ± 21
FBS 5%	1.63 ± 0.11	1.52 ± 0.09	198 ± 7	2.29 ± 0.16	467 ± 37
NBS 5%	1.66 ± 0.27	1.32 ± 0.13	155 ± 20	2.61 ± 0.16	347 ± 25

The data are mean values ± SD of 4 replicate cultures.

FBS: Fetal Bovine Serum (GIBCO/Invitrogen ref no 10099-141, lot no 6792117Y)
NBS: Newborn Bovine Serum (GIBCO/Invitrogen ref no 16010-159, lot no 6330496D)
FBS and NBS were heat-denaturized (30 min, 56 °C) prior to use.

Table 3: Effect of either bovine lipoprotein fractions prepared from FBS or lipid-deficient FBS, in comparison to FBS, on neuronal and glial maturation in aggregating brain cell cultures

AGENTS added DIV 8-20	Total Protein and Total Enzymatic Activities determined at DIV 21				
	Total Protein [mg/sample]	CNP [U/sample]	GS [mU/sample]	GAD [mU/sample]	ChAT [µU/sample]
NONE	2.26 ± 0.39	3.39 ± 0.54	78 ± 16	1.87 ± 0.27	249 ± 45
FBS 5%	2.51 ± 0.14	6.60 ± 0.65	174 ± 18	2.78 ± 0.28	722 ± 97
FBS dial 5%	1.67 ± 0.51	5.67 ± 0.27	150 ± 2	3.11 ± 0.17	640 ± 58
bVLDL	1.26 ± 0.10	3.15 ± 0.35	71 ± 7	1.74 ± 0.23	228 ± 26
bLDL	1.19 ± 0.10	3.59 ± 0.16	82 ± 5	2.13 ± 0.13	291 ± 15
bHDL	2.15 ± 0.13	3.21 ± 0.26	69 ± 4	1.94 ± 0.12	266 ± 24
FBS–LP 5%	1.21 ± 0.07	6.65 ± 0.68	128 ± 10	2.88 ± 0.34	506 ± 46

The data are mean values ± SD of 3 - 4 replicate cultures.

FBS: Fetal Bovine Serum (GIBCO/Invitrogen ref no 10099-141, lot no 6792117Y)
FBS dial: Fetal Bovine Serum as above, dialyzed as described below
bVLDL: Very Low Density Lipoprotein fraction (d = 1.019, containing also IDL) prepared from above FBS
bLDL: Low Density Lipoprotein fraction (d = 1.063) prepared from above FBS
bHDL: High Density Lipoprotein fraction (d = 1.21) prepared from above FBS
FBS–LP: LP-deficient FBS, remaining from the preparation of the lipoprotein fractions

FBS, FBS dial, and FBS–LP were heat-denaturized (30 min, 56 °C)

FBS dial, FBS–LP and the three lipoprotein fractions were dialyzed in 170 mM NaCl, containing 0.1 mM EDTA and 0.5 mM HEPES pH 7.3. All dialyzed fractions were sterile filtered (0.2 µm filters) prior to use.

The amount of lipoprotein added to the medium was equivalent to 1.8-fold the theoretical amount expected to reside in the active fraction if all stimulatory activity of serum was to be found in that fraction. For example, a lipoprotein fraction isolated from 66 ml of bovine serum or human plasma in a volume of 3.6 ml was diluted 200-fold in the culture medium.

Table 4: Effect of human lipoproteins on neuronal and glial maturation in aggregating brain cell cultures

AGENTS added DIV 8-20	Total Protein and Total Enzymatic Activities determined at DIV 21				
	Total Protein [mg/sample]	CNP [U/sample]	GS [mU/sample]	GAD [mU/sample]	ChAT [µU/sample]
NONE	1.19 ± 0.16	2.75 ± 0.27	85 ± 10	1.88 ± 0.24	175 ± 17
hVLDL	1.40 ± 0.39	2.86 ± 1.18	83 ± 25	2.13 ± 0.67	182 ± 49
hLDL	1.29 ± 0.35	2.19 ± 0.77	77 ± 24	1.89 ± 0.52	169 ± 46
hHDL	1.27 ± 0.12	2.73 ± 0.49	77 ± 11	1.81 ± 0.19	165 ± 13

The data are mean values ± SD of 4 replicate cultures.

hVLDL: Very Low Density Lipoprotein fraction (d = 1.019, including IDL) from human blood plasma
hLDL: Low Density Lipoprotein fraction (d = 1.063) from human blood plasma
hHDL: High Density Lipoprotein fraction (d = 1.21) from human blood plasma

The three lipoprotein fractions were prepared from human blood plasma, dialyzed individually in 170 mM NaCl, containing 0.1 mM EDTA and 0.5 mM HEPES pH 7.3, and sterile filtered (0.2 µm filters).

The proportions of lipoprotein added to the culture media were analogous to those stated in the legend to Table 3.

3R-Project 110-08

Development of an in-vitro assay for the screening of antischistosomal drugs

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Keywords: *human; rodents*; *infectious diseases*; *cell cultures: parasites*; *replacement*; *drug screening*

Duration: 1 year **Project Completion:** 2010

Background and Aim
Schistosomiasis is a so-called neglected tropical disease, but it affects over 200 million people and is of considerable public health significance.

The treatment and control of schistosomiasis virtually relies on a single drug, praziquantel. The pressing need to develop new antischistosomal compounds has been stressed, particularly in view of the blanket application of praziquantel within the frame of preventive chemotherapy, a strategy that might select for drugresistant parasites (1). The current drug screening in-vitro screening protocols are based on juvenile and adult schistosomes, obtained by portal perfusion from the mouse or hamster, hence rely on infected rodents and allow only a small number of molecules to be tested (2). Our goal was to develop a reliable in-vitro antischistosomal drug sensitivity assay, with high sensitivity for screening large numbers of compounds based on schistosomula, which can be obtained from infected snails, as depicted in Figure 1.

Method and Results
We have compared 4 different published methods (mechanical, chemical and skin transformation of cercariae) (3, 4) for the production of schistosomula (Figure 2) with regard to quantity of schistosomula obtained, quality (worm motor activity and morphology of schistosomula) and its ease of use. We found that the most successful and convenient transformation method to artificially produce large amounts of schistosomula was the vortex transformation method. Schistosomula obtained by vortex transformation are now an important part of our drug screening cascade. For example, we have successfully used vortex produced schistosomula to describe the antischistosomal properties of mefloquine and its isomers (6, 7). In line with our findings, Abdulla and colleagues have also recently described the development of a medium throughput assay based on schistosomula, which they describe as an attractive parasite stage since adaptable to the 96-well plate format and quickly and easily transformed from cercariae that are harvestable in great numbers from vector snails (8).

In order to determine whether the effect of drugs on schistosomula obtained by vortex transformation is similar to the effect on adult *Schistosoma mansoni* (the worm stages which need to be targeted by drugs) in vitro, we have tested the effect of a variety of compounds, with known in vivo antischistosomal activity (e.g. praziquantel, oxamniquine, artesunate or mefloquine) against adult schistosomes and schistosomula (Figure 3). The effect of drugs was analyzed based on motility disturbances (e.g. activity or paralysis), morphological changes (relaxation, shrinkage, curling, tegumental disruption, worm disintegration) and worm death. In addition, we have used for the first time isothermal microcalorimetry to study drug effects on schistosomes.

Similar sensitivities on schistosomula and adult schistosomes were observed for praziquantel and mefloquine, while slight differences in the drug susceptibilities of the two development stages were noted with oxamniquine and artesunate. Overall, we concluded that isothermal microcalorimetry is a useful tool for antischistosomal drug discovery, which should be further validated. In addition, our data support the use of schistosomula in in vitro schistosome drug assays (9). Mansour et al. have recently developed a schistosomula assay based on inhibition of Alamar Blue reduction, a redox indicator of enzyme activity, which has been successfully used for colorimetric or fluorometric determination of viability of a number of protozoan parasites. They found that this assay was very effective in detecting severely damaged and dead schistosomula and with a sufficiently long culture period (7 days) was able to detect most of the known schistosome active compounds tested (10).

Conclusions and Relevance for 3R
In conclusion, we have achieved our goal and developed a drug sensitivity test based on schistosomula, which can be obtained from infected snails, which is able to replace the adult schistosome in vitro assay. Hence, our technique reduces and replaces live animals in experimental methods in accordance with the 3Rs animal protection principles. In addition, the schistosomula screen is cost-effective and allows screening a large set of compounds (medium throughput assay),

hence shortens the duration of assay turnaround times, a distinctively advantageous feature of an ideal screening assay given the urgency in discovery of new compounds.

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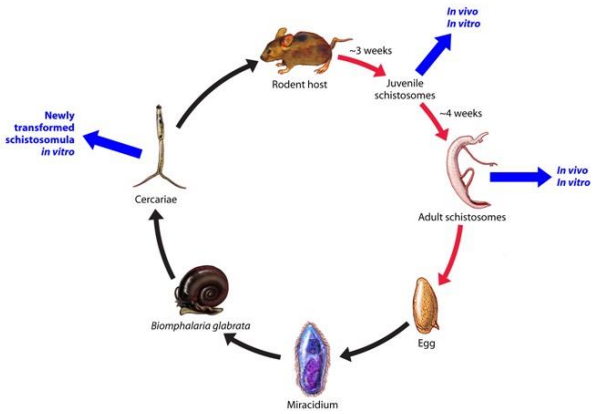

Figure 1: Schistosomiasis - Life Cycle: Human-Snail phase.

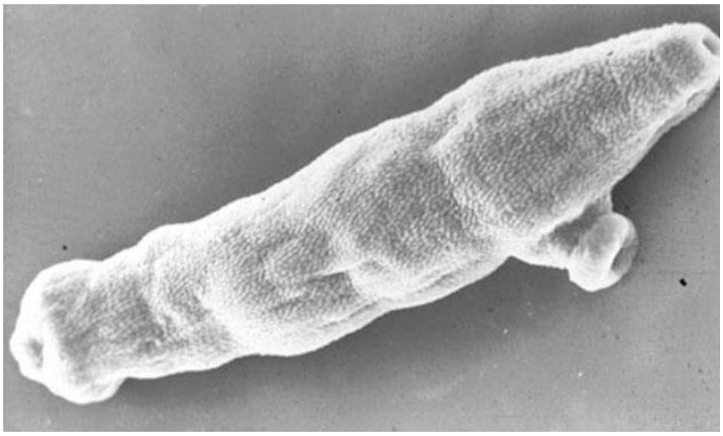

Figure 2: Picture of schistosomula

3R-Project 111-08

Establishment of an organ ex-vivo tissue slice model for cardiovascular research in particular for therapeutic atherosclerosis targeting

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Keywords: *human; mice; rodents; transgenic mice*; *blood: vessel*; *atherosclerosis; infectious diseases*; *cell cultures: intact tissue*; *reduction; replacement*; *drug screening*

Duration: 2 years **Project Completion:** 2011

Background and Aim
It is desirable to reduce and replace animal experiments in the life sciences, while still being able to acquire biologically relevant data. Two-dimensional cell cultures offer easy maintenance and manipulation as well as high throughput compatibility. Their drawback is the important gap between such cultures and live tissue, as encountered *in vivo*. *In vivo* tissue consists of a complex three-dimensional arrangement of and interactions between a multiplicity of different cells and extra-cellular matrix components which are connected to the rest of the body by blood and lymph vessels. A step towards *in vivo* conditions is the use of three-dimensional cell co-cultures, where some approaches are also compatible with high throughput.

In vascular biology, the gap between *in vivo* conditions and cell cultures can be bridged using *ex vivo* blood vessel models. These models preserve the complex tissue structure, while allowing selected biochemical processes to be continuously monitored for milliseconds up to days. This means that data can be gathered at many points in time, which in classical animal models would be represented by sacrificing one animal for one time end-point. In atherosclerosis research, knock-out mice (apoE-/-) are widely used as animal models. Current *ex vivo* artery models involving such mice use peripheral arteries, whereas in classical *in vivo* atherosclerosis research it is mainly the aorta which is analysed.

In mouse atherosclerosis models, the aorta usually has multiple regions of diseased tissue, whereas for example carotid artery bifurcations normally do not have more than two regions, which also develop later. The novel method we present here therefore offers the advantages of (a) gaining more data in one experiment (compared with carotid artery models), thus sparing a certain number of laboratory animals, and (b) providing data which is directly comparable with existing data from classical *in vivo* experiments using aortas, therefore obviating the need for establishing a body of reference *in vivo* data for carotid arteries and consequently reducing the number of animals required.

Method and Results
We established a model for long-term culture including perfusion of the mouse aorta using PDMS (polydimethyl siloxane) as an embedding matrix (5). PDMS is biocompatible, transparent in the visible light domain and gas permeable. This enabled us to visualise the aorta using confocal time-lapse microscopy. The ApoE-/- mouse aorta was exposed using microsurgery. A conventional vein catheter was inserted into the mouse aorta from each side (aortic sinus through left ventricle and abdominal aorta). The aorta was then removed and embedded in the PDMS. After polymerisation for two hours, the aorta-PDMS block (Figure 1B) was linked to the specifically developed perfusion system (Figure 1A) and mounted in the culture chamber of an LSM710 confocal microscope.

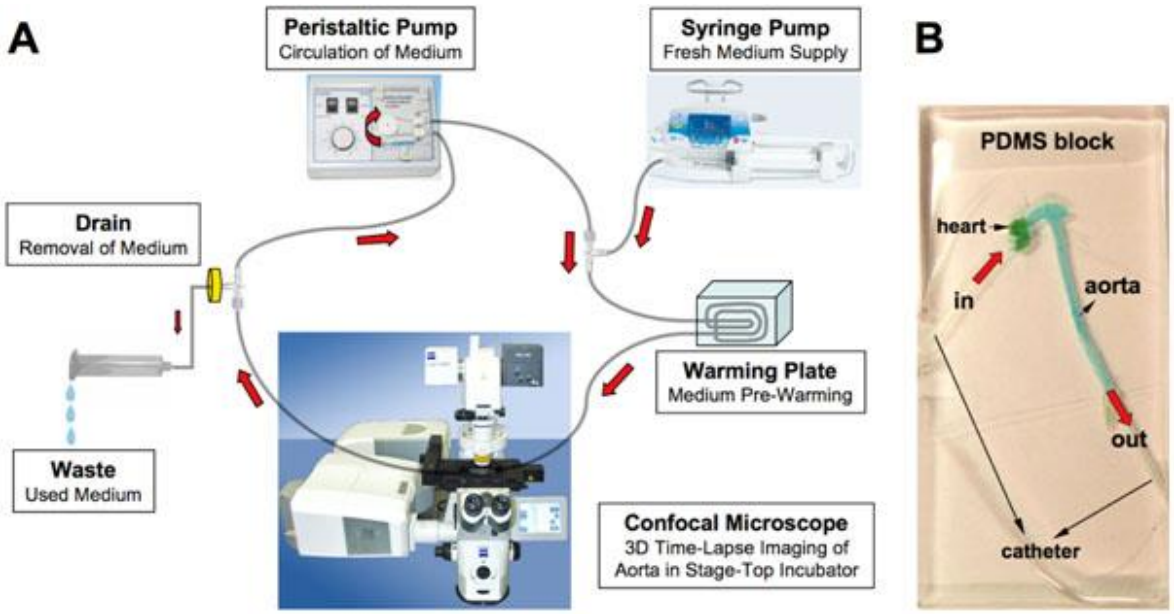

Figure 1: Diagram of artificial perfusion system (A): the aorta-PDMS block (B); the aorta was stained with blue food colouring.

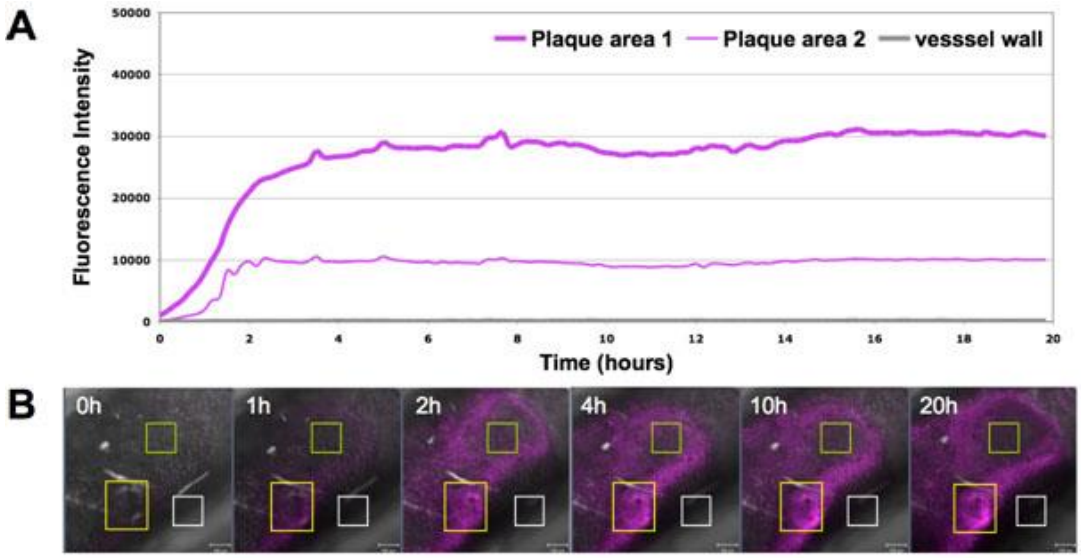

Figure 2: **(A)** The Cy5-conjugated aptamer was circulated in the perfusion system and the fluorescent signal was observed under a confocal microscope for 20 hours. (B) The intensity of the fluorescence at one point (magenta) at different times varies and can be recorded. We defined three regions of interest in the image data generated from one experiment. The yellow rectangle corresponds to 'plaque area 1' on the time-line graph, the green one to 'plaque area 2'. The white rectangle serves as a negative control and corresponds to non-plaque vessel wall (grey line on the time-line graph). The scale bar is 100µm.

Immunohistochemical analysis of aorta cryosections revealed that the aortic tissue was still alive after 24 hours of artificial *ex vivo* perfusion with a cell-culture medium. To test the targeting of a cell receptor on mouse aortic tissue we perfused the atherosclerotic aorta of an ApoE -/- mouse with pre-warmed cell-culture medium containing 500 nM Cy5-conjugated aptamer for 20 hours. This aptamer is known to bind to a receptor on macrophages that reside in atherosclerotic lesions. We observed an increase of the fluorescent signal in the plaque regions, whereas the signal in non-plaque regions remained at a background level. The plaque signal curve reached a plateau after 3 hours (Figure 2A, **2B**).

Conclusions and Relevance for 3R

We established an artificial perfusion system using aortas extracted from ApoE deficient mice in order to reduce the number of animals required for atherosclerosis studies (5). With this model it was possible to visualise in 3 D multiple atherosclerotic plaques in single isolated aortas using time-lapse confocal microscopy. This method thus enabled us
(i) to study the ligand-receptor interaction on the plaque area at different stages of severity,
(ii) to test the acute toxicity on the vascular tissue, and
(iii) to pre-select the concentration range for later tests *in vivo*, thus reducing the number of animals required to a minimum. Based on the success of this new model, it can be applied in efficacy, toxicity and biodistribution studies of nanomaterials in larger trials in the future, replacing conventional mouse experiments.

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3R-Project 112-08

A novel in-vitro model for the holistic assessment and optimisation of engineered tissue for functional cartilage repair

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Keywords: *human*; *bone, cartilage*; *cell cultures: bioreactor; tissue engineering*; *reduction; replacement*

Duration: 2 years **Project Completion:** 2011

Background and Aim
Tissue engineering offers potential solutions to the clinical challenges of cartilage repair and regeneration. Animal models are still widely used in evaluation of tissue engineering strategies for the repair of damaged human articular cartilage. However, there is no consensus on the most appropriate animal model and none of the species used replicate the anatomical, cellular and biomechanical properties of human articular cartilage. A further major problem is the poor integration of newly implanted engineered cartilage with adjacent healthy tissue (1, 2). Without full integration into native cartilage, the implants will experience abnormal mechanical stress during their post-surgical maturation and remodeling, potentially leading to further damage to both new and adjacent tissue and thereby limiting the efficacy of the repair. Application of mechanical stimulation to engineered tissue has been reported to enhance cartilaginous matrix formation and improve tissue growth (3-5). However, despite reports of improvements in the quality of *in vitro* engineered cartilage tissue, there is a lack of information about whether these 'better quality' engineered tissue constructs ever achieve successful lateral integration between repair tissue and adjacent cartilage after implantation.

A major aim of this study was to contribute to a reduction in animal experimentation by establishing a novel, holistic in vitro model to investigate tissue induction, remodelling and lateral integration to adjacent cartilage after cell-scaffold constructs are implanted in cartilage defect sites. This model would be used as an ***in vitro screening capability for newly developed technologies*** intended for cartilage repair, thereby not only reducing the numbers of animal procedures but also, in some cases, replacing entirely certain specific procedures that are currently carried out *in vivo*.

Method and Results
We developed a novel 3-D co-culture model for in-vitro evaluation of tissue-engineering techniques intended to provide constructs for cartilage repair. This model consists of a cartilage explant in the form of an annular disk, into which is inserted the engineered tissue construct (implant) intended for cartilage repair. Each construct, maintained sterile in culture, was exposed to cyclic compressive loads (CCL) over prolonged periods in our specially designed bioreactors. The preliminary loading regime used was within the physiological range at 0.2Mps, with a frequency of 0.5 Hz, applied for 30 minutes per day, 5 days per week for up to 9 consecutive weeks. Identical constructs were cultured in the bioreactor chamber without application of CCL and used as controls. Cell viability was assessed with confocal microscopy. Maturation of the implant tissue was evaluated biochemically and histologically. Mechanical properties of the constructs were measured in the form of their compressive modulus. The integration between engineered tissue and surrounding cartilage was also evaluated histologically.
Over 160 bovine cartilage explants were seeded on to scaffolds to provide constructs for testing in the above model. Two types of scaffolds were tested within the constructs:

(1) *Non-absorbable scaffold* made of non-woven filamentous polyester (PET). Chondrocytes from bovine knee cartilage were seeded onto PET scaffolds, encapsulated with 1.2% alginate gel and implanted into the central holes of cartilage rings and subjected to CCL at the above loading regime for 9 weeks. Identical constructs were cultured in the bioreactor chamber without application of CCL (controls). Constructs were investigated at 4 and 9 weeks.

(2) *Bioabsorbable scaffold* made of Type-VII low melting agarose (Sigma, St. Louis, MO) was dissolved in culture media,

autoclaved, then mixed with chondrocytes at 37°C to a final gel concentration of 2% (w/v). The resulting constructs were implanted into cartilage rings and were subjected to CCL as described previously in (1) above.

Constructs formed from non-woven PET (1) showed greater amounts of tissue formation after CCL compared with non-loaded controls (Fig. 1) after 4 weeks of co-culture.

Confocal microscopy showed partial integration between the implants and adjacent cartilage with greater integration in the CCL group at both 4 and 9 weeks (Fig. 2).

GAG synthesis within agrose constructs ± CCL after 4 and 8 weeks in culture, expressed as total GAG per microgram of DNA. (n=6): *, $p<0.05$. CCL increased GAG synthesis at both time points.

Cell viability in both the construct and cartilage ring was 95% and 90% at 4 weeks and 9 weeks respectively. GAG synthesis increased by 32% and 40% after CCL application for 4 weeks and 9 weeks respectively (Fig.3).

Hydroxyproline content of PET constructs ± CCL, expressed as total hydroxyproline per microgram of DNA (n=6). No significant change after CCL application at both time points (Fig. 4).

No significant change after CCL was observed in total collagen content per µg DNA as determined by hydroxyproline assay (Fig.4).

Mechanical testing of PET scaffold constructs after co-culture. Modulus was measured as tangent to the stress strain curves at strain values of 20 %. CCL significantly increased construct stiffness at week 9 (p,0.05) (Fig. 5).

Confocal microscopy of agarose constructs in co-culture at week 4, better integration was observed after CCL (A) compared with controls (B) (Fig. 6).

Histochemical staining indicated that chondrocytes had maintained their phenotype during the 8 week period of the experiment. However, the overall level of GAG within constructs was low, with positive GAG staining mainly pericellular with less stain in the extracellular matrix. Histology also showed relatively higher levels of GAGs after CCL compared with controls. Immunohistochemical staining for type II collagen (Fig.9) also suggested slightly elevated matrix levels in the CCL group. There were no significant differences in the mechanical properties between the test and control constructs.

Conclusions and Relevance for 3R

The ultimate aim of this study was to develop an *in vitro* approach to screening various materials and constructs used in tissue engineering leading to the reduction in the number of animals used in pre-clinical trials of different implants and methods proposed for cartilage repair. We used a 3-D co-culture model, comprising a cartilage disc in the form of a ring, into which are placed different types of constructs intended for cartilage repair. These rings were then subjected to compressive cycling loading in sterile culture medium to investigate the effect of mechanical stimulus on the quality of repair tissue. The cyclic loading was applied in a bespoke bioreactor over a period of 9 weeks. Two types of constructs were tested.

Our data demonstrated that the quality of repair tissue was improved after CCL in terms of amount of tissue formed, biochemical and histological maturation, and the maintenance of cell phenotype. Differences in mechanical properties related to the two scaffolds used were also discerned; mechanical stimulus had a positive effect when PET scaffolds were used, improving construct mechanical properties but not when agarose gel scaffolds were employed.

These results indicate that our model has been successfully developed and it now available for future use as an effective tool to screen constructs that are made from different biomaterials and intended for cartilage repair. The screening of more promising constructs and materials at an early stage would reduce the number proceeding to further investigation in animal models and in turn would result in a substantial reduction of animals used in pre-clinical trials.

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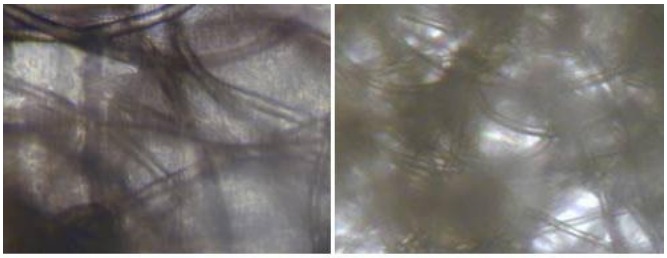

Figure 1: Tissue formation within the PET scaffold constructs after 4 weeks co-culture. There were greater number of chondrocytes and more tissue formation within the constructs after 4 weeks application of CCL **(A)** than within the control group **(B)**.

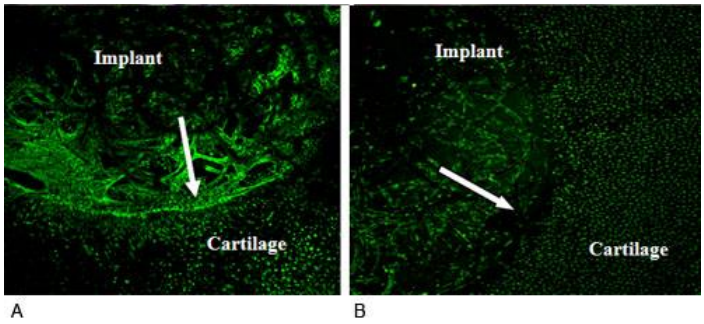

Figure 2: Integration between the PET scaffold contructs and surrounding cartilage observed by confocal scanning microscopy showing more tissue integrated into surrounding cartilage in the CCL application group (A) than the Control group (B) after 4 weeks co-culture. Arrow shows the boundary between the implant and surrounding cartilage and there were gaps between the implant and cartilage at some sites. 95% cell viability within the implant was achieved on both CCL application group and control group.

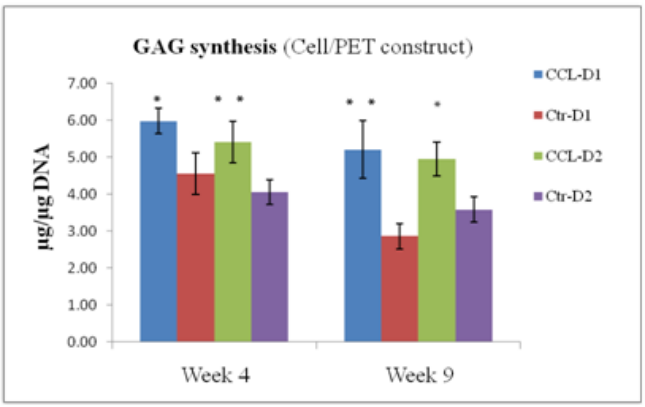

Figure 3: GAG content of PET constructs ± CCL after 4 and 9 weeks in culture, expressed as total GAG per microgram of DNA. (n=6); * p<0.05; **p<0.01. CCL increased GAG synthesis at both time points.

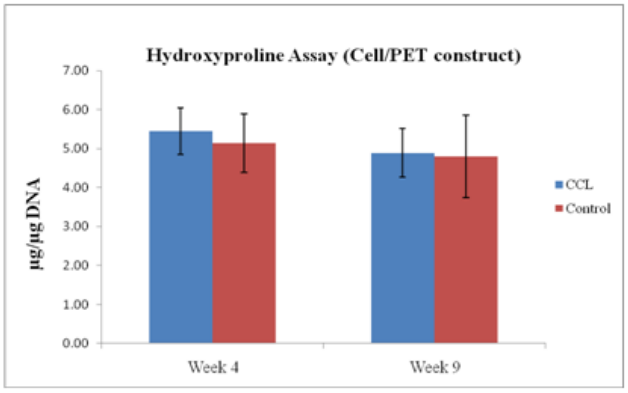

Figure 4: Mechanical property (modulus) of the constructs at 9 weeks was 1.7 times greater in the CCL group than the control (p<0.05, Fig.5).

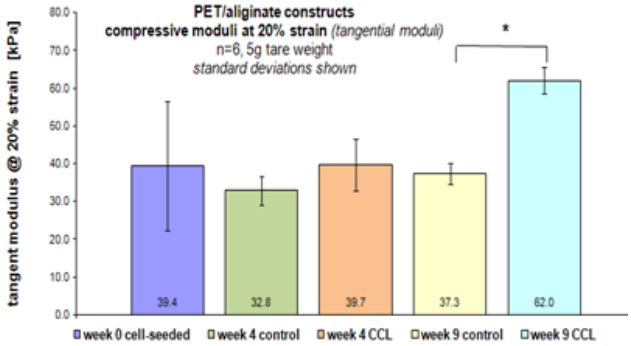

Figure 5: Constructs formed from bioabsorbable agarose (2) showed good cell growth. Confocal microscopy revealed cell viability of 91% and 88% at 4 weeks and 8 weeks respectively. Some integration between the constructs and surrounding cartilage was observed at 4 weeks after CCL application although the boundary between the two components was clearly seen (Fig.6).

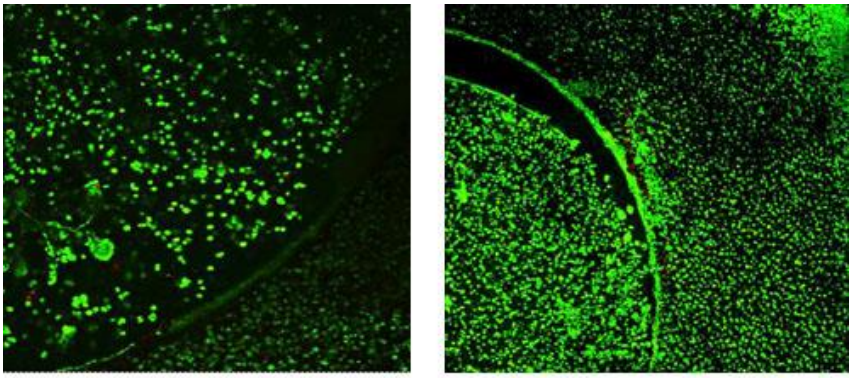

Figure 6: Histological analysis confirmed only partial integration between constructs and adjacent cartilage for both groups, though some of the CCL samples had better integration compared to the controls (Fig.9).

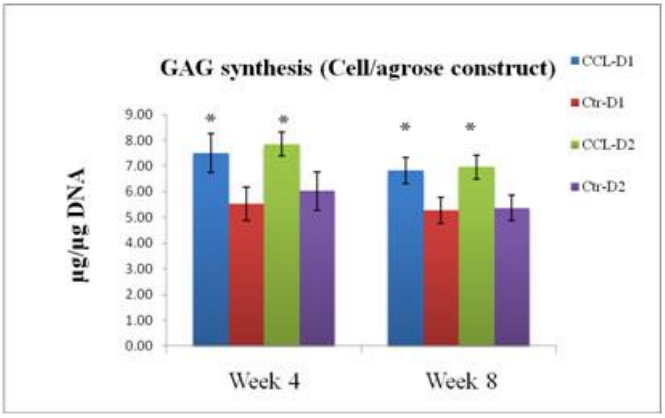

Figure 7: GAG synthesis within agrose constructs ± CCL after 4 and 8 weeks in culture, expressed as total GAG per microgram of DNA. (n=6): *, p<0.05. CCL increased GAG synthesis at both time points.

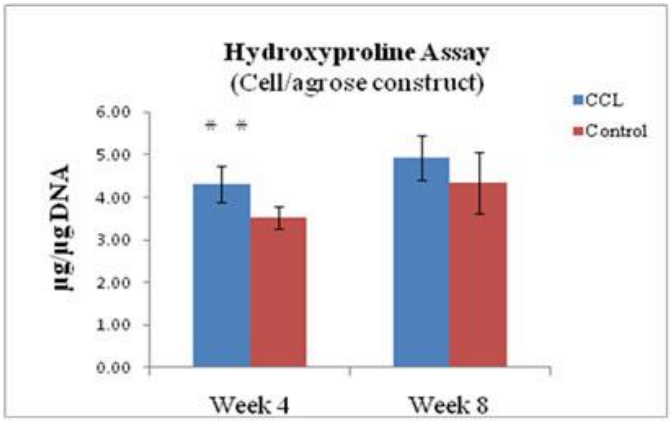

Figure 8: Hydroxyproline (HYP) content of agrose constructs ± CCL after 4 and 8 weeks in culture, expressed as total HYP per microgram of DNA (n=6). **P<0.01. CCL increased HYP at week 4.

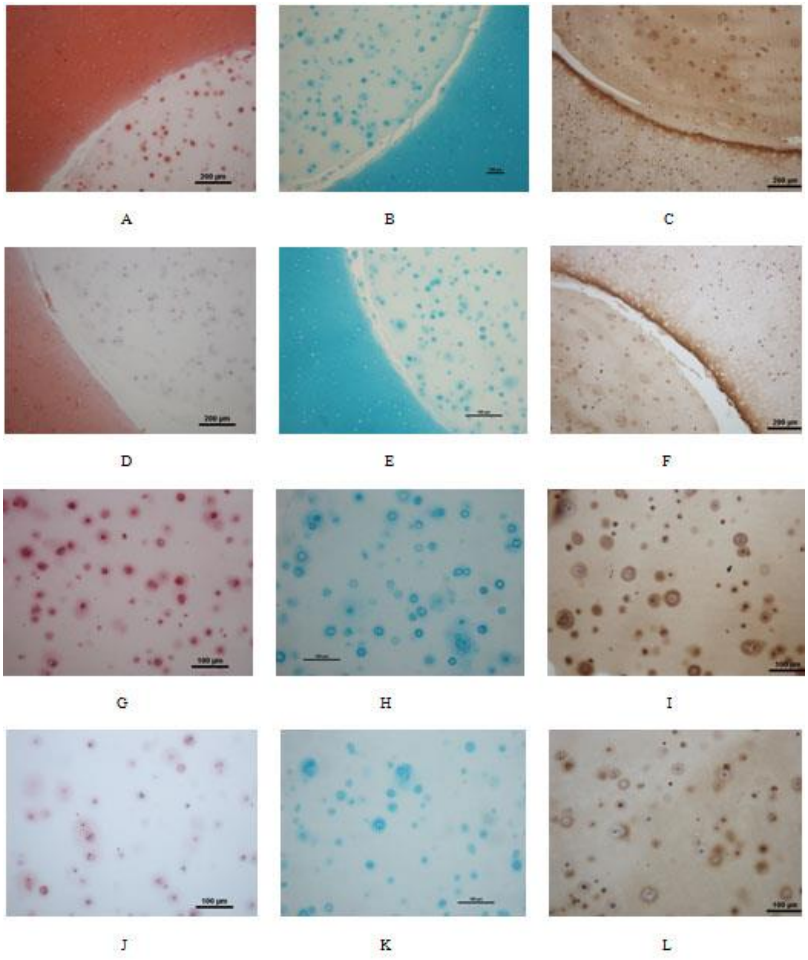

Figure 9: Histological analysis of agarose-constructs at week 8 in co-culture: Safranin O stain (A,D,G,J), Alcian blue stain (B,E,H,K) and immunohistochemical stain of type II collagen (C,F,I,L) showing implant and surrounding cartilage (A-F) and constructs only (G-L). The expression of GAG and type II collagen was higher in the CCL group (A-C, G-I) compared with controls (D-F, J-L). Partial integration between implants and adjacent cartilage was observed (A-F).

3R-Project 113-08

Generic in-vitro evaluation assay for immunological correlates of protection to replace animal challenge infections

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Keywords: *live stock*; *vaccination*; *infectious diseases*; *reduction; replacement*; *diagnostic approaches: vaccines*

Duration: 3 years **Project Completion:** 2012

Background and Aim
Vaccination against foot-and-mouth disease (FMD) represents an essential element in controlling and combating outbreaks, which would otherwise have disastrous consequences. This is pertinent to regions in large parts of the developing world in which the FMD virus (FMDV) is endemic, as well as during an epidemic in FMDV free areas such as Europe. Nevertheless, successful vaccination against FMDV requires selection of the appropriately matching vaccine strain providing protection against a particular circulating field virus. This problem originates from the existence of seven known serotypes of FMDV, with which high antigenic variation of virus is observed. In addition, subtypical antigenic variation within a serotype is under constant evolutionary change due to the high mutation rate of FMDV. For these reasons, continuous vaccine testing and modification in the light of recent antigenic changes to the virus is required. Currently, vaccines are tested and selected using vaccination-challenge experiments in cattle. Such procedures are not only extremely expensive, but are also environmentally and ethically problematic, considering the severe animal suffering associated with disease development, and the requirement that all animals be slaughtered at the end of the experimentation.
Accordingly, the proposed solution is to develop realisable and robust *in-vitro* alternatives to such challenge infection tests. To this end, the present project integrates with the EU project FMD-DISCONVAC, from which the necessary samples will be obtained. Through this integration with the FMD-DISCONVAC project, we will be fulfilling the objectives of the EU call KBBE-2008-1-3-02: To substitute vaccine potency tests in animals by assays enabling a correlation of in-vitro tests based on immunological principles of antibody-based effector immune responses operating in vivo.
Although vaccine-induced protection can be predicted when high levels of virus neutralising antibodies are induced, this does not apply for vaccinates with relatively low levels of antibodies. So far, no alternative tests applicable to such sera have been developed and systematically applied to vaccine testing. Thus, the approach selected will go beyond virus neutralization tests, and also enable the functional analysis of non-neutralizing antibodies with respect to FMDV destruction and the induction of antiviral activity. Developed tests will have the potential of serving as improved correlates of protection against homologous or closely related FMDV vaccine strains. In addition, the tests have the potential to identify correlates of heterologous protection induced by vaccination.

Method and Results
Based on immunological evidence obtained from previous work, we are developing *in-vitro* screening assays based on the relationship between protection and the avidity of specific antibody for binding to the pathogen in question. We are evaluating the relationship between protection and the capacity of a serum to promote Fc receptor-mediated phagocytosis of FMDV immune complexes by macrophages and dendritic cells in terms of virus destructionn, and Fc receptor-mediated IFN-alpha and cytokine responses in plasmacytoid dendritic cells (see Figure 1).

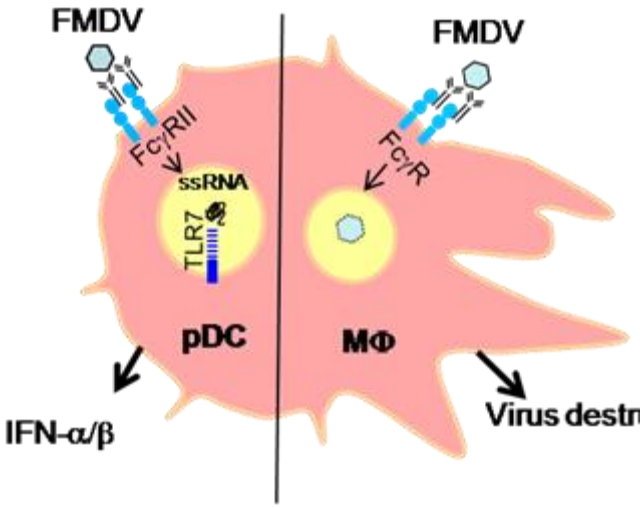

Figure 1: Immunological background: FcR-mediated anti-FMDV responses induced in plasmacytoid dendritic cells (pDC) and macrophages (MF) as examples of antibody-mediated effector functions independent of direct virus neutralization.

In various cellular system, to test the influence of immune complexes obtained from vaccinated cattle and pigs were established. These include an assay measuring enhanced infection of bovine monocyte-derived dendritic cells by immune complexed-FMDV and for the porcine system enhanced IFN-alpha responses by plasmacytoid DC. Our results demonstrate a high sensitivity of both assays and show that opsonising antibodies possess a broad cross-reactivity against various FMDV isolates within one serotype and in some cases even across serotypes.
A main conclusion from was that opsonizing activity was clearly much broader than neutralizing activity suggesting that non-neutralizing IgG could also complex with FMDV to infect bovine MoDC or activate pDC.
To systematically investigate the relationship of opsonising antibodies with protection using a large panel of sera, we also have established a reporter system to measure opsonizing antibodies against FMDV. This is based on bovine FcRII (CD32) expressing murine RAW 264.7 macrophage cells. The principle of this test is that the RAW 264.7 cells are resistant to FMDV infection in the absence of antibodies but get infected and die when the virus is complexed with antibodies. Our results demonstrate that this test is highly sensitive, similar to the test using bovine MoDC, and has potential as an additional measurement to determine immunological correlates of protections.

Conclusions and Relevance for 3R

The experimental systems established now permits the quantification of opsonizing antibodies at a very high sensitivity. We are even able to detect reactivity across serotypes which was unexpected considering that there is no cross-protection between serotypes. We are now testing a larger collection of sera to determine the relationship of opsonizing antibodies to protection and to conclude on the applicability of this test to measure vaccine quality without challenge infection.

Reference

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3R-Project 114-08

Reduction of the number of animals used in the Fish Acute Toxicity Test

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Keywords: *fish*; *ecotoxicology*; *data base*; *oecd-guideline; reduction; refinement*

Duration: 1 year **Project Completion:** 2010

Background and Aim
(OECD Guideline 203), Fish Acute Toxicity Test, states that at least five concentrations in a geometric series with a separation factor preferably not exceeding 2.2 should be used and at least 7 fish per concentration. However, it is not uncommon in fish tests to find just one concentration with a partial mortality (mortality >0 and <100%), i.e., a very steep dose-effect relationship, and little information is gained from the multiple test concentrations with no or complete mortality. Based on this observation, it appears that the present design could be modified to reduce the number of fish whilst still providing the LC values with a precision comparable to the present design. It is the aim of this project to analyse historical data and to use the findings for alternative testing approaches with a reduced number of fish.

Method and Results
Historical data of acute fish toxicity tests of two substance groups (agrochemicals and non-agrochemicals) from a database of the chemical industry were analysed to characterise the distribution of LC50 values and slopes of the dose-effect relationship. In a second step, data from the OPP Pesticide Ecotoxicity Database were examined as supportive data. The results of the analysis of the two substance groups were compared. The findings were used in a computer simulation study, in which the performance of different experimental designs with reduced separation factors, smaller numbers of test concentrations, and smaller numbers of fish per test concentration were compared. The results of the simulations demonstrate the potential for improved experimental efficiency and reduction of animals without loss of quality in the LC50 estimate.
During a workshop, attendees applied their expert judgement to develop alternative designs to reduce the number of animals in testing.
The summarised results of the project are:

There was no difference between either the LC50 values or the slopes of the dose-effect relationships between agrochemicals and non-agrochemicals.

30% of agrochemical tests and 50% of the non-agrochemical tests of the chemical industry database were limit tests.The impression of generally steep slopes (median: 13) was confirmed in the database of the chemical industry for which a factor between concentrations of 1.7 (OECD ≤2.2) may be adequate to get two partial mortalities for a precise LC50 estimation.

The OPP database did not confirm this finding; the median of the slopes was 6.5 for which a factor between concentrations of 2.1 may be adequate to get two partial mortalities.

The simulation studies showed that 6 fish per concentration yield the same quality of the LC50 value as does a minimum of 7 fish as presently required by the Guideline.

The resulting test strategies of the workshop were to use the fish embryo test proposed as the OECD Guideline, stepping down from the upper threshold concentration corresponding to the lowest value of algae and daphnia tests, followed by an in-vivo fish confirmatory test performed as limit test, or a full test if an LC50 value is required.

Conclusions and Relevance for 3R
The results of the project show ways of reducing the number of animals in the acute fish testing procedure. First by using 6 fish per concentration instead of at least 7 as stated in the Guideline. Secondly, by using juvenile fish only for a final confirmatory test whereas any previous tests are performed with fish embryos. In order to really save animals in practice,

however, Guideline 203 has to be adapted and a lead country has to submit a Standard Project Submission Format to the OECD.

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Figure 1: Bluegill sunfish (Lepomis macrochirus) are often used in bioassays including toxicity testing according to OECD guidelines.

3R-Project 115-09

Engineering of a human brain tumour model to replace animal experimentation

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Keywords: *human*; *brain; stem cells; tumour*; *tumourigenesis*; *cell cultures: co-cultures*; *reduction; replacement*

Duration: 3 years **Project Completion:** 2012

Background and Aim
Gliomas account for more than 70% of all brain tumours, and of these, glioblastoma is the most frequent and malignant histological type. Fewer than 3% of glioblastoma patients are still alive at 5 years after diagnosis. Recently, there has been important progress in our understanding of the molecular pathogenesis of malignant gliomas, and especially the importance of cancer stem cells. Models are central to allow the transition from scientific concepts to the understanding of the reality of a tumour in a person. They may be used for therapeutic screens, in preclinical trials, or to study the basic biology of brain tumours. Injection of glioblastoma cells either subcutaneously or directly into the animal brain, usually using immunodeficient strains such as Nude or SCID, or transgenic mouse models spontaneously developing brain tumours, allow in-vivo modelling with similar histopathology, etiology, and biology. These mouse models are widely used but, unfortunately, these available model systems are very aggressive for animals (infections, painful, severe motor and behavioural symptoms). In addition, such animal models do not reflect the real interaction between tumoural cells and the host that occurs in the human tissue. Indeed, human tumour cells are generally injected into an animal brain. Thus, new models that (i) replace animal experiments and (ii) provide a more relevant tumour/host interaction are needed.
Using the technology of embryonic stem cells (ESC), we propose to create an integrated and fully *in-vitro* model of a human glioblastoma developing within a human brain tissue. To achieve this goal, we will combine two technologies already available in our laboratory: (i) ENT technology: We recently developed in our laboratory the technology of Engineered Neural Tissues (ENTs). ENTs are reconstituted brain-like tissue pieces derived *in-vitro* from human embryonic stem cells (Preynat-Seauve, O. et al., 2008.). (ii) EGT technology: in parallel, using the same technology as ENTs, we have developed EGTs (Engineered Glial Tumors). EGTs are a model of reconstituted glioblastomas in three dimension derived *in-vitro* from patient brain tumour samples (Preynat-Seauve, O. et al., 2008.). We propose to combine ENT and EGT technologies in order to develop a fully *in-vitro* model of a human glioblastoma growing within a human brain-like tissue. This method will provide a powerful human model for the study of glioblastomas replacing animal models.

Method and Results
in progress (present status)
The technology to generate in vitro brain-like tissues (Engineered Neural Tissues or ENT) was already established in the laboratory, as well as the method to generate glioblastoma-like tissue (Engineered Glial Tumor or EGT) from patient tumor samples (gliomaspheres or GS). The aim of the first part of the project is to associate physically ENT with EGT in order to modelize tumor development within a host nervous tissue. To associated ENT with tumor samples, we have tested two approaches : (i) produce ENT and EGT separately before their association (ii) deposition of GS on ENT. To distinguish the tumor from the host tissue (ENT), GS were transduced with the red Tomato Fluorescent Protein (TFP) using the lentivector technology. In both cases, glioblastoma-derived cells survived in ENT and expanded, generating a red fluorescent cell mass within ENT. GS derived from three different tumor samples were tested: 2 glio-neuronal tumors otained at the university of Paris, France (TG1, TG6, Dr Hervé Chneiweiss), one high grade glioblastoma (GB1), obtained at the university of Nice, France (Dr Thierry Virolle and Dr laurent Turchi). The three types of GS survived and developped within ENT.
Sections from the obtained tissues were analyzed by histology. Several important features of glioblastomas and glio-neuronal tumors were observed.
In contrats to other brain tumors, glioblastomas and glio-neuronal tumors diffusely infiltrate the brain, rather than "push" the host tissue. Histological analysis of engineered ENT/tumors showed the presence of numerous individual single tumor cells (TFP+) infiltrating in a diffuse manner the host tissue. As a consequence, secondary TFP+ tumor sites at distance of the implantation site were systematically observed, mimicking the frequent local "metastasis" observed in patients.
The presence of large necrotic/apoptotic areas in constantly observed in patients and it is one important criteria for the

histopathological diagnosis of glioblastoma and glio-neuronal tumors. Hemalun/eosin coloration, TUNEL staining and immunoreactivity against activated caspase-3 confirmed the presence of large apoptotic areas around the tumor. Three different independant neuropathologists from different institutions analyzed the tissue samples and confirmed their strong similaritiesd with the in vivo situation . We have performed immuno-phenotyping of the engineered tissues and found in the TFP+ region numerous markers frequently expressed by the tumor in vivo (GFAP, vimentin, nestin, betaIII-tubulin, Sox-2, Musashi-1, EGFR, neurofilament, NeuN). Also, the tumor expressed the proliferation marker PCNA, indicative of a high mitotic index like the in vivo situation.

Conclusions and Relevance for 3R

The development of such model which:
- is new
- mimics significantly the in vivo situation
- includes cells only from the human species

is expected to reduce animal experimentation for the study of glioblastomas. It should be very promising to extrapolate such studies to other tumors of the central nervous system.

Reference

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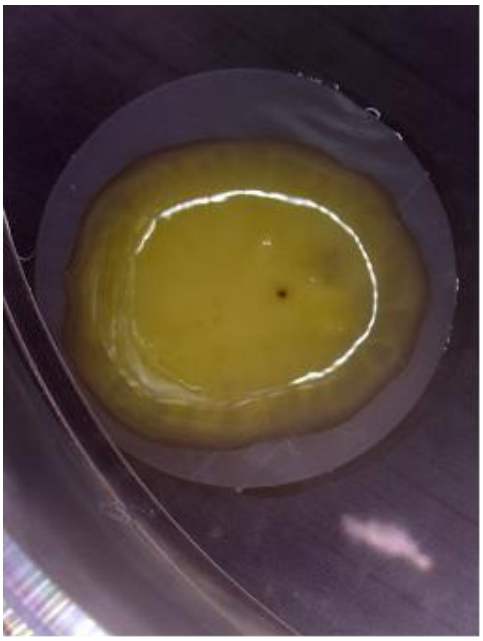

Figure 1: Macroscopic view of a human ENT derived in vitro from embryonic stem cells.

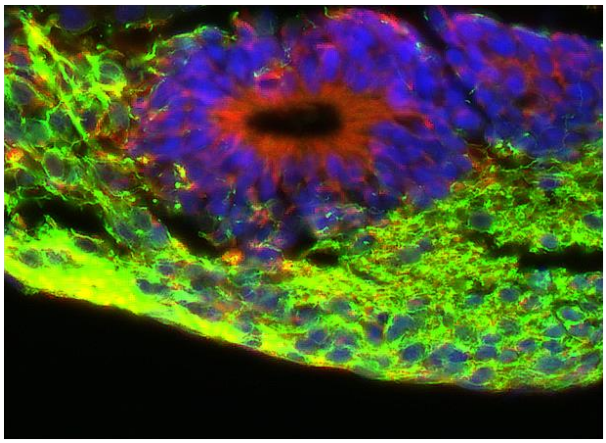

Figure 2: Coloration with fluorescent probes of an engineered neural tissue section. Blue staining indicate cell nuclei, showing the tissular organization. Red staining indicates germinal layers of neural stem cells generating the mature neuronal tissue (in green).

Coloration with fluorescent probes of an engineered neural tissue section. Blue staining indicate cell nuclei, showing the tissular organization. Red staining indicates germinal layers of neural stem cells generating the mature neuronal tissue (in green)

3R-Project 116-09

Organotypic brain-slice cultures derived from regularly-slaughtered animals as an *in-vitro* alternative for the investigation of neuroinfectious diseases in ruminants

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Keywords: *live stock*; *brain*; *infectious diseases; veterinary disease*; *cell cultures: organ-specific*; *reduction; replacement*

Duration: 3 years **Project Completion:** 2013

Background and Aim
Infectious disorders of the central nervous system (CNS) in livestock may have severe economic and public-health implications and are therefore of major concern. This was demonstrated dramatically in the mid 1990s, when it became evident during the upsurge of bovine spongiform encephalopathy (BSE) that the disease was transmissible from cattle to humans. Recently, atypical variants of transmissible spongiform encephalopathies (TSEs) have been detected in ruminants. As yet, it is not known whether they manifest inter-species cross-over potential. Listeriosis, which is caused by *Listeria* (*L*.) monocytogenes(LM), is another infectious and zoonotic CNS-disease of likewise high impact on livestock and humans.

Despite intensive research activity in the fields of TSEs and listeriosis during the past decades, very few *in-vitro* sytems are available for the modelling of their neuropathogenesis and host-pathogen interactions and for strain-typing. Studies depend largely on bioassays either in laboratory rodents or in the natural ruminant host, since these best reflect the intricate pathogenesis of CNS-infections. Given the highly invasive inoculation routes and the severity of the resulting disease, such experiments raise fundamental ethical concerns, and are awarded the highest severity grade (Schweregrad 3). In the case of TSEs, experiments may be protracted over several years until the time of sacrifice. Moreover, it is often unclear to what extent the results of rodent models can be extrapolated to the situation in the natural host. On the other hand, pertinent cell models of ruminant neuroinfectious diseases do not exist. Hence, our aim was to develop an ethically-sustainable, host-specific, organotypic brain-slice culture using material derived from regularly-slaughtered ruminants as an *in-vitro* system for the identification and investigation of neuroinfectious diseases, here exemplified for prion diseases and listeric encephalitis.

Method and Results
The hippocampus, cerebellum and brainstem were collected from sheep, goats and cattle at a slaugtherhouse, immediately after slaughter. Using a vibratome, 350-µm-thick brain-slices were cut and plated on membrane inserts in 6-well plates (Figure 1; for methodological details, please see Guldimann *et al*., 2012). We assessed the viability of the brain-slices by determining the difference between the number of dead cells and the total number of cells present in a slice (Figure 2, a and b). The presence of brain-cell types (neurons, microglia, astrocytes, oligodendrocytes) was assessed by double-immunofluorescence. We demonstrated that bovine brain-slices can be maintained in culture for up to forty-nine days and that all endogenous brain-cell populations, including neurons (Figure 3), are present. Because the viability of the brainstem and of the cortical slices was low, experiments with these regions were discontinued. The viability results of the hippocampal slices were consistently better than those of the cerebellar ones. Brain-slices that were derived from slaughtered sheep and goats were significantly less viable than were those stemming from cattle. Owing to the disintegration of the tissue, they could not be maintained for extended periods of time in culture. For these reasons, we used only bovine hippocampal slices for the infection assays with LM. To allow time for the explantation trauma to subside, the slices were incubated for seven days prior to the performance of the infection assays. We demonstrated that this *in-vitro* system is susceptible to LM-infection and that it replicates features of natural rhombencephalitis in ruminants (Figure 5; Guldimann *et al.*, 2012).

To investigate whether specifically neurovirulent LM-strains can be identified and distinguished from those of other sources in the brain-slice model, infection assays with forty-seven LM-strains from different sources (rhombencephalitis,

abortion, gastroenteritis, environment, food, human infections) and genetic complexes were performed on cultured bovine hippocampal slices (Guldimann *et al*., in preparation). To measure bacterial replication in the brain-slices, CFU's were determined 48 hours post-infection. To evaluate the extent of bacterial spreading, the slices were fixed in paraformaldehyde, and immunofluorescence for LM was then performed. The total size of all bacterial foci within a slice was measured and expressed in μm^2. All LM-strains but one were capable of infecting and of replicating in bovine hippocampal slices. Although the infection assays in brain-slice cultures cannot clearly distinguish between encephalitogenic, non-encephalitogenic and environmental LM-strains, they nevertheless reveal inter-strain differences in neurovirulence that are linked to the genotype. MLVA (Multilocus Variable Number of Tandem Repeats Analysis) complex-A LM-strains replicate (Figure 4) and spread more efficiently in hippocampal slices than do complex-C ones (Balandyte *et al.* ,2011).

Brain slices for infection assays with scrapie prions were obtained from slaughtered lambs and caprine kids. Sets of tissue-slices were prepared from the hippocampus and the cerebellum. The slices were categorized as being either susceptible or resistant to classical scrapie, depending on the genotype of the animal as determined by the polymerase-chain reaction. Slices derived from classical scrapie-susceptible animals were microinjected with classical scrapie prions, whereas those derived from classical scrapie-resistant (and hence atypical scrapie-susceptible) animals were microinjected with atypical scrapie material. At least one slice per animal was injected with phosphate-buffered saline (negative control). Slices were then harvested at weeks 0, 3, 4, 5 and 6 post-infection. After homogenization, they were treated with Proteinase K to distinguish between PrPc and PrPsc. Protein extracts were then analyzed by PrPsc-Western blotting. Prion replication was not observed in slices that had been incoculated with atypical scrapie material. In the Western-blot analysis that was conducted at the 4-week juncture, the classical scrapie-inoculated brain-slices of two caprine kids and of one lamb revealed bands that reacted with the two prion-protein-specific antibodies. However, their molecular masses did not match with the profiles that were indicative for the disease-associated prion protein. Moreover, the signals were at the detection limit and only poorly reproducible when analyzed in replicates. It was not possible to analyze the slices of these three animals that were harvested at later time-points owing to their extensive disintegration. The other classical scrapie-inoculated brain-slices were negative in the Western-blot analyses.

Conclusions and Relevance for 3R

Although the suitability of the system for long culturing times lends itself to the investigation of slow-growing agents such as prions, we were not able to efficiently propagate scrapie prions in ovine and caprine brain-slices. However, there may be some scope for improvement by combining tissues from highly genetically-susceptible sheep or goats with scrapie strains that are specifically adapted to these genotypes.

Slaughterhouse-derived brain-slices from bovine animals were sufficiently viable and could be successfully infected with LM (Guldimann *et al*., 2012). Results from our infection assays with LM indicate that strains from all three genetic complexes (Balandyte *et al.,* 2011) are capable of evoking encephalitis and that all LM-strains have to be considered as potentially neurovirulent. However, together with our MLVA-data (Balandyte *et al*., 2011) - according to which most encephalitic strains from ruminants cluster strongly in the genetically-homogenous complex A - our infection assays revealed the latter to be particularly neurovirulent.

Organotypic brain-slices that are derived from slaughterhouse material appear to be an adequate *in-vitro* model to study the pathogenesis of the intracerebral phase of LM-infection and to screen LM-strains for neurovirulence. We are confident that such organotypic brain-slice cultures represent an ethically-sustainable and host-specific *in-vitro* system for the investigation of neurolisteriosis. Brains from slaughtered cattle are available on a regular basis and relevant brain regions that are adequate for culturing purposes (such as the hippocampus) are easily accessible on site. Currently, we are using bovine organotypic brain-slices to study the bacterial determinants of intracerebral replication and spreading, which are crucial for an understanding of the neuropathogenetic process. The use of brain-slice cultures not only helps to spare the life of animals, but also offers the combined advantages of reflecting the organ-specific microarchitecture of the brain and of working under controllable conditions.

Our results on CNS-listeriosis might encourage other scientists to consider this *in-vitro* approach as an alternative to animal experiments in their own research. We are confident that in the era of next-generation sequencing (NGS) – in which novel neuroinfectious agents will be identified - this system has the potential to substitute animal models of neuroinfectious diseases with an impact on veterinary and public health.

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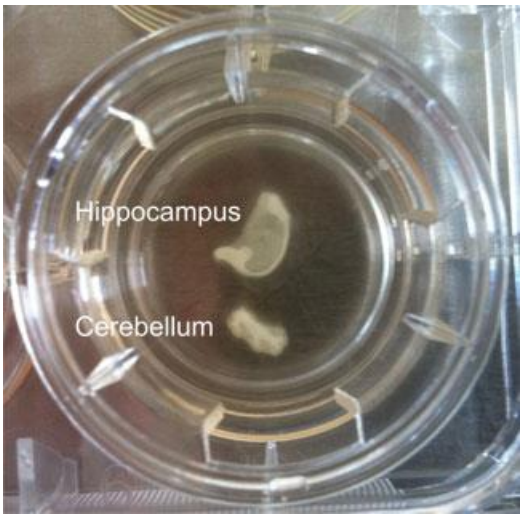

Figure 1: Hippocampal and cerebellar brain slice culture from a bovine. Anatomical architecture is maintained and at the edges cells grow out.

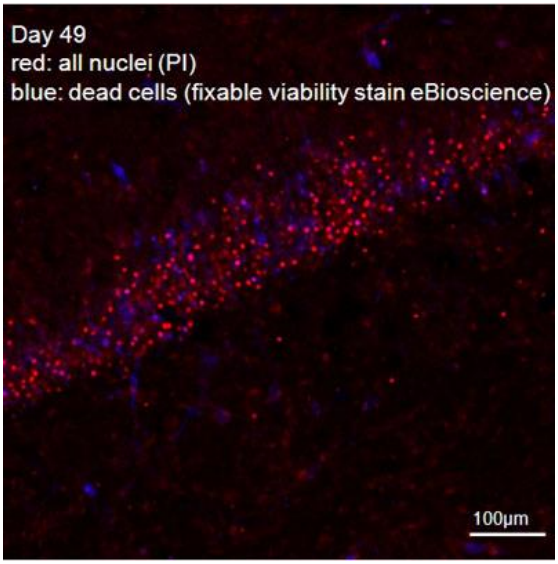

Figure 2: Hippocampal slice, combined fixable-viability (blue, nuclei of dead cells) and PI (red, all nuclei) stain.

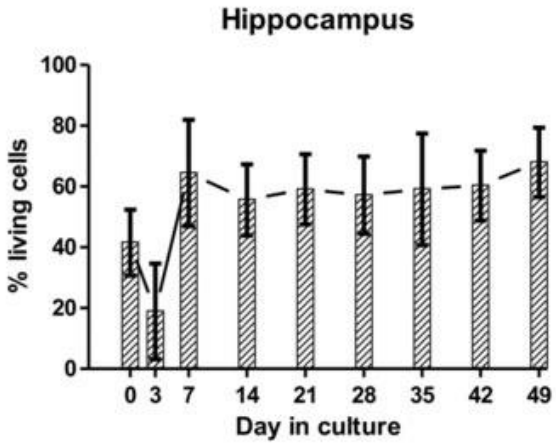

Figure 3: The average proportion of viable cells as estimated from dead cell and total cell counts are indicated at different time points for hippocampal and cerebellar tissue-slice cultures from six different calves (error bars represent standard deviations).

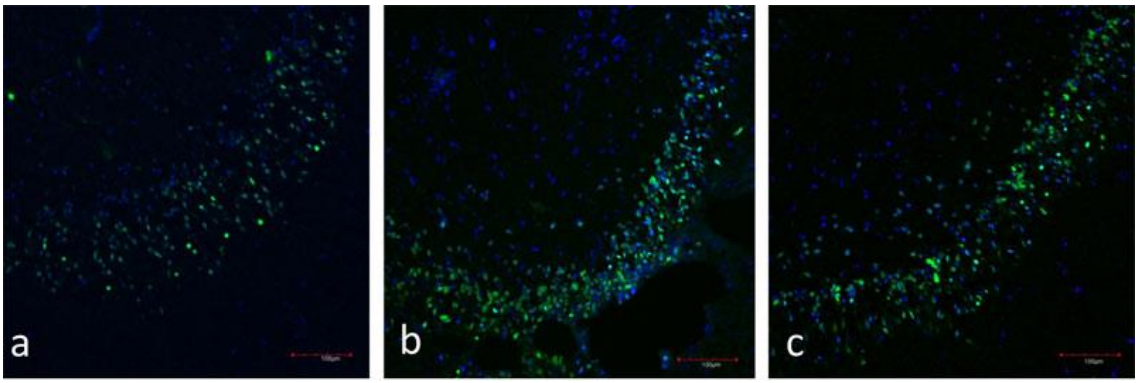

Figure 4: a) Immunofluorescence of the hippocampal dentate gyrus (day 7 in vitro). Neurons are stained in green (NeuN), nuclei are stained in blue (TOTO-3).
b) Immunofluorescence of the hippocampal dentate gyrus (day 28 in vitro). Neurons are stained in green (NeuN), nuclei are stained in blue (TOTO-3).
c) Immunofluorescence of the hippocampal dentate gyrus (day 49 in vitro). Neurons are stained in green (NeuN), nuclei are stained in blue (TOTO-3).

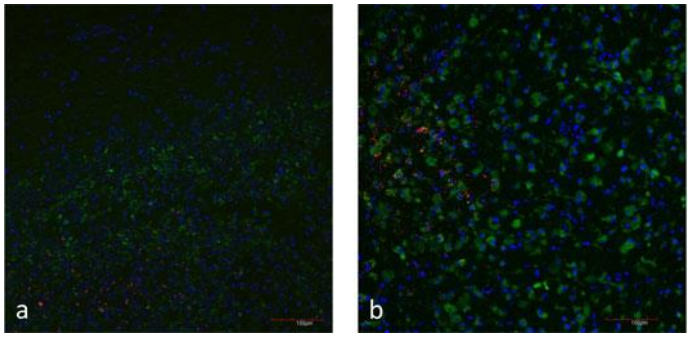

Figure 5: a) Natural case of listeric rhombencephalitis, double-immunofluorescence for *L. monocytogenes* (red) and microglia cells (CD68 in green). Focal replication of *L. monocytogenes*.
b) Brain slice inoculated with *L. monocytogenes* (red). Microglia cells are stained with CD68 (green). Focal replication of *L. monocytogenes*.

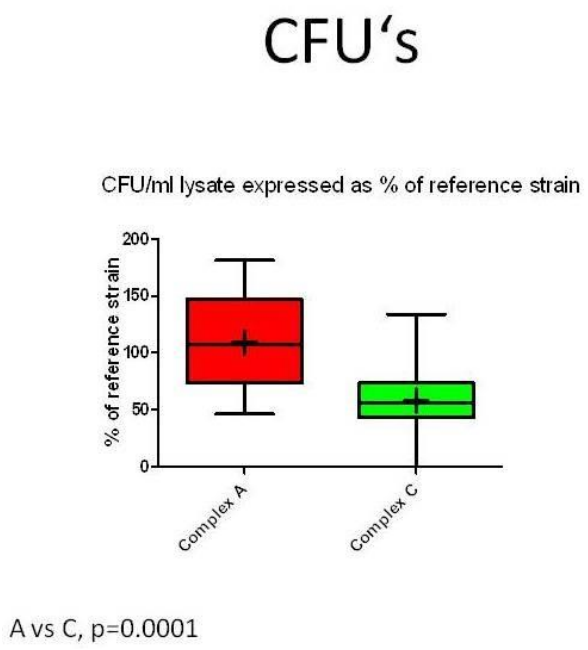

Figure 6: CFU’s recovered from lysed hippocampal slices inoculated with *Listeria monocytogenes*strains (n=47) from MLVA complexes A and C. Values are expressed as % of an internal reference strain (L104). Complex A strains have significantly increased CFU’s compared to complex C strains indicating they replicate more efficiently in hippocampal slices.

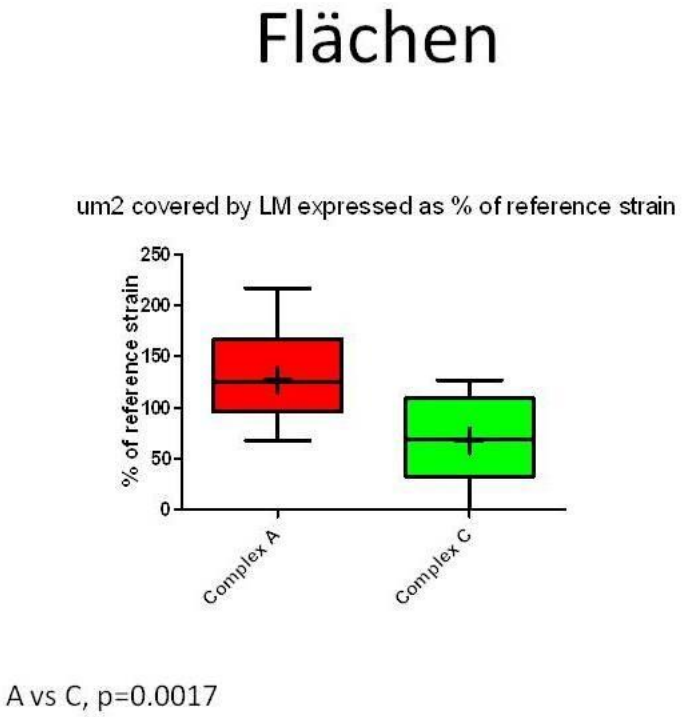

Figure 7: µm^2 area of hippocampal slices invaded by *Listeria monocytogenes* strains from MLVA complexes A and C. Values are expressed as % of an internal reference strain (L104). Areas occupied by complex A strains are significantly larger than areas occupied by complex C strains indicating complex A strains spread more efficiently between cells in hippocampal slices.

3R-Project 117-09

Embryonic stem-cell-derived *in-vitro* model of tissue inflammation following confrontation with implant materials

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Keywords: *rodents*; *stem cells*; *angiogenesis*; *angiogenesis; inflammation*; *cell cultures: 3d cultures; cell cultures: co-cultures*

Duration: 2 years **Project Completion:** 2012

Background and Aim
Artificial implants are now commonly and routinely used in clinical practice, for instance, in dentistry, orthopaedics and cardiology. In recent years, biocompatible and biodegradable polymers have been developed for a variety of clinical applications, for example, as artificial heart valves, artificial blood vessels and occluders. Biodegradable polymers support the healing process in so far as they undergo degradation within the targeted biological milieu. The immunocompatibility of implant materials is currently tested in animal models by an analysis of immunorejection. In these animal models, typical signs of an inflammatory response are analyzed, for instance, diarrhea, loss of hair, a hooked posture, lethargy and an increase in the circulating population of leukocytes. Conventional biocompatibility testing is also performed *in vitro* using cultures of usually one cell type (e.g., fibroblasts). After the cells have been plated on the polymeric material to be tested, their adhesiveness, proliferative capacity and viability are evaluated. However using this conventional in-vitro testing system, the inflammatory response that is evoked *in vitro* by the interaction of the polymeric material with the host tissue cannot be assessed.

The aim of this project was to develop an *in-vitro* system to assess the biocompatibility of newly-developed implant materials, which could be implemented as a viable alternative to testing in living animal models.

Method and Results
In this project, embryonic stem cells were used to generate an immunocompetent vascularized tissue (Inflaplant) which displays a cellular inflammatory response to specific materials, thereby permitting the gleaning of information appertaining to the biocompatibility of the tested product. The interaction between the embryonic stem-cell-derived tissue (embryoid bodies) and the particles of the polymeric material was achieved in “hanging-drop“ cultures (Figure 1, A-C). The polymeric materials were developed and provided by Innovent Inc. (Jena, Germany).

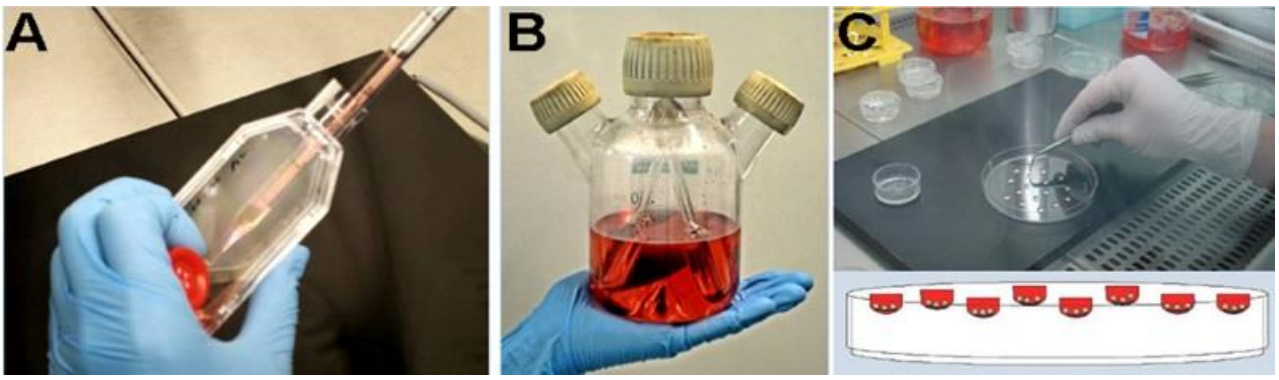

Figure 1: Generation of INFLAPLANT. Confrontation cultures derived from murine embryonic stem cells and particles of a polymeric material. The embryonic stem cells were dissociated and seeded into a spinner-flask culture (A). In this cell culture, the cells form embryoid bodies (B). On day 10, the embryoid bodies were removed from the spinner flask and inoculated into the “hanging drop” containing polymeric particles of similar size (C). Following an adhesion time of 48 hours, the adherent cells and the particles of the material were transferred to cell-culture dishes.

Using a material that is deemed to be poorly biocompatible, such as PVC-lead, a substantial reduction in the CD31-positive area was revealed (Figure 2A). Using one that is believed to be highly biocompatible, the CD31-positive area was well differentiated and well connected (Figure 2B). Furthermore, using another material that was suspected of being poorly biocompatible, the percentage of dead cells – assessed using an ethidium-homodimer – increased (Figure 2C). The dimensions of the maximal and minimal extensions of the embryoid bodies that were co-cultivated on the tested polymer varied in accordance with the postulated biocompatibility of the material during a defined time-window (Figure 2D). Also the differentiation and the behaviour of the inflammatory cells (e.g., the CD68-type, which is a marker for macrophages) is potentially suggestive of the biocompatibility of the material that undergoes embryoid-body-based compatibility testing (Figure 2, E and F). A material that induces an inflammatory response potentially leads to the stimulation of cytokine secretion in the medium, as shown in Figure 3 (A-F).

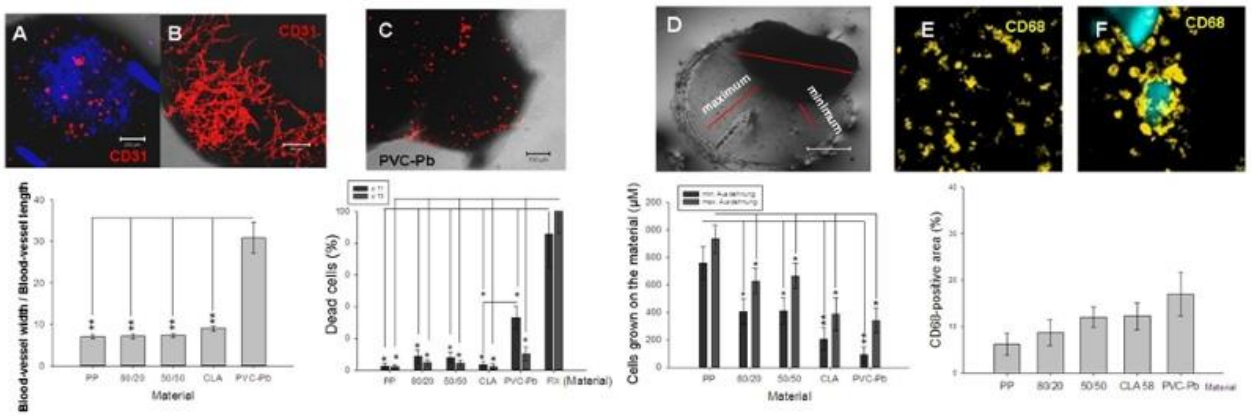

Figure 2: Interaction between the embryoid bodies and the polymeric material. Representative images and quantitative analysis of the PECAM-1(CD31)-positive cells after their interaction with either a poorly biocompatible (A) or a highly biocompatible material (B). The lethality of the cells in the embryoid bodies was measured after their confrontation with the polymeric particles in the hanging drops (C). The maximal and minimal extensions of the embryoid bodies that were co-cultivated with the tested materials were quantified (D). The percentage area that was occupied by CD68-positive cells (monocytes/macrophages) in the embryoid bodies was measured after their confrontation with the polymeric particles (E, F). The fluorescent areas in the respective optical sections were evaluated by the image-analysis software of the confocal laser-scanning microscope (LSM 510, Zeiss, Jena, Germany).

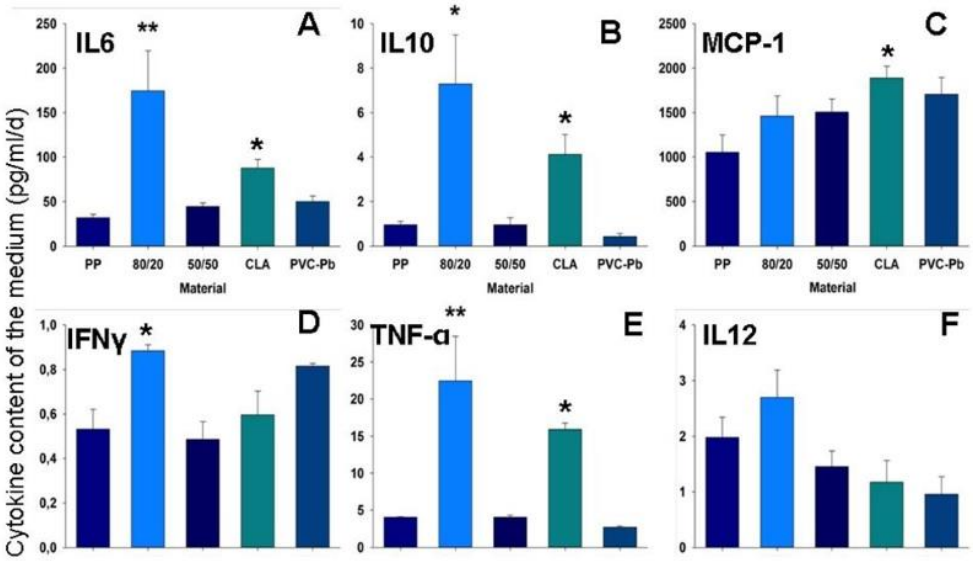

Figure 3: Inflammatory response. Graphs depicting the levels of various cytokines, which were monitored in the medium surrounding the embryoid bodies upon their interaction with the different test-materials.
A: *interleukin-6 (*IL-6); B: interleukin-10 (IL-10); C: monocyte chemotactic protein 1 (MCP-1); D: interferon-gamma (IFN-

γ); E: tumour necrosis factor alpha (TNF-α); F: interleukin-12 (IL-12). * Significantly different from a highly biocompatible material *(p < 0.05);* ** highly significant *(p < 0.01).*

The *in-vitro* embryoid-body system is suitable for examining the influence of biomaterials on living structures. The elevated differentiability, and hence the high reactivity of the embryoid bodies, permit statements to be made concerning changes in the cells after their confrontation with the material in the hanging drop.

Conclusions and Relevance for 3R

The development of a 3-dimensional and multicellular *in-vitro* model for the testing of a tissue's inflammatory response and of cellular retraction during its exposure to biodegradable polymers will help to cut down on the number of animals that are currently employed for testing purposes under *in-vivo* conditions.

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3R-Project 118-10

Engineering of an in vitro hepatocyte tissue system for malaria liver infection research

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Keywords: *human*; *hepatocyte; parasites*; *infectious diseases; malaria*; *reduction; replacement*; *infectiosity*

Duration: 1 year **Project Completion:** 2011

Background and Aim
Malaria continues to be an important global infectious disease for which mortality levels remain unacceptably high. The illness is caused by the Plasmodium parasite, most often *Plasmodium falciparum*, which is transmitted to humans in sporozoite form by infected Anopheles mosquitoes. Drug therapy represents an important intervention in controlling the disease. However the parasite has become increasingly resistant to treatment with the current line of available drugs. In certain regions tolerance is now exhibited even to artemisinin based therapy (Wongsrichanalai et al., 2008), which is widely considered to be the antimalarial of last defence. There is therefore an increasing urgency to develop new approaches and technologies that speed up the creation of effective new drugs and, ultimately, vaccines against the disease. Despite advances in our understanding of *P. falciparum* and its intra-erythrocytic development, significantly less is known about the parasite's development in hepatocytes. A major reason underlying this shortfall has been the unavailability of suitable *in vitro* models through which to study parasite-host hepatocyte development on a large-scale. Consequently, surrogate animal models based on related parasite species are widely employed for this purpose. Through joint collaboration between the CSIR Biosciences and Material Sciences and Manufacturing units, work has been initiated to engineer a novel 3D polymer-based hepatocyte tissue culture system. This development is driven by a need to create new *in vitro* systems that correlate more closely with the *in vivo* liver state, providing a more reliable predictive model for research, in particular for investigating parasite-host interaction. Through the successful establishment of such a system (3), we envisage the possibility of perfomring detailed molecular investigations of *P. falciparum* hepatocyte development, leading to new opportunities in drug and vaccine discovery.

Method and Results
Through the use of conventional non-woven polymers and smart polymer technology (3), it has been possible to develop a proprietary three-dimensional scaffold system that is capable of supporting the growth of hepatocyte (and other anchorage dependent) cells in an enhanced manner. In particular, the system preserves proteins and glycans expressed on the extracellular matrix that are critical, together with other factors, to facilitating malaria sporozoite invasion and development. Primary hepatocyte cell lines (e.g. hNHEPS and HC04 (Sattabongkot et al., 2006)) will be employed to facilitate infection by *Plasmodium falciparum* sporozoites that have been isolated from Anopheles mosquito hosts. Conditions will be optimized throughout to achieve maximum rates of infection and development, culminating in the assessment of merozoite viability by exposing mature merozoites from the new system to conventional red blood cell cultures. Successful merozoite invasion of red blood cells, and subsequent intra-erythtrocytic development in these cells, will confirm the validity of the new *in vitro* model.

Conclusions and Relevance for 3R
This project aims to develop a 3D-based hepatocyte culture system that facilitates large-scale molecular analysis of the human form of malaria, *P. falciparum*, in this tissue. To date, this research has largely relied on the use of surrogate animal models, that utilize related species of this parasite. New opportunities in malaria drug and vaccine discovery research are anticipated from the new system.

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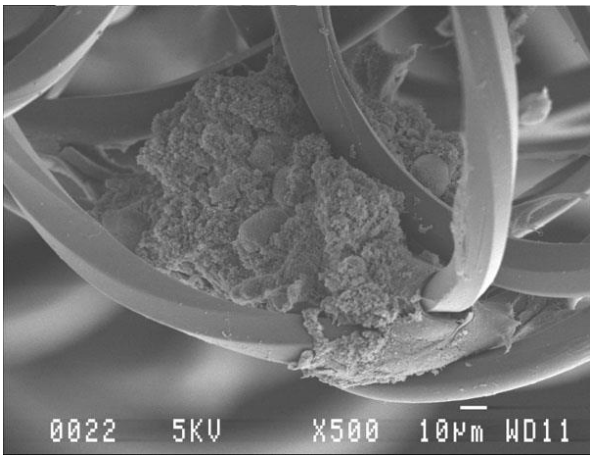

Figure 1: Electron micrograph of differentiated HepG2 hepatocytes grown on the 3D scaffold surrounded by extracellular matrix.

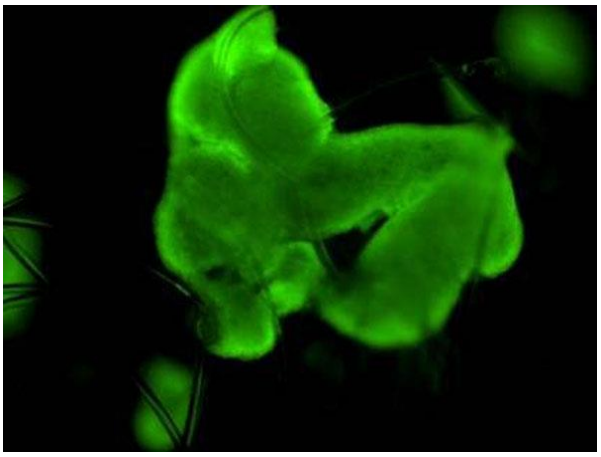

Figure 2: Fluorescence confocal image at 400X magnification illustrating differentiated HepG2 hepatocyte cell viability grown on the 3D scaffold following fluorescein diacetate staining.

3R-Project 119-10

Non invasive electrical monitoring of the population spiking activity in the central nervous system

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Keywords: *human; primates*; *brain*; *refinement; replacement*

Duration: 3 years **Project Completion:** 2013

Background and Aim

Much of the knowledge about the dynamic functioning of the central nervous system (CNS) has come from invasive animal experiments in which parts of the nervous system are electrically monitored, stimulated or destroyed. For obvious ethical reasons, there is an increasing interest to replace the invasive approaches by less invasive ones.
While several non-invasive neuroimaging modalities have been developed in the last years, none of them is yet able to substitute invasive animal recordings aimed to monitor electrical activity at the single cell/population level. High spatial resolution techniques such as the fMRI lack temporal resolution and fail to provide a complete, clear account of spiking activity as measured in animals. The Electroencephalogram (EEG), likely to be the most direct correlate of neural activity that can be obtained non-invasively, lacks spatial resolution providing information limited to the scalp surface. The issue of the spatial resolution of the EEG can be circumvented by the use of physico-mathematical approaches capable of providing direct estimates of the neural intracerebral activity at the population level [1]. While this is yet far away from the information contained in the spiking activity recorded with neural implants, recent studies using simultaneous invasive recordings of spiking activity and scalp EEG suggest that there is a link between the phase of oscillations recorded at the macroscopic level and the spiking activity of cell populations. One goal of this project is to confirm this relationship in humans or in other structures different from the visual cortex in primates. This could serve to significantly enhance the spatial resolution that can be ultimately achieved with a method termed ELECTRA [2], which we have previously developed to estimate intracerebral activity from scalp EEG.

Method and Results

in progress (present status)

Recent technological advances are starting to allow simultaneous recordings of neural activity at different spatial scales in behaving animals. In one of this studies [3] published at the end of 2009 by Whittingstall and Logothetis, it has been shown that MUA in the primary visual cortex of monkeys can be predicted from the increases in the power of gamma band activity recorded at the scalp surface (i.e., EEG) occurring during the negative phase of the delta band oscillations. The fact that the phase of slow oscillations in the delta/theta band is a key element to understand spiking activity is indeed not new and has been locally observed in diverse structures in animals [4] and humans [5]. The main merit of the Whittingstall and Logothetis study is indeed to show that such links between the phases and power of the oscillations to spiking activity of populations still holds true for scalp EEG measurements. To combine this recent experimental evidence with the ELECTRA approach proposed in our group to estimate field potentials from scalp measurements (Figure 1), we need to verify that this relationship also holds for other brain structures in humans and primates.

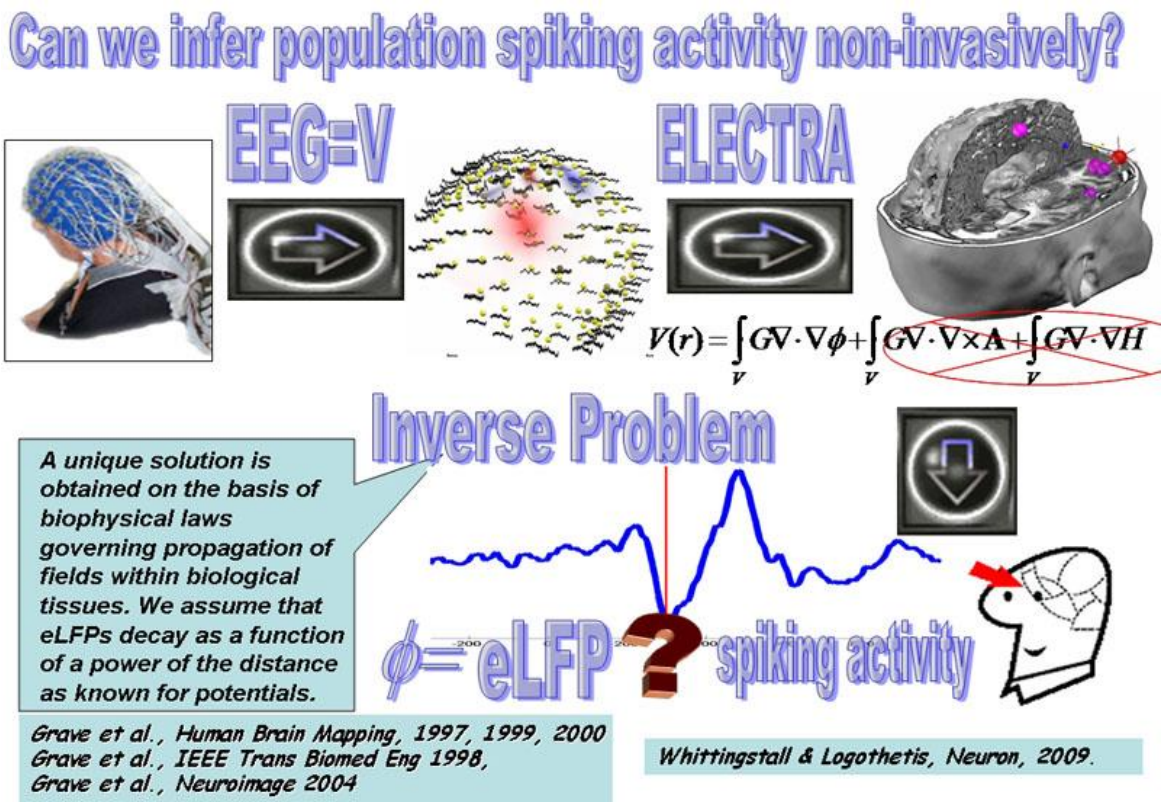

Figure 1: *Main schema of the project:*

The scalp recorded EEG provides the most direct correlate of neural activity that can be obtained non-invasively but lacks spatial resolution providing information limited to the scalp surface. However, it can be mathematically proven that scalp recorded potentials (V) relate to intracerebral field potentials (F) proportional to the invasively recorded Local Field Potentials (LFP) in animals. This relationship is at the basis of a source model called ELECTRA that can be combined with physical and mathematical constraints to solve an ill-posed inverse problem and estimate LFPs within the brain (eLFP). While this is yet far away from the information contained in the spiking activity recorded with neural implants, recent studies using simultaneous invasive recordings of spiking activity and scalp EEG suggest that there is a link between the phase of oscillations recorded at the macroscopic level (eLFP) and the spiking activity of cell populations. We would like to confirm this relationship in humans and in other structures different than the visual cortex in primates to evaluate whether and how well could we estimate population spiking activity non-invasively.

Figure 2: Relationships between delta phase (z-score) and amplitudes within different frequency bands recorded by an electrode in the central nuclei of the amygdala and MUA activity recorded in the basal nuclei of one awake primate (maccaque mulata). Note that modulations in the delta phase and gamma power in the central nuclei accompany MUA decreases in the basal nuclei. There is no clear increase in gamma power. This finding confirms a dissociation between delta phase and MUA.

Figure 3: Relationships between delta phase (z-score) and amplitudes within different frequency bands recorded by an electrode over the occipital cortex in a healthy human subject performing a visual discrimination task. Note that changes in delta phase accompany stimulus onset but there are no significant increases in gamma power.

The analysis of simultaneous EEG/LFP and MUA recordings in rats and primates indicate that the relationship between delta phase changes, increases in gamma power and MUA activity is not ubiquitous. As shown in Figure 2, there can exist significant changes in the phase of delta oscillations (here shown for the central nuclei of the amygdala of awake primates) that bear no relationship with significant increases in gamma power but instead correlate with decreases in spiking (MUA) activity (within the basal nuclei).Modulations of the phase of delta oscillations at the time of stimulus (varied images of different emotional content) onset (zero time) are however significant and last for around 200 ms.

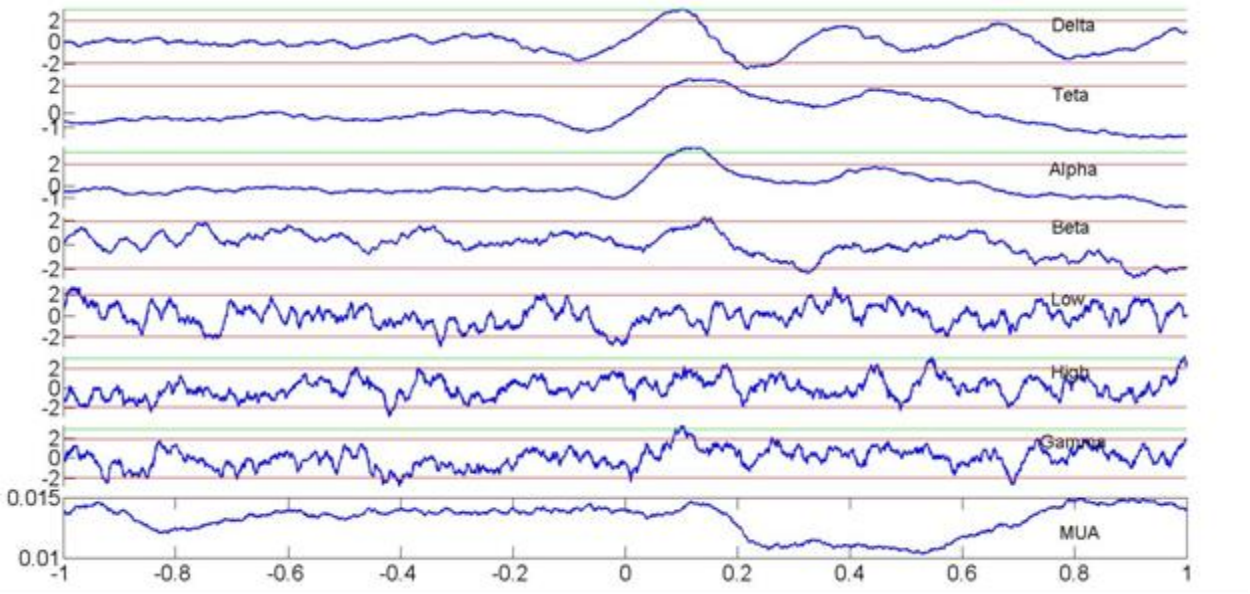

Figure 2: A similar situation is observed in humans. As shown in Figure 3, recordings over the occipital cortex in a healthy volunteer performing a visual discrimination task (onset f the stimuli at zero) indicate that changes in the phase of delta oscillations are a systematic marker of evoked neural responses. However, no significant increases are seen in the power of other frequency bands.

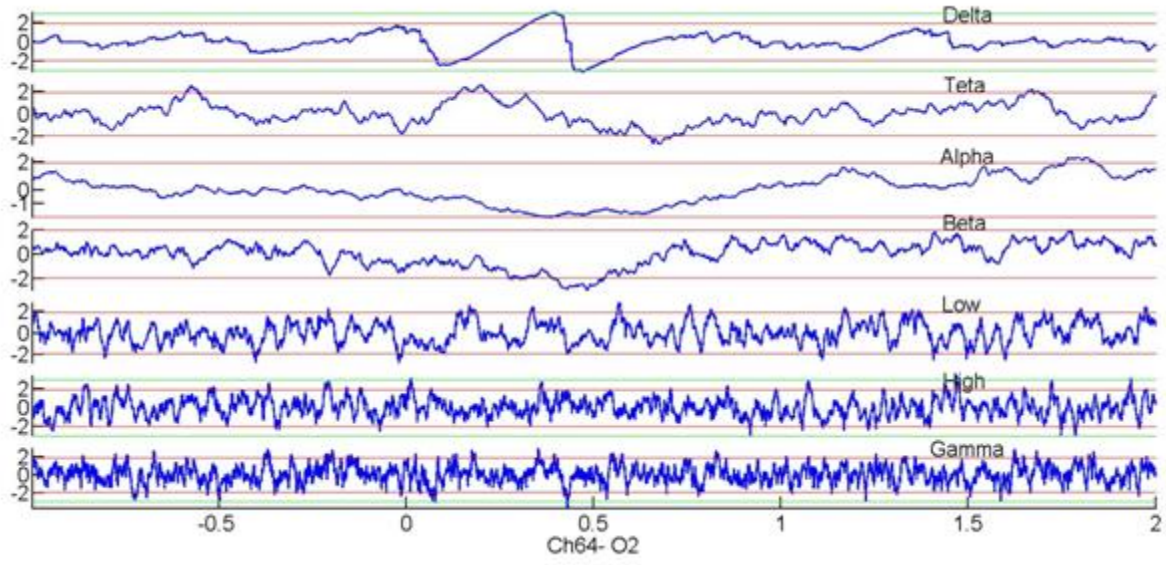

Figure 3: The main idea behind the project is to combine ELECTRA approach to determine intracerebral field potentials with the link between phase/power of neural oscillations and MUA disclosed in [3] to obtain non-invasive estimates, even if rough, of population spiking activity. This is certainly an ambitious goal and several steps are required before we can claim that this is indeed feasible. The first aspect that needs to be evaluated is whether the same or different relationships between frequency bands and MUA holds true in other neural structures (e.g., amygdala) or different species (humans instead of monkeys). Otherwise, we will need to identify the valid relationships through the analysis of concomitant invasive recordings of field potentials and MUA in different structures and species.

To confirm the link phase-power of the oscillations with spiking activity we will rely on simultaneous LFP/MUA recordings in primates' amygdala and invasive recordings in epileptic patients. In parallel, we will compute intracranial field potentials from scalp EEG data using ELECTRA source model. The estimates of field potentials will be transformed into the time frequency domain using the S-transform to compute phase/power relationships over the gray matter (see [2] for details). To investigate the resolution achieved in the non-invasive estimates, we will initially assume that the Whittingstall and Logothetis proposed relationship always holds true. We will therefore consider that a voxel carries substantial information

about MUA when a significant increase in gamma power accompanies the negative-going phase of the delta oscillations. Resolution will be then estimated from the spatial dispersion around the voxel showing maximal activation for each area (most active voxel). The spatial dispersion is characterized by the spatial extent occupied by voxels fulfilling the Whittingstall and Logothetis condition. If novel relationships between LFP phase/power and MUA are disclosed by the analysis of intracranial data in monkeys and humans, we will repeat the resolution analysis previously exposed. This procedure will then allow us to assess whether and how well can we estimate population spiking activity non-invasively.

Conclusions and Relevance for 3R

The analyses of data from different animal species including humans indicate that there is a dissociation between delta phase-gamma power and MUA activity. To account for the observed experimental results we are currently developing a biophysical model to explain the generation of the macroscopic EEG/LFP signals and their relationship to action potentials. In the new formulation we drop the quasistatic approximation [6] and replace it by a dispersive model of neural tissue. The preliminary simulations indicate that this model is compatible with the electrophysiological recordings and explain the systematic phase locking of action potentials to ongoing oscillations in the short spatial scale as well as current experimental data. On the light of this model we will need to modify the whole physical-mathematical formalism behind the formulation of the EEG which serves to determine its sources via the solution of the inverse problem. However, according to the proposed formalism the EEG conveys more direct information about action potentials than currently thought. This observation could therefore significantly enhance the probability of imaging non-invasively the spiking activity in the CNS.

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3R-Project 120-10

Development of non-invasive strategies to study corticospinal neuron disease, injury and repair

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Keywords: *rodents*; *spinal cord*; *spinal cord repair / diseases*; *non-invasive*; *refinement*

Duration: 1 year **Project Completion:** 2011

Background and Aim
Motor pathways of the cerebral cortex and spinal cord progressively degenerate in amyotrophic lateral sclerosisand these neurons are lesioned by spinal cord injury. There is currently no available treatment for these conditions, which are the focus of intense worldwide research.
Spinal cord and cortical interventions in mice and rats have been widely used for such research, but these surgical procedures are very invasive, time consuming, have the negative effect of high per- and post-operative mortality, and often require extensive breeding of transgenic animals over prolonged period. Although stereotactic surgeries are widely used to target specific regions of the nervous system in adult rodents, they are not applicable to spinal cord interventions and lack precision in young animals. Here we propose a pauci-invasive alternative to these surgeries by using high-resolution ultrasound guidance to target the spinal cord and specific cortical targets via percutaneous microinjections.
To develop and validate this approach, we will determine the most efficient experimental parameters to (1) label spinal cord neurons and specific cortical sensory and motor areas in vivo in mice and (2) overexpress a genetic construct in corticospinal neurons.

Method and Results
High-resolution ultrasonography (VEVO 770, Visualsonics, Canada) will be used to target the microinjection of different volumes of a plasmid or viral construct at cervical, thoracic and lumbar levels at P0, P5 and P20, in C57/Bl6 mice, as well as at P0 in the neocortex. We will use an expression vector in which a gene of interest is expressed from a strong CMV/β-actin promoter followed by an IRES-Gfp reporter cassette, which will be electroporated using different voltage intensities (Lai*, Jabaudon* et al., Neuron, 2008).
Twenty-four to 48 hours after injection, the mice will be put down and the nervous tissue collected for identification of labeled neurons. Axial 60 um thick sections will be obtained from paraformaldehyde-fixed spinal cords and brains, and neuronal labeling will be determined to identify the most efficient injection protocol.

Conclusions and Relevance for 3R
The formal validation (6) of an experimental protocol to target the developing cortex and spinal cord and the adult spinal cord in mice using ultrasound-guided percutaneous injections work towards reducing and refining animal procedures in the field of spinal cord research. Furthermore, the development of novel efficient approaches (6) to deliver gene products works towards the reduction in the use of transgenic lines.

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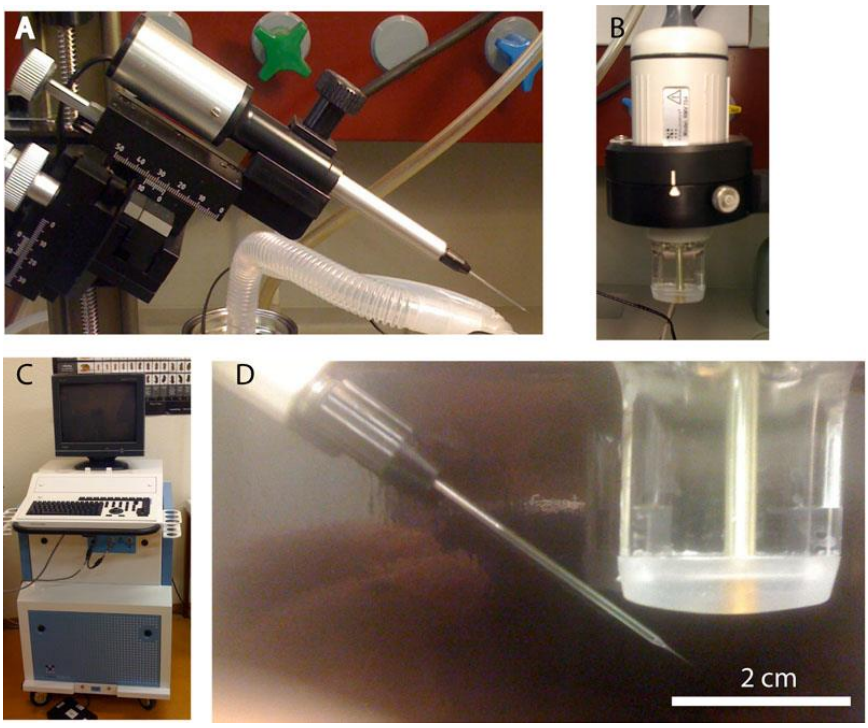

Figure 1: Microinjection and high resolution ultrasound setup: (A) microinjcetion device (B) high resolution ultrasound scanhead (C) high resolution imaging device (D) high magnification showing a borosilicate micropipette fitted onto the microinjection device, right together with the ultrasound scanhead.

3R-Project 121-10

Organotypic spinal cord slices to study SCI and MS like lesions

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Keywords: *mice*; *spinal cord*; *spinal cord repair / diseases*; *reduction; replacement*

Duration: 2 years **Project Completion:** 2012

Background and Aim
Spinal cord injury (SCI) or diseases such as multiple sclerosis (MS) are very debilitating and need the development of new therapeutic strategies. It is a topic of intense basic and applied research. Animal models have allowed a number of findings in understanding the mechanisms involved in SCI and MS, and in testing potential therapeutic strategies. However, such *in vivo* models are extremely distressful for animals. Moreover, the use of a wide variety of models, and their variability and complexity, limit the ability to identify new therapies. We present a new model based on organotypic cultures of mouse spinal cord longitudinal slices ("slice model") that we recently developed (Bonnici and Kapfhammer, 2008). This "slice model" allows to generate reproducible SCI-like and MS-like lesions and to study axonal regeneration.

Method and Results
We implemented a neuroinflammation/demyelination model on the slices by co-treating them with interferon gamma (IFNγ) and lipopolysaccharide (LPS) (Defaux et al. 2010). Using different readout including immunohistochemistry, Western blotting, qPCR, ELISA, and enzymatic assays, we characterized the features of this model and compared it to MS and SCI lesions. We observed significant demyelination with an early and progressive decrease in myelin gene expression and protein levels. Microglia was activated becoming macrophagic and proinflammatory cytokine expression and secretion were highly increased demonstrating neuroinflammation. Interestingly, astrogliosis seemed very limited. More likely as a consequence of the neuroinflammation, we also observed neurodegeneration indicated by axonal lesions, and quantitative (e.g. decrease of glutamate decarboxylase activity) and qualitative (e.g. change in phosphorylation state of neurofilament heavy chain) alterations in neuronal markers.

Conclusions and Relevance for 3R
The developed model allows *in vitro* reproduction of important features of both SCI and MS making it a relevant and multivalent model to study mechanisms or treatment for these diseases. Therefore, the "slice model" may substantially help to reduce animal experimentation in the field of SCI and MS where any *in-vivo* model is by definition extremely debilitating.

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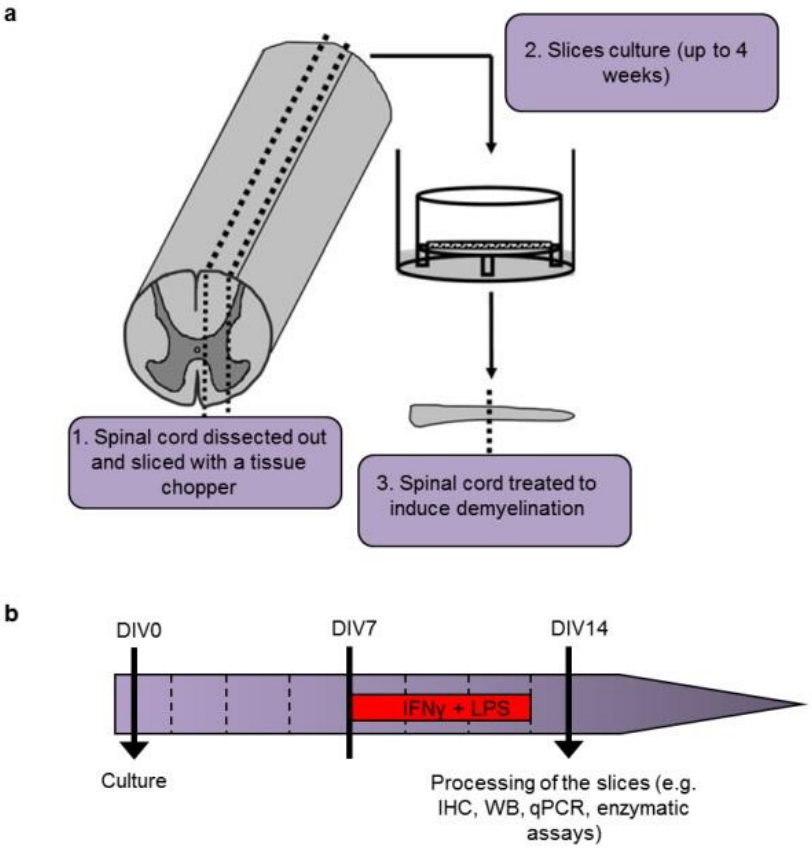

Figure 1: Induction of neuroinflammation/demyelination in our slice model.

a) schematic representation of organotypic cultures of mouse spinal cord sagittal longitudinal slices (modified from Bonnici and Kapfhammer).

b) timeline summarizing the process to induce neuroinflammation/demyelination with IFNγ (100 U/ml) and LPS (10 µg/ml). Medium is changed every 2 days (dotted lines).

3R-Project 122-10

Improved perioperative analgesia and reduced stress during recovery for the experimental animal: ultrasound-guided sciatic and femoral nerve block in sheep and quantitative assessment of block quality

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Keywords: *sheep*; *animal welfare; mechanobiology; orthopedics; pain / stress*; *animal welfare; refinement*

Duration: 3 years **Project Completion:** 2014

Background and Aim
Due to a lack of alternative methods, sheep are widely used for biomechanical research purposes, e.g. for studies concerning the knee joint. Until now, sheep have usually received a combination of analgesics at the time of induction of anaesthesia and again during the post-operative phase by intravenous or intramuscular injection. Based on knowledge of surgery in other species, the assumption may be made that such treatment might be insufficient and these sheep may still feel severe pain.
Regional anaesthesia as an adjunct to general anaesthesia may markedly improve the wellbeing of these experimental animals during the post-operative period [1, 2]. A possible side effect of neuraxial (spinal or epidural) anaesthesia is a bilateral blockade of motor fibres beside the blockade of sensitive fibres. Especially in flight animals, as sheep are, this bilateral paralysis may again cause severe distress. Treating only one side – the side of surgery – using peripheral perineural administration of local anaesthetics helps to prevent this source of stress. The reliability of peripheral perineural blockade can be increased by reducing the failure rate if performed using ultrasound guidance. [3].
Species-specific quantification of post-operative pain is mandatory to evaluate the efficacy of the analgesic technique used. In sheep, as in other flight animals, pain recognition and quantification are particularly difficult. The nociceptive withdrawal reflex (NWR) has been extensively used for the study of experimental nociception in animals and humans [4, 5]. A close relationship between pain threshold and NWR threshold has been identified [6, 7].
Behavioural pain assessment combined with the evaluation of mechanical and thermal nociceptive thresholds in the operated area and a quantification of the NWR will provide a complete way to assess the efficacy of the proposed local anaesthetic technique.

Method and Results
in progress (present status)
The technique of ultrasound-guided blockade of the sciatic and the femoral nerves has been modified and adapted to the ovine anatomy. First, the method was tested in sheep cadavers. In a next step, blockade of the sciatic nerve and the placement of a perineural catheter under sonographic control were evaluated in healthy sheep. The repeated evaluation of the NWR in combination with tests of cutaneous sensation and a determination of the motoric function of the limb allowed an adequate evaluation of quality and duration of loss of sensation after perineural injection of ropivacaine to the target nerve. The duration of effect was longer than expected but the animals could cope with the loss of sensation as well as the motoric function after some time of adaptation.
The clinical application of the method remains to be performed in sheep undergoing invasive hind limb surgery.

Conclusions and Relevance for 3R
If we are successful in the description and optimization of ultrasound-guided single shot and catheter-based sciatic-femoral nerve blocks in sheep, these techniques may become standard treatment for hind limb surgeries in experimental sheep to provide efficacious intra and post-operative analgesia. Due to the remote location from the surgical area, regional anaesthesia techniques will not influence the surgical procedure.

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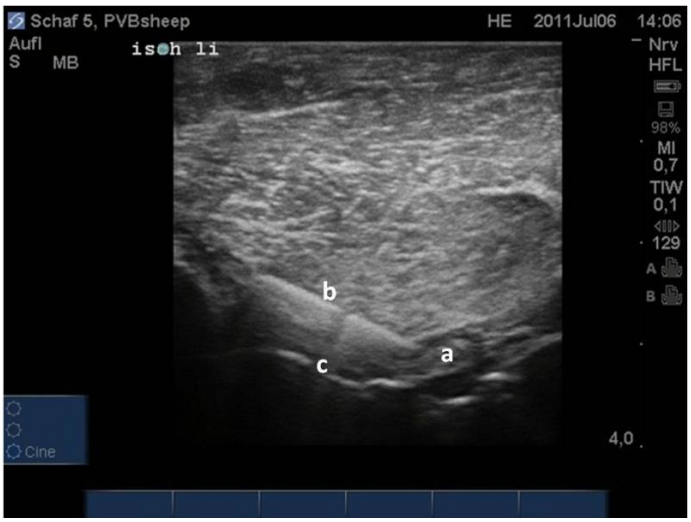

Figure 1: Ultrasonographic image of the left sciatic nerve (a). The position of the needle tip was identified on the basis of reflector marks. (b) Ultrasound needle with its reflector mark (c) Ilium.

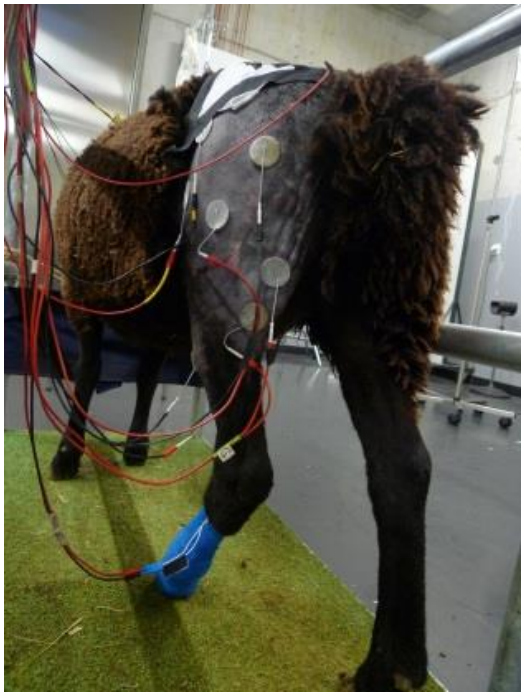

Figure 2: Sheep with a catheter at the sciatic nerve an EMG electrodes at 4 muscles of the left hind limb.

3R-Project 123-10

Use of "moribund" stage in the acute fish toxicity test according to OECD guideline 203 and its effect on LC50 values

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Keywords: *fish*; *ecotoxicology*; *data base*; *oecd-guideline; reduction; refinement*

Duration: 2 years **Project Completion:** 2013

Background and Aim
In the acute fish toxicity test according to OECD guideline 203, LC50 is assessed in terms of the concentration of a test substance at which 50% of the fish die within an exposure period of 96h. 7 fish per sample are exposed to 5 different concentrations plus a control. The criterion for death is defined as (§ 19): "no visible movement, no response when touching the tail." A total of at least 6 x 7 = 42 fish are exposed to severity degrees ranging from 1 to 3.
The duration of suffering could be reduced by using the criterion of ”moribund” commonly applied in rodent acute toxicity testing. Fish might be declared moribund if no visible movement is observed and are removed from the test as soon as this occurs. Dictionary definitions of moribund include words and phrases such as “dying”, ”at the point of death”, “in the state of dying”, or “approaching death” (there is no official definition for moribund stage in fish). The criterion has already been introduced to ecotoxicological testing in the UK, and is being used by some laboratories in Germany and Switzerland. It not only leads to a deviation from the OECD guideline but, and more importantly, also affects main study outcomes, in particular the LC50 values, which may be lower when moribund is applied. Accordingly the present study has several goals. 1) to find a suitable and commonly acceptable definition for the state moribund in fish, 2) to assess the magnitude of the potential decrease of the LC50 value, 3) to estimate the range of shortening the suffering of the fish, and 4) to provide information on how the test guideline should be adapted to reduce the subjectiveness introduced by the use of a the state moribund.

Figure 1: Fish in moribund stage

Method and Results

in progress (present status)

In a first step, moribund state was defined. A first definition (Definition A Table 2) arises from the reporting style of existing studies of an industry laboratory (observations of five symptoms, Swimming Behaviour, Loss of Equilibrium, Respiratory Function, Pigmentation and Exophthalmus as suggested in the OECD guideline)#Table 2# Definition A is based on observations of at least two of the following sub-lethal symptoms Swimming Behaviour, Loss of Equilibrium, and Respiratory Function over 48h with severe symptoms (severity degree 3 in a range from 0 to 3).

The effect on the LD50 values was investigated by a retrospective analysis of 328 studies of an industry laboratory. The analysis was based on definition A of moribund. This allows determining the magnitude of the LC50 based on moribund fish, $LC50_{moribund}$, the frequency of a reduction of the $LC50_{moribund}$ compared to the conventional LC50, and the number of fish affected by a shortened duration of exposure and/or suffering.

A typical example of the symptoms and degrees of severity as reported by the industry laboratory is given in Table 3. #Table 3# In this study, fish showed severe symptoms expressed in terms of altered swimming behaviour and loss of equilibrium from 24h to 96h (i.e. over a period of 72h). The fish would be declared moribund after 48h and removed from the test, thereby reducing degree 3 severity of suffering by 48h in concentrations of 0.88, 1.7, 3.3 and 6.5 mg/L (28 fish in total). Accordingly LC moribund would be 100% in the 4 concentrations whereas the "death" (lethal) criterion results in only 14, 14, 29 and 43% mortality within a 48h time span. Hence, the application of the moribund criterion results in a LC50 moribund of 0.66 compared to 4.4 mg/L using the conventional method (factor 6.7 lower), and to a classification of the chemical into the acute category 1 for hazardous to the aquatic environment (acute (short-term) aquatic hazard).

The procedure was repeated based on a second definition (Definiton B Table 2) of moribund deducted from the reporting style of other industry laboratories. Finally, the outcome of three additional definitions of moribund (Definiton C, D and E, Table 2) based on the reporting style of 10 different laboratories in Europe and the U.S. was evaluated both on the 328 fish acute toxicity tests of the industry laboratory and the 111 tests from the other laboratories.

In the studies evaluated, 10 to 23% of the fish were declared as moribund reducing the suffering of severity grade 3 (severe distress) by up to 92h. The median of the decrease of $LC50_{moribund}$ in relation to the LC50 was by a factor of about 2; the maximum factor observed was 15.7. While the reduction of the suffering is desirable, a decrease of the measured toxicity endpoint has consequences on the classification and on risk assessments.To produce comparable results between laboratories when moribund is used requires the following specifications in an updated test guideline: 1) A unique, generally accepted definition of the moribund state in fish, 2) specification on the type of visible abnormalities to be reported (symptoms), and 3) specifications on the degree of the effects (e.g. how many single observations per concentration).

Conclusions and Relevance for 3R

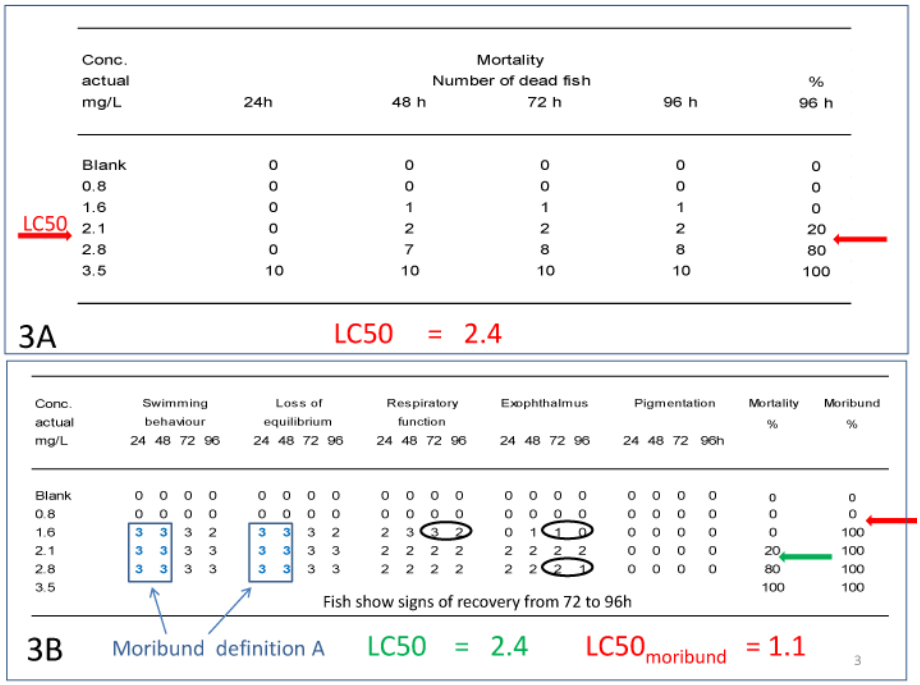
The potential of the project is in refinement. In 2009 and 2010, 46,000 and 36,000 fish were used for testing in Switzerland, about 13,000 and 10,000 of which in acute and chronic toxicity tests, respectively. REACh requires approx. 1.2 million fish each year, with a considerable proportion used in toxicity tests. The fish suffer degree 3 severity at least in the highest dose. A successful inclusion of the moribund criterion in the OECD guideline would mean that fish would be exposed to stress for a shorter period of time, and suffering would be reduced accordingly.

Reference

Rufli H., Introduction of moribund category to OECD fish acute test and its effect on suffering and LC50-values. Environmental Toxicology and Chemistry, Vol. 31, No. 5, pp. 1107–1112, 2012.

Definition A:	swimming behaviour and loss of equilibrium over ≥48h
Definition B:	swimming behaviour over ≥48h, and at least single observation of complete loss of equilibrium or strong ventilation
Definition C:	swimming behaviour over ≥48h, and at least single observation of complete loss of equilibrium, strong ventilation, or strong discoloration
Definition D:	at least single observation of swimming behaviour over ≥24h, and complete loss of equilibrium or strong ventilation
Definition E:	at least single observation of swimming behaviour over ≥24h, and complete loss of equilibrium, strong ventilation or strong discoloration

Figure 2: Definitions of “moribund” stage (all effects with severity degree 3)



Conc. actual mg/L	Mortality Number of dead fish 24h	48 h	72 h	96 h	% 96 h
Blank	0	0	0	0	0
0.8	0	0	0	0	0
1.6	0	1	1	1	0
LC50 2.1	0	2	2	2	20
2.8	0	7	8	8	80
3.5	10	10	10	10	100

3A LC50 = 2.4

Conc. actual mg/L	Swimming behaviour 24 48 72 96	Loss of equilibrium 24 48 72 96	Respiratory function 24 48 72 96	Exophthalmus 24 48 72 96	Pigmentation 24 48 72 96h	Mortality %	Moribund %
Blank	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0	0
0.8	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0	0
1.6	3 3 3 2	3 3 3 2	2 3 3 2	0 1 1 0	0 0 0 0	0	100
2.1	3 3 3 3	3 3 3 3	2 2 2 2	2 2 2 2	0 0 0 0	20	100
2.8	3 3 3 3	3 3 3 3	2 2 2 2	2 2 2 1	0 0 0 0	80	100
3.5						100	100

Fish show signs of recovery from 72 to 96h

3B Moribund definition A LC50 = 2.4 $LC50_{moribund}$ = 1.1

Figure 3: Comparison between LC50 and $LC_{moribund}$ in a real-life study:

3A: Mortaility reported, 7 fish per concentration, LC50 calculated.

3B: Sublethal symptoms observed

The severity degrees 3 of Swimming Behaviour and Loss of Equilibrium are marked within the blue frame as these indicate moribund status according to the definition A.

Explanation of symptoms: 0: none; 1: light; 2: moderate; 3: severe.

3R-Project 124-10

Comparative in vitro and in vivo testing on biofilm formation on the surface of bone grafts

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Keywords: *orthopedics*; *replacement*; *infectiosity; toxicity testing: biomaterials / implants*

Duration: 2 years **Project Completion:** 2013

Background and Aim

Infections associated with orthopaedic implants rarely occur, but represent one of the most devastating complications with high morbidity and substantial costs (1). These implant-associated infections are typically caused by micro-organisms that grow in biofilms (1). Extensive research has been performed in recent years to determine the propensity of medical devices to sustain biofilm formation (2). We recently established an in-vitro setting to analyse biofilm formation on bone grafts and bone graft substitutes (3), and showed in-vitro the importance to monitor (i) material properties (3), and (ii) incubation conditions (4) when investigating biofilm formation on bone grafts. Importantly, it remains unclear whether these in-vitro data are meaningful for in-vivo biofilm formation.

The planned experiment will compare the data of our in-vitro biofilm experiments on bone grafts with data from biofilms produced in-vivo in an established animal model for foreign body infections (5-7). In-vivo data obtained in the guinea-pig model can be directly transferred for predicting efficacy in humans (8), making this model an appropriate and validated tool to investigate implant-associated infections.

The aim of this project is (i) to compare in-vitro and in-vivo biofilm formation on the surface of calcium phosphate (CaP, Fig. 1) bone grafts, and (ii) to establish an in-vitro setting for biofilm formation to reduce the number of animals needed in preclincial trials for testing infect susceptibility of bone grafts in general.

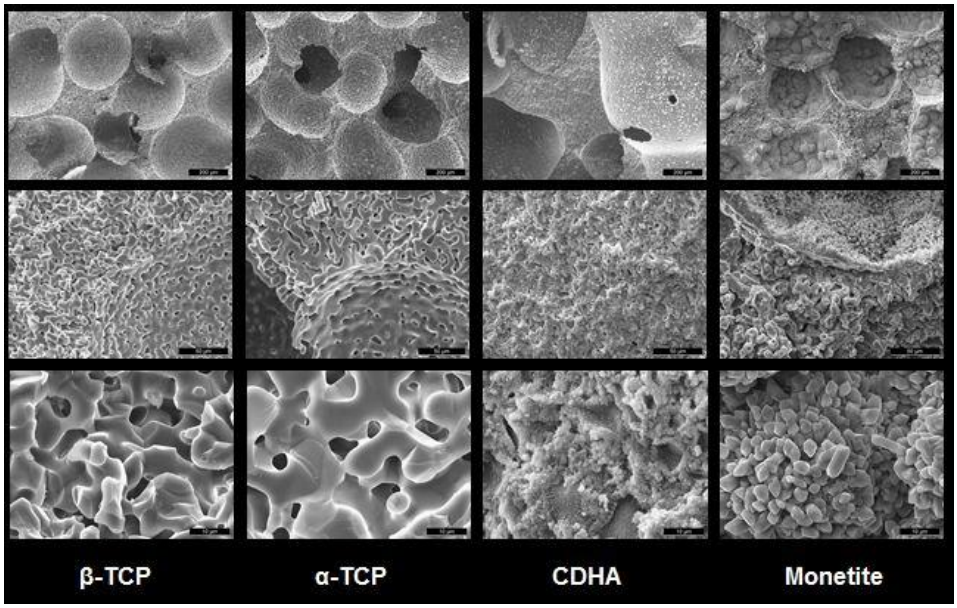

Figure 1: CaP bone grafts used for comparison: alpha tri-calcium phosphate (α-TCP), beta tri-calcium phosphate (β-TCP), calcium deficient hydroxyapatite (CDHA) and Monetite. The used bone grafts can be produced out of each other by means of thermal and chemical transformation (For further details see Bohner M 2000 Injury 31 Suppl 42 and Galea L 2008 Biomaterials 29).

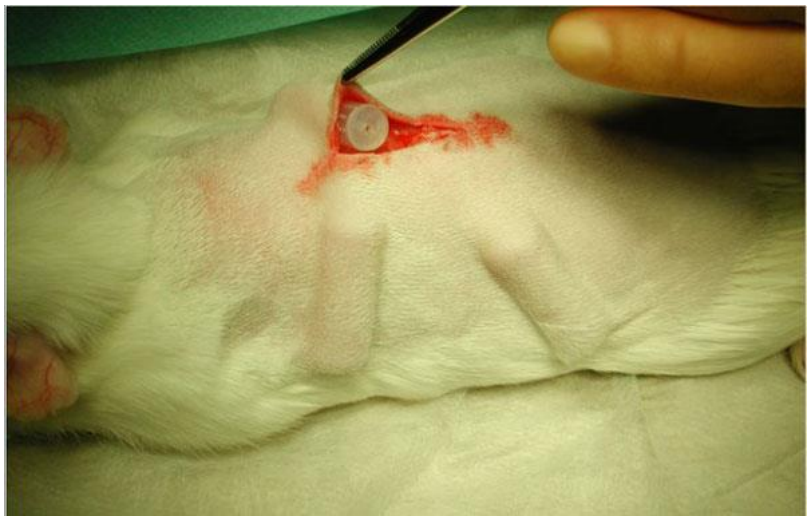

Figure 2: Guinea pig modell with ongoing implantation of tissue cages pre-filled with CaP bone grafts. (Courtesy of Prof. W. Zimmerli, Liestal)

Method and Results

In-vitro biofilm formation of staphylococcal strains on CaP bone grafts: CaP bone grafts will be incubated in pooled human serum. Then samples will be infected by inoculation with 1 x 105 colony-forming units (cfu) of either *S. aureus* ATCC 29213 (MSSA), *S. aureus* ATCC 43300 (MRSA) or *S. epidermidis* RP62A ATCC 35984 (MSSE). Samples with adjacent biofilm will be harvested at defined time points (4).

In-vivo biofilm formation of staphylococcal strains on CaP bone grafts: Biofilm will be formed in tissue cages in the back of guinea pigs. For this purpose four sterile polytetrafluorethylene (Teflon) tubes will be prefilled with CaP grafts and sterilised. They will then be implanted into the flanks of albino guinea pigs (Fig. 2).

These tissue cages, which have been prefilled with CaP grafts, will be infected by inoculation with 1 x 105 cfu of either *S. aureus* ATCC 29213 (MSSA), *S. aureus* ATCC 43300 (MRSA) or *S. epidermidis* RP62A ATCC 35984 (MSSE). Identical time intervals will apply to infecting the grafts in-vivo and in-vitro. The animals will be killed and CaP samples harvested.

Comparative biofilm analysis:

CaP bone grafts will be transferred to a 48-channel batch microcalorimeter (thermal activity monitor, model 3102 TAM III; TA Instruments, DE, USA) and Heat Flow measured at 37 ± 0.0001°C with a sensitivity of 200 nW for 24h. The microcalorimetry procedure has recently been described in detail (3). In brief, samples will be transferred to sterile 4 ml microcalorimeter ampoules pre-filled with 1 ml TSB, and transferred to the microcalorimeter.

Conclusions and Relevance for 3R

In-vitro biofim formation on bone grafts will be compared with *in-vivo* biofilm formation. It is hypothesized that *in-vitro* results can predict the *in-vivo* behaviour. The results should form a basis to develop a valid *in-vitro* screening test for biofilm formation on the surface of medical devices, helping to reduce the number of animals needed for definitive pre-clinical *in-vivo* tests.

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3R-Project 125-11

Nerve-cell mimicking liposomes as in vitro alternative to potency-testing of toxins with multistep pathways, such as Botulinum neurotoxins

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Keywords: *mice*; *toxicology*; *molecular biology: receptors*; *replacement*; *toxicity testing: neurotoxins; toxicity testing: pharmaceuticals; toxicity testing: receptor mediated*

Duration: 2 years **Project Completion:** 2013

Background and Aim

Botulinum neurotoxins (BoNT), produced by the Gram-positive bacterium *Clostridium botulinum*, are the most lethal toxins known to man. Similar to other toxins with multiple active sites, BoNT exerts a complex function in the human body. In the latter case, the toxin binds via its binding domain (HC_C = heavy chain binding domain) to specific cell-surface receptors on motoneurons. The translocation domain (HC_N = heavy chain translocation domain) facilitates translocation of the endoproteinase domain (LC = light chain) into the nerve cell lumen, where the latter specifically cleaves distinct SNARE proteins. As a result, neurotransmitter release is inhibited and the adjacent muscle cells are paralysed. If untreated, this leads to cumulative paralysis, and eventually death by respiratory arrest. If applied in minute doses, however, the toxin exerts a locally constrained paralysing effect, which is employed in medical applications for the treatment of a wide range of diseases, disorders and in aesthetic surgery.

In the case of BoNT, the mouse LD_{50} test is so far the preferred method for batch control of BoNT-containing pharmaceutical products, such as BOTOX, Myobloc and others [1,2]. Hence, with an increasing number of medical applications, more than half a million mice are used each year for routine potency testing [3,4]. Owing to the often complex nature of multi-domain toxins such as BoNT, most existing cell-free *in vitro* methods fail to detect the entire toxic activity, instead measuring either merely part of their toxicity or only the toxins present, e.g. by immunological methods. Although cell culture assays have been established for use in toxicity testing, many of these assays still require expensive and difficult maintenance, qualified personnel and the appropriate cell culture facilities. In addition, if stem-cell derived cells are used, long differentiation times of up to four weeks can be necessary [5].

Here, we describe the groundwork for an *in vitro* alternative for testing the entire toxic activity of toxins with multiple active sites, taking BoNT type B (BoNT/B) as an example, using liposomes with integrated nerve cell receptors to imitate the motoneuron membrane. BoNT/B binds via its HC_C to the presented receptors on the liposome surface. If the pH in the surrounding medium is lowered, the HC_N translocates the LC into the liposomal lumen, where the latter exerts its endoproteinase activity by cleaving a peptide substrate with specific cleavage sites and a quenched fluorophore pair. Upon cleavage, the fluorophore pair is unquenched and its fluorescent signal can be detected, allowing for quantification of the actual potency of BoNT in the system. With the use of well defined components, the liposomes may be produced under reproducible conditions. Moreover, liposomes can be stored long term at little expense and reconstituted on demand [6,7]. Furthermore, if the respective binding molecules (i.e. receptors) and substrates are available, the modular character of the presented system may also allow for testing of other toxins with multistep pathways which exert complex (chain reactions) functional changes, which are difficult to measure, in cells and the body.

In the Motoneuron: BoNT (in red and blue) is bound to the motoneuron via specific receptors integrated into its membrane. Subsequently, the toxin is passively taken up via endocytosis and becomes encapsulated into an endosome.

During endosome-lysosome transition the interior of this vesicle becomes acidified. The drop in pH triggers the active transmembrane translocation of the toxin's light chain (blue) via its heavy chain (red). Once in the cytosol, the light chain specifically cleaves SNARE-proteins. This leads to an inhibition of acetylcholine secretion. The following muscle cell becomes paralysed.

In the liposomes: Similar to the motoneuron, BoNT is bound to receptors integrated into the liposome membrane. Via acidification of the outer milieu, the heavy chain translocates the light chain of the toxin into the liposome lumen. There, it cleaves a peptide reporter with a fluorophore and a quencher molecule attached to the opposite ends of its peptide chain. Upon cleavage of the peptide chain, the fluorophore is dequenched and yields a quantifiable fluorescent signal.

Method and Results

in progress (present status)

So far, reporter assays have been established for the sensitive detection of BoNT/A and BoNT/B, even under liposome-compatible conditions. Fully assembled liposomes (FAL) for detection of BoNT/A and BoNT/B have been successfully produced with lipid compositions similar to those found in mammalian nerve cells. FAL contain high amounts of the peptide reporter molecules and the respective receptors integrated into the liposomal membrane. First results indicate that the proposed detection system is functional (proof-of-concept). Currently, experiments are in progress to characterize the observed reaction kinetics and to optimize the system's robustness and sensitivity.

Conclusions and Relevance for 3R

If the proof-of-principle is to be successful, then exact validation of the system's capability will be the following step. If this demonstrates that the assay is sensitive and robust enough it may be used to replace the mouse LD_{50} test for batch control of pharmaceutical products that contain BoNT. Although the proof-of-concept aims to test for BoNT/B (active compound e.g. in Myobloc), modification of the assay may provide the means of detecting additional relevant BoNT types, especially BoNT/A (the active compound in BOTOX and others). Also, if the necessary receptors and substrates are available, it may theoretically also be used to detect Tetanus toxin, and even other toxins with multiple active sites which act in a way similar to BoNT.

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Figure 1: *Mouse LD50 test:* For batch control of BoNT containing pharmaceutical products, different dilutions of the product are injected intraperitoneally into mice. The mice are observed for typical botulism symptoms. If the dose is high enough, mice die of suffocation within 96 hours.

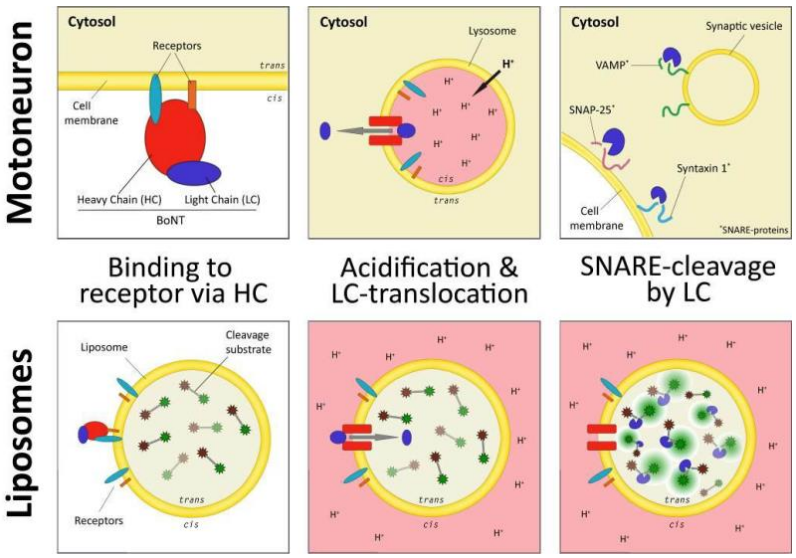

Figure 2: *BoNT toxic activity and its effect at motoneurons (panels above) and the model of detection, usingfunctionalised liposomes (panels below).*

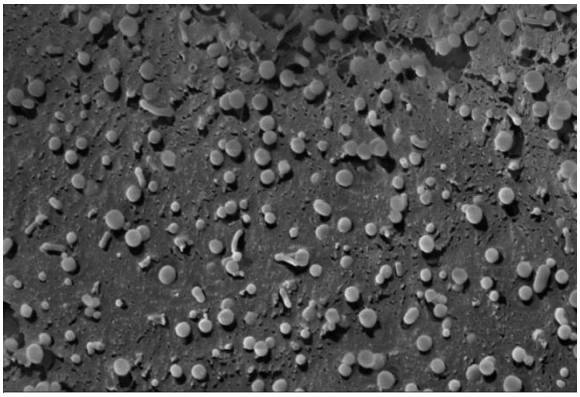

Figure 3: Figure 3: EM-characterisation of Fully Assembled Liposomes (FAL). Cryo SEM-picture of FAL taken in collaboration with Electron Microscopy ETH Zürich (EMEZ). Scale bar is 200 nm.

3R-Project 126-11

Development and validation of a model to investigate myeloid-cell homeostasis

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Keywords: *mice; transgenic mice*; *neutrophils*; *inflammation*; *cell cultures: transgenic*; *reduction*

Duration: 2 years **Project Completion:** 2013

Background and Aim
Every single day, the bone marrow of an adult human produces 100 billion (10^{11}) neutrophils to replace the rapidly aging circulating population. Differentiating from haematopoietic stem cells and developing in the bone marrow, neutrophils play a critical role in combating infections: they are recruited *en masse* to kill and eliminate microbes. The neutrophil-killing machinery includes powerful enzymes, such as proteases, whose activities are indiscriminate and, if unregulated, lead to substantial inflammatory responses, which may be injurious to the host cells and tissues.

An understanding of the molecular mechanisms underlying neutrophil homeostasis is thus of importance in the development of new therapeutic strategies that aim to modulate inflammatory and immune responses.

A major challenge in the field of neutrophil biology is posed by the extremely short lifespan of these cells. *Ex vivo*, they survive no more than a few days; under these conditions, genetic and molecular manipulations are not conceivable. Studies that have been conducted with transgenic and knock-out mice have yielded fundamental information appertaining to the functions of individual proteins in neutrophil biology under steady-state and pathological conditions. Since mice have but a small number of circulating neutrophils, functional assays have been largely performed either *in vivo* or on mature cells that have been isolated from the bone marrow of transgenic and knock-out animals, for which purposes many mice are consumed.

It was the purpose of this project to test the utility of *in-vitro*-immortalized line of neutrophil progenitors (MyPh8) (Wang et al. 2006), which can be conditionally induced under culturing conditions to differentiate into unlimited numbers of mature cells. The availability of an approach of this kind would reduce the need for isolating primary neutrophils from living mice and thus help to cut down on the consumption of animals for experimental purposes. It was also our aim to validate the methodology by investigating the function of the intracellular serine protease inhibitor serpinB1 in neutrophil homeostasis, as previously demonstrated not only *in vitro* for isolated bone-marrow cells, but also *in vivo* (Benarafa *et al*. 2007; 2011).

Method and Results
We first established and characterized immortalized wild-type (WT) and serpinB1-deficient myeloid progenitors (MyPh8), which were generated by the lentiviral transduction of the oestrogen-receptor-mediated expression of HoxB8 in the presence of stem cell factor. Upon withdrawal of oestrogen and treatment with G-CSF, myeloid progenitors differentiated into cells that manifested the characteristic features of mature neutrophils, including the expression of Ly-6G and MMP-9 and the ability to produce reactive oxygen species. SerpinB1-defcient MyPh8-cells were more susceptible to death than their WT-counterparts, as previously reported for primary neutrophils (Benarafa *et al*. 2011).

Our studies traced the premature death of serpinB1-deficient neutrophils to a cell-intrinsic death pathway that is mediated by cathepsin G, a known target of serpinB1 (Baumann *et al*. 2013). To further validate these data, we generated MyPh8-cell lines from cathepsin-G-deficient (CG-/-) and double-knock-out (CG.sB1-/-) mice. However, the rates of spontaneous death of the MyPh8-cells prior to induction increased in proportion to the duration of culturing, thereby compromising the interpretation of the data gleaned at different passaging times. When generated in parallel, the cell-death kinetics of the CG.sB1-double-deficient MyPh8-derived neutrophils and their WT-counterparts were similar.

Conclusions and Relevance for 3R
We have investigated an approach involving the differentiation of myeloid progenitors into cells bearing the characteristic features of mature neutrophils with the objective of helping to reduce the number of mice that are used for neutrophil studies. We have confirmed existing *in-vitro* and *in-vivo* data appertaining to bone-marrow-derived neutrophils, which

reveal the regulation of neutrophil death to be mediated by cathepsin G and to be inhibited by serpinB1. However, as an alternative to primary neutrophils, MyPh8-cells that are derived from various genetically-modified mice are of limited potential, owing to the discovery that the rates of spontaneous cell death increase as function of culturing duration, which compromises the interpretation of data gleaned at different passaging times. This is a particularly inconvenient caveat when studying cell-death pathways.

In conclusion, the potential of the investigated approach to help in reducing the number of experimental mice used in neutrophil studies is not as great as originally hoped. The methodology will nevertheless be valuable in studies involving mice with severely-compromised late-embryonic or postnatal phenotypes. In such animals, the foetal liver could be used as a source of haematopoietic stem cells for the derivation of an MyPh8-line. It has been recently shown that by combining oestrogen-regulated Hoxb8 with the Flt3-ligand instead of SCF, the pluripotent progenitor status of the MyPh8-cells could be enhanced (Redecke *et al*. 2013).

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3R-Project 127-11

Establishing a novel system for quantitative production of murine basophils in vitro

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Keywords: *mice; transgenic mice*; *basophils*; *immunology*; *allergy*; *cell cultures: transgenic*; *reduction; refinement*
Duration: 2 years **Project Completion:** 2014

Background and Aim
Basophilic granulocytes (basophils) are a rare leukocyte population, constituting ~0.5% of peripheral blood leukocytes. Recently, several high impact studies have identified previously unrecognised roles for basophils in allergic responses as well as in immune regulation, indicating that the role of basophils may have been underestimated so far (1-5) The study of basophils is particularly challenging as no suitable cell culture model for human or mouse basophils exists to date. Moreover, *in vitro* differentiation of basophils from bone marrow is very inefficient. Therefore, large numbers of mice have to be sacrificed in order to isolate very limited numbers of primary basophils from blood. For those reasons, we have recently established a novel method to generate conditionally immortalised, basophil-committed progenitor cell lines, which can be differentiated *in vitro* in near-unlimited amounts into mature basophils. The main aim of the 3R project is the full phenotypical and functional characterisation of this, to our knowledge, unique cellular mode for murine basophils.

Method and Results
Basophil-committed progenitor cell lines are generated from murine bone marrow (or foetal livers) using conditional Hoxb8 by a significantly modified protocol published for the quantitative production of mouse neutrophils/macrophages (6). The most important changes include the use of different cytokines and a novel lentiviral system for conditional Hoxb8 expression. (7) Once immortal cells lines are established, they can be easily handled and manipulated and maintained in culture for prolonged times (Figure 1A). Differentiation into mature basophils is achieved 'on demand' by shutdown of exogenous Hoxb8 expression (Figure 1B). Within 6 days, an enriched population of cells resembling mature basophils is obtained. Mature Hoxb8 basophils are end-differentiated (non-cycling), have lobulated nuclei and a granular appearance and display a surface expression profile of a number of membrane bound, cell type specific surface proteins (FcεRIhighCD11b+ckitnegGr1negIL5RnegCCR3neg, see Figure 1C, D and not shown). The high affinity receptor for IgE, FcεRI, is functional, as receptor crosslinking leads to degranulation and release of N-acetyl-β-D-hexosaminidase (Figure 1E).

Conclusions and Relevance for 3R
We have established a method to generate basophil-committed progenitor cell lines using conditional Hoxb8. These cells can be differentiated in quantitative amounts into mature basophils *in vitro*, making it, to our knowledge, a novel and unique cellular model for murine basophils. The basophil lines described here are genetically stable, can be generated from any genetically modified mouse strain and can easily be manipulated (e.g. knockdown by short-hairpin RNA, introduction of transgene, etc.) and progenitors can be differentiated into mature basophils in near unlimited numbers. As large amounts of mice are required to isolate primary basophils, we would like to promote this cellular model as an alternative method to study basophil biology, in particular to answer questions requiring large numbers of basophils (e.g. biochemical work, study of signalling pathways, etc.). Additionally, Hoxb8 basophil lines can be generated form foetal livers (E12-E14) of mouse strains displaying severe phenotypes. The main relevance for 3R of this project therefore lies in its potential to reduce the number of sacrificed mice and the need to grow mice with severe phenotypes to adulthood.

Publications
i) directly related
Gurzeler U, Rabachini T, Salmanidis M, Brumatti G, Ekert PG, Echeverry N, Bachmann D, Simon HU, and Kaufmann T. In vitro differentiation of near-unlimited numbers of functional mouse basophils using conditional Hoxb8, Allergy (2013) 68: 604–613; highlighted in editorial (Gibbs & Nilsson, same issue).
Reinhart R, Wicki S, and Kaufmann T. In vitro differentiation of mouse granulocytes, Methods Mol Biol (2016),1419:95-107.
ii) subsequent publications using “Hoxb8 basophils”
Morshed M, Hlushchuk R, Simon D, Walls AF, Obat-Ninomiya K, Karasuyama H, Djonov V, Eggel A, Kaufmann T, Simon HU, and Yousefi S. NADPH oxidase-independent formation of extracellular DNA traps by basophils, J Immunol (2014), 192: 5314-5323.
Yousefi S, Morshed M, Amini P, Stojkov D, Simon D, von Gunten S, Kaufmann T, and Simon HU. Basophils exhibit antibacterial activity through extracellular trap formation, Allergy (2015) 70(9):1184-8.
Reinhart R, Rohner L, Wicki S, Fux M, and Kaufmann T. BH3 mimetics efficiently induce apoptosis inn mouse basophils and mast cells.Cell Death Diff, doi:10.1038/cdd.2017.154. [Epub ahead of print].

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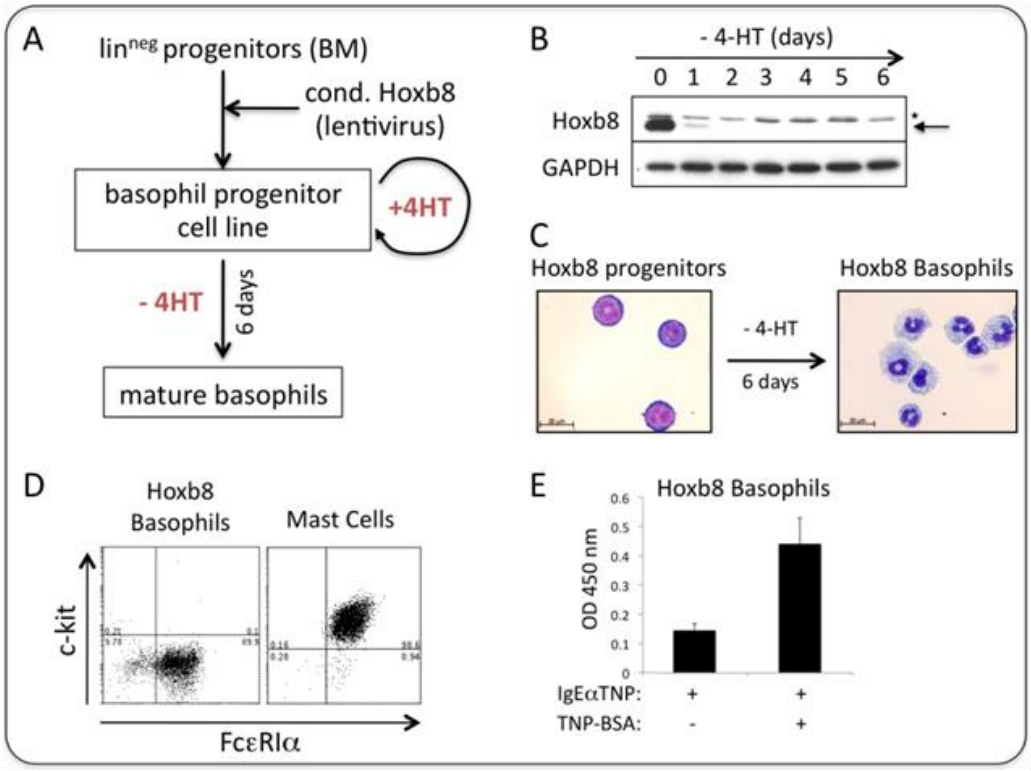

Figure 1: Generation of murine basophils in vitro using conditional Hoxb8
(A) Simplified scheme of generation of basophil committed progenitor cell lines from bone marrow.
(B) Western blot showing rapid shutdown of exogenous Hoxb8 upon removal of 4-hydroxytamoxifen (4HT).
(C) DiffQuik staining of immature and mature Hoxb8 basophils.
(D) c-kit (CD117) and FcεRI surface expression profile by FACS of mature Hoxb8 basophils and bone marrow derived mast cells.
(E) N-acetyl-ε-D-hexosaminidase release (as a measure for degranulation) upon FcεRI crosslinking. Phenotypical and functional characterisation was performed in immature progenitors as well as differentiated basophils. We investigated the expansion and clonogenic potential of Hoxb8 basophils and extended the characterisation of basophil specific surface markers and enzymes (e.g. mast cell protease 8 and 11, see Ugajin et al.8). Functionality of Hoxb8 basophils was tested by crosslinking FceRI9,10 followed by the analysis of induction and/or release of lipid mediators (e.g. leukotriene C4), histamine and cytokines (IL-4, IL-13). Treatment with the protein fragment C5a (released from complement component C5) was used in parallel as an immunoglobulin-independent stimulus. Furthermore, chemotaxis, transmigration behaviour and oxidative burst upon activation with various stimulants was or is being investigated.

3R-Project 128-11

Genetic modification of the human airway epithelium – a paradigmatic system to study host responses to human respiratory viruses

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Keywords: *human; viruses*; *epithelia; epithelia; lung*; *lung diseases*; *cell cultures: organ-specific; cell cultures: transgenic*; *reduction; replacement*; *infectiosity*

Duration: 2 years **Project Completion:** 2013

Background and Aim
The epithelium of the human airways is a port of entry for many respiratory viruses, such as the respiratory syncytial virus, rhinoviruses, influenza viruses, human parainfluenza viruses, adenoviruses, human metapneumoviruses and human coronaviruses. Recent advances in the cultivation of primary human airway epithelia (HAE) have permitted the study of human respiratory viral infections within a culturing system that morphologically and functionally resembles the human airways *in vivo*. Notwithstanding these recent technical achievements, the analysis of basic virus-host interactions at the molecular level is still preferentially performed in animal models. This is particularly true in the case of viral infections for which a murine model is available. The main reason for this preference is that when using a murine model of viral infection, advantage can be taken of widely available transgenic or knock-out mouse strains. Thus, in a murine model, and in contrast to primary human cells, individual genes can be genetically deleted or trans-complemented, in the mouse model, thereby permitting detailed analyses of virus-host interactions at the molecular level. To overcome the limitation in the human case, we endeavoured to render the HAE-culturing system amenable to genetic modifications. The research objectives of this project included: (i) the generation of lentiviral vectors encoding reporter proteins and/or shRNA-sequences that are expressed upon induction, and (ii) optimization of the transduction of primary HAE-cultures and establishment of efficacious affinity-selection methods to obtain HAE-cultures with a high level of lentiviral vector-based gene expression.

Method and Results
Construction of lentiviral vectors

To render the HAE-culturing system amenable to genetic modification, we constructed lentiviral vectors encoding the Cherry-Picker transgene and containing an insertion site for a shRNA-sequence that is under the regulation of an inducible human U6-promoter element that is actively repressed by the lactose-repressor protein. This basic organization of our lentiviral vectors has two advantages. Firstly, the Cherry-Picker gene encodes a chimeric red fluorescent protein which facilitates the detection of transduced cells. By virtue of its cell-surface expression profile and by making use of a Cherry-Picker-specific antibody, transduced cells can be sorted. Secondly, the inducibility of shRNA-expression permits a controlled knock-down of targeted genes, including those that may interfere with the differentiation of HAE-cultures.

The Cherry-Picker gene was fused via a 2A-autoprotease sequence to the LacL-gene. Unfortunately, our attempts to select the transduced cells by MACS-sorting were repeatedly unsuccessful. Further analyses revealed that the Cherry-Picker protein appeared to be retained within the cytoplasm of the transduced cells. Similar cytoplasmic retention has occasionally been described for genes that are cloned upstream of the 2A-autoprotease sequences. We have, therefore, exchanged the 2A-autoprotease sequence for an internal ribosomal entry site. Since we have already cloned shRNA-sequences that are directed against GFP and human CD13 (receptor of the human coronavirus 229E), we now proceed with the Cherry-Picker-based selection.

The overall strategy - assessing transduction efficacy - cellular composition of transduced epithelia

Lung epithelial cells were isolated from human tissue by protease digestion and were then expanded in two-dimensional cultures. The cells were either cryo-preserved or seeded in liquid-liquid interface cultures. The apical medium was subsequently removed, whereby the apical side of the confluent cell layer was exposed to air and an air-liquid interface culture established (Figure 1). To render the HAE-culturing system amenable to genetic modifications, a lentiviral transductionmethodology had to be developed. During the establishment of differentiated HAE-cultures, the primary cells can be transduced at three different stages:

a. after their isolation;
b. in an undifferentiated state during liquid-liquid interface culturing;
c. in a differentiated state during air-liquid interface culturing.

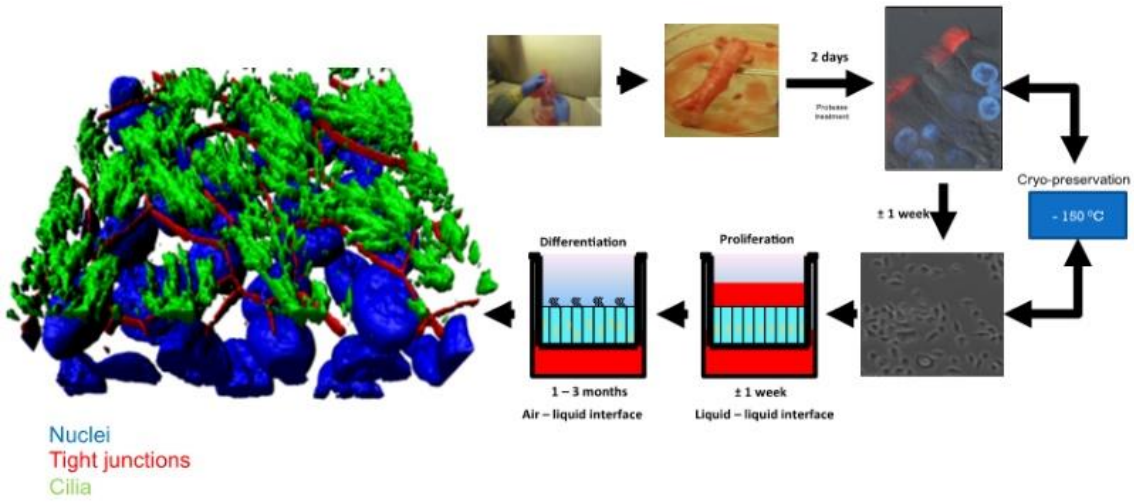

Figure 1: Generation of HAE-cultures: We have assessed the transduction efficacies at these different stages. In suspension, undifferentiated epithelial cells were observed to be most efficiently transduced and expanded as conventional cultures. At a rather low multiplicity of infection (MOI) of 5, we were able to detect transgene expression in 5-30 % of the cells. Importantly, although the cells were transduced during the undifferentiated stage, the transduced cells (i.e. those expressing the transgene) can undergo full differentiation into a pseudostratified epithelial layer (Figure 2). We used a number of marker proteins (Mucin1, EpCAM, NGFR, Tubulin) to assess the cellular composition of cell types that are represented within the epithelium (Figure 2, A&B). These analyses revealed that the cellular compositions are similar between different donors, when different MOIs are used. Moreover, the cellular composition of transgene-expressing cells remained indistinguishable from the non-transduced HAE-cultures.

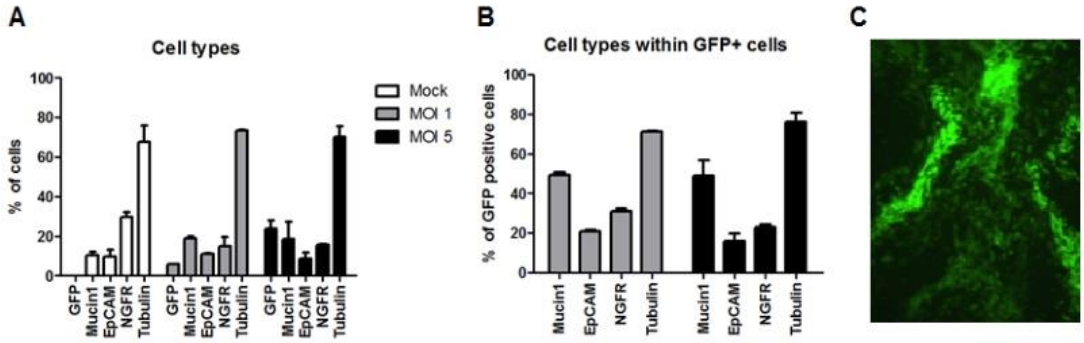

Figure 2: Analysis of transduced and fully-differentiated HAE-cultures: The cellular composition of the HAE-cultures was assessed by flow cytometry (A, B); and transgene expression (GFP) by the transduced and the fully-differentiated HAE-cultures was evaluated by fluorescence microscopy (C).
Increasing the passage number of undifferentiated cells using the Rho-kinase inhibitor Y-27632

In order to obtain epithelial cultures containing (almost) exclusively transduced cells, we employ MACS-sorting in combination with the cell-surface expression of the Cherry-Picker protein. However, one limitation of this approach is that following the initial isolation of the primary human epithelial cells, only 2-3 passages are possible before the cells lose their ability to re-differentiate and to form intact pseudostratified epithelial layers. Since the expression of the Cherry-Picker protein and the cell-sorting process require at least 1-2 passages we established the use of the Rho-kinase inhibitor Y-27632, that has been described to prolong the basal cell phenotype of primary epithelial cells in culture. Using this approach, we were indeed able to propagate primary epithelial cells *in vitro* for an extended period of time. Most importantly, Y-27632-treated cells were still capable of fully differentiating into pseudostratified epithelial layers in late passages, whereas non-treated cells failed to form fully-differentiated layers after passage 3 (Figure 3). This approach represents a significant improvement, since it is now possible - without any passage or time restriction - to assess the expression of the selection gene (e.g., the Cherry-Picker gene), to select for transduced cells, and even to extend the period of proliferation of the transduced cells to obtain larger quantities (e.g., for storage).

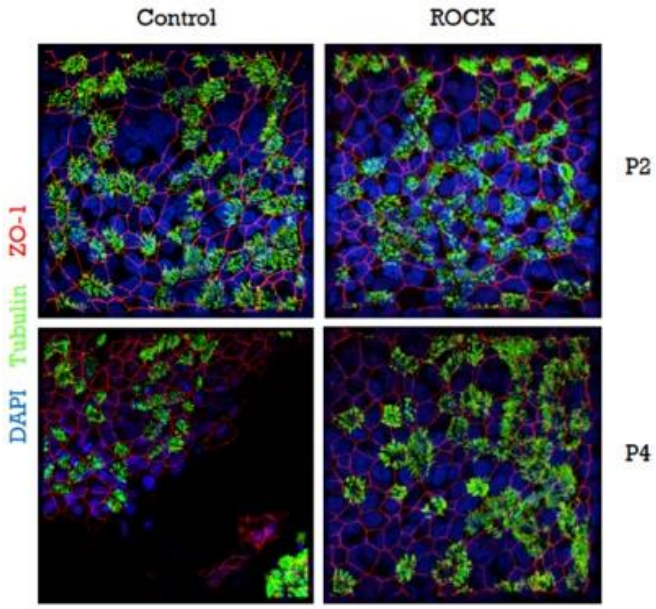

Figure 3: Y-27632-treated cells retain the ability to form intact and fully-differentiated HAE-cultures after passage 3.

Conclusions and Relevance for 3R

The availability of an HAE-culture system that permits to efficiently inactivate the expression of particular genes or the expression of genes of interest *in trans* renders possible the replacement of many animal experiments that are based on the use of specific transgenic and knock-out mouse strains. An HAE-culturing system that is amenable to the genetic modification of host gene expression is likely to find a wide application in the analysis of host-pathogen interactions appertaining to many human respiratory pathogens.

In addition, the emergence of the MERS-coronavirus during this project afforded us the opportunity of demonstrating the usefulness of primary HAE-cultures for experimental *in vitro* studies on newly emerging respiratory pathogens. The HAE-culture system could be used to demonstrate the growth kinetics and the cellular tropism of the MERS-coronavirus (1) and to identify its cellular receptor on differentiated human lung epithelia (2).

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3R-Project 129-11

The use of microfluidic chambers to study axonal transport in PTEN and SOCS3 dependent axonal regeneration

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Keywords: *mice*; *axons; neurons; spinal cord*; *spinal cord repair / diseases*; *perfusion chamber*; *reduction; replacement*

Duration: 1 year **Project Completion:** 2013

Background and Aim

Rodents are extensively used to study nerve injury. The mouse spinal cord injury model, widely used in nerve injury research, is extremely debilitating. *In vivo* studies have allowed major advancements in the comprehension of the incapacity of adult central nervous system axons to regenerate. Notably, *in vivo* studies have shown that axonal regeneration after nerve injury was possible in adult mice if PTEN or SOCS3 were deleted in knock-out mice (1,2,3). However, studying *in vivo* mechanistic adaptation at the cellular level remains challenging. Axonal transport is an important cellular mechanisms evidenced by the numerous neurodegenerative diseases that have been related to an axonal transport impairment. Improving axonal transport in the injured and diseased central nervous system has been proposed as a promising strategy to improve neuronal repair. However, the contribution of each cargo to the repair mechanism is unknown. Because the transport of specific cargos after axonal insult has not been examined systematically in a model of enhanced regenerative capacity, it is unknown whether the transport of all cargos would be modulated equally in injured central nervous system neurons. In order to test the transport adaptation of cellular cargos during axonal regeneration, an *in vitro* system allowing straightforward manipulation and analysis is required. We used microfluidic chambers (4) to mimic nerve injury and regeneration *in vitro*. This method allowed us to (i) injure axons without affecting the cell body, (ii) manipulate neuron cell bodies and axons specifically, and (iii) analyze axonal transport during axonal regeneration at a single axon resolution.

Method and Results

Using a microfluidic culture system we compared neurons co-deleted for PTEN and SOCS3, an established model of high axonal regeneration capacity, to control neurons. We measured the axonal transport of three cargos (mitochondria, synaptic vesicles and late endosomes) in regenerating axons and found that the transport of mitochondria, but not the other cargos, was increased in PTEN/SOCS3 co-deleted axons relative to controls. The results reported here suggest a pivotal role for this organelle during axonal regeneration and validate the microfluidic culture system to identify cellular mechanisms occurring in regenerating axons.

Conclusions and Relevance for 3R

A lab testing the regenerative capacity of axons using the spinal cord injury model (transgenic mice, drug treatment) will use roughly 5,000 mice per year. Some of these mice are used to test hypotheses that will not give any satisfactory results. To increase the chance of obtaining positive results *in vivo* while decreasing the number of mice used, we propose to validate the microfluidic chambers as an *in vitro* system that would be a primary test to establish promising hypotheses worth testing *in vivo*, if possible. We estimate that by first testing the hypotheses in a reliable *in vitro* system would save one third of the mice used per year. We hope that our study will establish microfluiding chambers as a gold standard *in vitro* system in the field of the study of spinal cord injury/axonal regeneration.

Publication

Cartoni, R., Pekkunaz, G., Wang, C., Schwarz,T.L., He, Z. An Elevated Mitochondrial Transport Rate characterizes high Regeneration Capacity Neurons in CNS neurons (2017). *PLoS One 12(9).*

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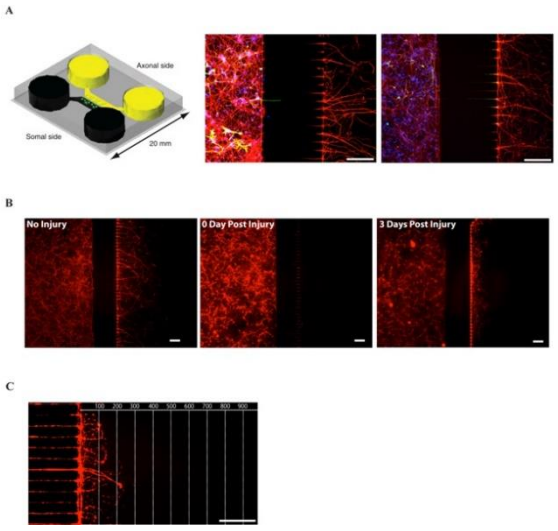

Figure 1: The microfluidic culture system
(A) First panel: Schematic view of a microfluidic chamber. Adapted from (4). Neurons plated in the somal side will project their axons through the 450 µm long microgrooves and reach the axonal side. Second and third panel: immunohistochemistry of E18 mouse cortical neurons culture (DIV7) in microfluidic chambers. Second panel: Red: anti-3-Tubulin (axonal marker), Green: anti-GFAP (glial marker), Blue: DAPI (nuclear marker). Third panel: Red: anti-3-Tubulin, Green: anti-MAP2 (dendrites marker), Blue: DAPI. 450 µm microgrooves allow a pure isolation of neurons. (B) Tuj1 immunohistochemistry of E18 mouse cortical neurons culture (DIV7) in microfluidic chambers. No Injury (first panel), immediately after injury (second panel) and 3 days after injury (third panel). (C) Regrowing axons can be observed 3 days after injury.

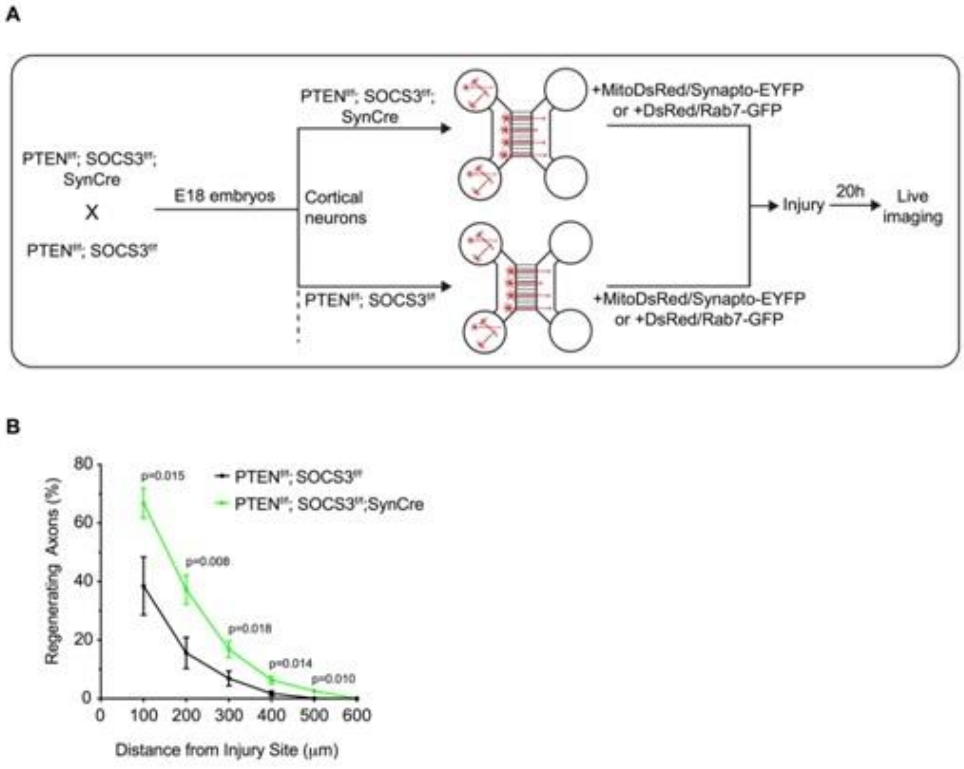

Figure 2: Deletion of PTEN and SOCS3 improved axonal regeneration of E18 cortical neurons
(A) Schematic of the *in vitro* platform to study the axonal transport in regenerating PTEN$^{-/-}$; SOCS3$^{-/-}$ cortical neurons. PTEN$^{-/-}$; SOCS3$^{-/-}$ E18 cortical neurons were obtained by breeding PTEN$^{f/f}$; SOCS3$^{f/f}$ mice with the PTEN$^{f/f}$; SOCS3$^{f/f}$; Synapsin Cre (SynCre) mice. The cortex of each embryo was processed individually so that each microfluidic chamber was seeded with neurons from a single embryo. Thereby PTEN$^{f/f}$; SOCS3$^{f/f}$; SynCre neurons were compared to PTEN$^{f/f}$; SOCS3$^{f/f}$ from littermate embryos. All neurons were cotransfected with MitoDsred2 and EYFP-Synaptophysin or Rab7-GFP. (B) Quantification of *in vitro* axonal regeneration of PTEN$^{-/-}$; SOCS3$^{-/-}$and controlcortical neurons 20h post injury. n=9-11 microfluidic cultures of individual embryos from 5 independent experiments. Two tailed Student's Unpaired *t*-test.

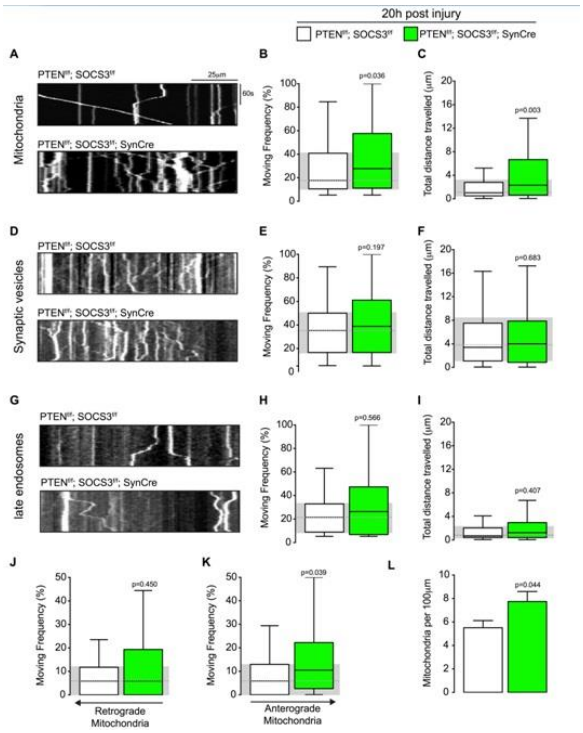

Figure 3: Mitochondrial transport is increased in regenerating axons co-deleted for PTEN and SOCS3.
(A) Representative kymographs from live imaging of mitochondria in regenerating axons from PTEN$^{f/f}$; SOCS3$^{f/f}$ and PTEN$^{f/f}$; SOCS3$^{f/f}$; SynCre neurons 20 h post injury. Consecutive line scans of the axon were arrayed top to bottom so that the y-axis of the kymograph represents the time and the x-axis the position of the object studied. Stationary objects therefore appear as vertical lines and motile ones as diagonals. (B, C) Box plot showing the moving frequency of motile mitochondria (B) and their distance travelled (C) in regenerating axons of the indicated genotypes. Mann-Whitney *U* test on the number of mitochondria. n=136-223 mitochondria from 18-22 axons and 4-6 individually cultured embryos from 2 independent litters. (D-F) Representative kymographs (D) and quantification of the moving frequency (E) and distance travelled (F) from live imaging of synaptophysin-positive synaptic vesicle precursors in regenerating axons of the indicated genotypes. Mann-Whitney *U* test. n=153-226 synaptic vesicles, 12-17 axons, 4-5 individually cultured embryos from 2 independent experiments. (G-I) Representative kymographs (G) and box plots of moving frequency (H) and total distance travelled (I) from live imaging of late endosomes in regenerating axons of indicated genotype. (Mann-Whitney *U* test. n=69-105 late endosomes, 13-14 axons, 5-4 individually cultured embryos from 2 independent experiments. (J and K) Retrograde *(J)* (toward cell body) and anterograde *(K)* (toward axon's tip) moving frequencies of the mitochondria analyzed in (A). Mann-Whitney *U* test. (L) Mitochondrial densities in the axons analyzed in (A). Two tailed Student's Unpaired *t*-test. Data in all the box plots are represented with a box that delimitates the lower (Q1) and the upper quartile (Q3) of the distribution. Horizontal line indicates the median (Q2) and whiskers indicate the maximum and minimum of the distribution.

3R-Project 130-11

Establishment of an in-vitro organ-slice defect model for meniscal repair in orthopaedic research

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Keywords: *bovine*; *bone, cartilage*; *mechanobiology; orthopedics*; *explants*; *reduction; replacement*

Duration: 2 years **Project Completion:** 2014

Background and Aim
The aim of this project is to develop a novel, simple and inexpensive organ-slice culture system to simulate the repair of meniscal lesions from synovial tissue *in vitro*. The model would be established using the menisci of bovine cows slaughtered at a local abattoir. In the field of orthopaedic research, appropriate*in-vitro* systems have been developed to simulate the repair of cartilage and bone, but not that of meniscal tissue. And even in the engineering of the former two tissue types, an *in-vitro* system is usually employed merely to establish the feasibility of the contemplated repair strategy, viz., to demonstrate that the selected growth factors can induce the targeted precursor cells to differentiate and elaborate a tissue-specific extracellular matrix.
The meniscus is an intra-articular fibrocartilaginous disc. By distributing the stresses to which a joint is normally exposed over a broad area of the cartilage surface, this organ exerts a chondroprotective effect [1, 2]. It is comprised of solid and fluid phases. When the meniscus is compressed, fluid is forced through the solid phase. Since frictional drag is exerted on the fluid by the low permeability of the solid phase, the fluid becomes pressurized, thereby assisting in the carriage of compressive loads. The bulk of the meniscal tissue is avascular; only the peripheral portion is vascularized. With respect to knee-joint pathology, the menisci, next to the ligaments, are the structures most frequently injured [3]. Meniscal injuries can occur secondary to overt trauma, and can also arise from the alterations in knee-joint function that are associated with aging, osteoarthritis, rheumatoid arthritis, disturbances in gait [4-8] and obesity. Sixty per cent of individuals over 50 years of age manifest some degree of meniscal pathology.
If an appropriate organ-slice culture system could be established to model the entire process of cell differentiation, tissue formation and defect healing *in vitro*, then it would be possible to develop and refine a therapeutic principle for the repair of meniscal tissue without having recourse to living animals except at an advanced stage, namely, when it has been established beyond reasonable doubt that the strategy is likely to be valuable in the clinical treatment of human patients. The availability of such an organ-slice culture model would permit a very significant reduction in the requisite number of living animals, and would also curtail the time expended on pre-clinical investigations as well as their expense. These substantial benefits would render the model highly attractive to academic institutions, industrial partners and contract-research organizations.

Method and Results
in progress (present status)
The medial meniscus will be harvested from the knee joints of bovine cows slaughtered at a local abattoir (Fig.1). Synovial tissue will be excised from the medial side of the same joints. Slices of meniscal tissue, approximately 3 mm in thickness, will be produced using a specially designed tool.

Having successfully developed a suitable slicing tool and established that meniscal slices of reproducible thickness can be produced without inducing apoptotic cell death (and/or necrosis), we will then design and manufacture hollow cylindrical punchers with different diameters (satisfying the same cutting criteria as the slicing tool) to prepare discs of meniscal tissue from the slices and to produce an inner chamber that represents the defect (Fig.2)
Two synovial discs with the same diameter as the meniscal defect will be punched out and respectively affixed by gluing with Histoacryl (medical grade) to the upper and the lower surfaces of the chamber (defect) to ensure that its dimensions are maintained during the course of culturing (6 weeks). Having successfully produced the “empty” constructs of meniscal and synovial tissue, we will then establish appropriate conditions for their culturing (Fig.3).

Conclusions and Relevance for 3R
If our conceived model of a meniscal defect could be practically realized on a reproducible basis, then it would afford not

only a means of establishing almost exclusively *in vitro* the stimulation conditions necessary for the lesion`s repair from synovial tissue, but also a welcome opportunity to cut down on the number of experiments with living animals, which, incidental to the important ethical benefit, would also be financially advantageous.

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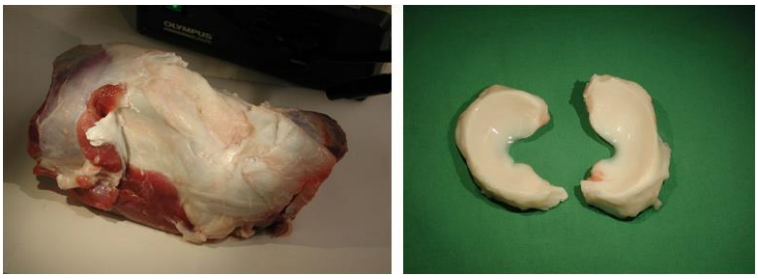

Figure 1: Bovine knee joint (left) together with the excised menisci (right).

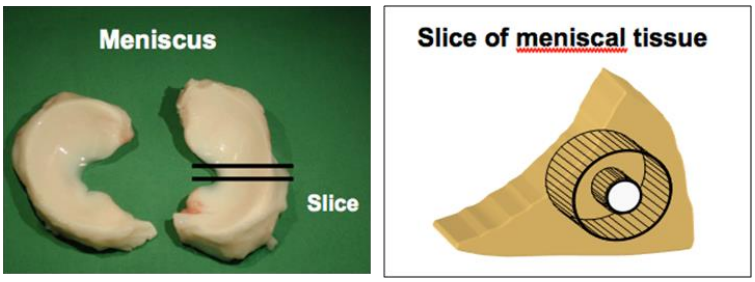

Figure 2: Preparation of the chamber (defect) model from a slice of meniscal tissue.

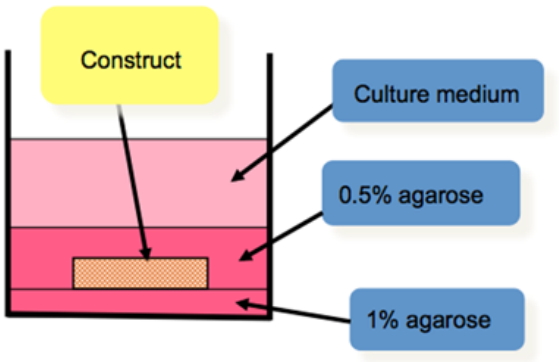

Figure 3: Culturing of the meniscal-chamber construct in agarose gel (cross-section).

3R-Project 131-12

Antibody-phage-selection strategy for application in non-specialized laboratories

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Keywords: *human*; *phage display*; *reduction; replacement*

Duration: 2 years **Project Completion:** 2014

Background and Aim

Antibodies are widely used in research for the detection or purification of proteins. Although antibodies with tailored binding specificities can be generated by *in-vitro* methodologies such as the phage-display technique (1, 2), most of the antibodies that are used in research are still produced by the immunization of animals. For technical and economical reasons, *in-vitro* techniques are not broadly applied to generate monoclonal antibodies. Phage-display involves many experimental and error-prone steps. Moreover, it is costly to establish the complex method in new laboratories. Another limitation is that many of the existing antibody–phage-display libraries have intellectual-property constraints.

Method and Results

We have cloned a large scFv-antibody phage-display library that is available to users free of charge and without intellectual-property constraints. The library was designed to contain V_H- and V_L-antibody domains, in the orientation V_H-linker-V_L, which are connected by a flexible $(GGGGS)_3$-linker. As an acceptor framework, human V_H 3-23- and V_L kappa 1-33-germline genes were chosen. 4-10 amino acids were randomized in V_H CDR3 and 4-7 in V_L CDR3. The library is comprised of 1.4 billion clones.

In addition to generating the scFv-phage library, we developed a phage-display-selection strategy that involves significantly fewer experimental steps than is usual, and which should facilitate the *in-vitro* generation of affinity ligands by non-experts. In brief, a phage-display library is subjected to a single round of affinity panning. The DNA of the isolated clones is sequenced using a next-generation sequencing technology, which companies now provide at an affordable price. We applied the approach for a peptide-based phage-selection of ligands. The procedure has been published in Nucleic Acid Research (4).

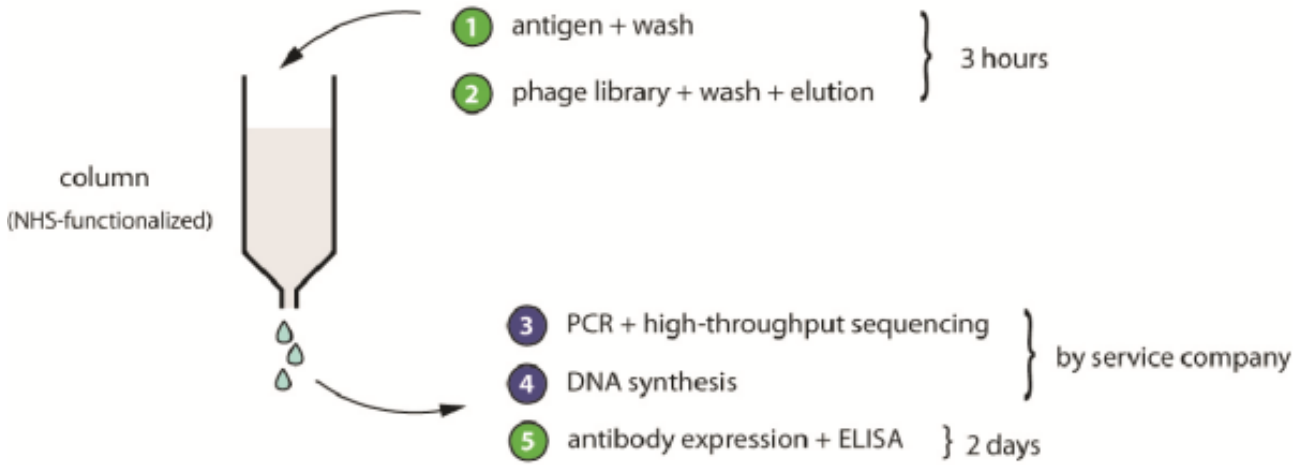

Figure 1: Schematic representation of the proposed phage-selection approach. Steps with green numbers are performed in-house by research laboratories; steps in blue are outsourced to service companies.

Conclusions and Relevance for 3R

The proposed methodology should replace animal experiments that are commonly performed to develop polyclonal and monoclonal antibodies. Monoclonal antibodies are typically produced by the repetitive injection of antigens into mice or other animals. The scFv-phage-display library has already been applied in several laboratories - including our own - to generate monoclonal antibodies. The antibodies were generated without having recourse to the immunization of animals. The novel methodology, which combines *in-vitro* evolution with high-throughput sequencing, is technically less complex than the classical phage-display technique. It should improve the accessibility of phage-display to non-experts.

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3R-Project 132-12

Identification of predictive in vitro markers of hematopoietic stem cell function

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Keywords: *human; mice*; *stem cells*; *transplantation*; *reduction; replacement*

Duration: 1 year **Project Completion:** 2013

Background and Aim
Although hematopoietic stem cells (HSC) are the most successfully used clinical stem cells (1), their transplantation to treat hematological malignancies remains a risky procedure with major complications including infections and graft-versus-host disease. Moreover, the limited availability of human leukocyte antigen (HLA)-matched donors and the low numbers of transplantable HSCs that can be isolated from a donor are major issues that could potentially be overcome if efficient *in vitro* HSC expansion methods were available. Yet, as soon as HSCs are removed from their native microenvironmental niches in the bone marrow and put in standard cell culture, they lose their characteristic functions by undergoing rapid differentiation.
Progress to discover robust *in vitro* systems capable to expand HSCs has been hampered by a lack of prospective markers that predict reconstitution activity of cultured HSCs. Indeed, the only functional assay to probe long-term HSC multipotency remains transplantation, a method that is time-consuming, entails animal suffering and thus raises ethical concerns. Classical *in vitro* assays to assess hematopoiesis such as colony formation in a semi-solid matrix that contains cytokines are a powerful means to assess clonogenicity and differentiation potential of an unknown population of stem/progenitor cells. However, such assays are retrospective and fail to predict long-term reconstitution activity. Furthermore, it is well accepted in the field that immunostaining *in vitro*-cultured HSCs does not faithfully mark functional stem cells.
Given these problems, the main goal of this 1-year project is to identify predictive metabolic markers of the HSC state and to validate these markers on stem cells exposed *in vitro* to self-renewal and differentiation culture conditions, as well as different oxygen tensions. In contrast to the widely used 'immunophenotyping' of stem and progenitor cell populations via antibodies against specific receptors, we will focus on metabolic markers of the stem/progenitor cell state, since recent studies have shown that HSC quiescence and self-renewal are at least partially controlled through regulation of their metabolic state (e.g. (2)).

Method and Results
in progress (present status)

Long-term HSCs in mice are exclusively marked by low metabolic activity (preliminary data)
In a preliminary experiment, we tested whether progenitor/stem cells having lower metabolic activity represent long-term repopulating (i.e. functional) HSCs. To this end, Lineage^{negative} Sca-1^{positive} c-kit^{positive} (LKS) cells were purified by FACS based on mitochondria activity using tetramethylrhodamine methyl ester (TMRM), a cell-permeant fluorescent dye that is sequestered by active mitochondria (Figure 1).

Strikingly, upon transplantation of 1000 cells of each population into lethally irradiated mice, we detected long-term reconstitution activity exclusively in the cell population with low mitochondria activity. This strongly suggests that mitochondria stainings might be predictive markers of the HSC state that could also be applied to *in vitro* cultured cells, for example to identify stem cell expansion conditions.

Probing mitochondria activity by microscopy-based techniques
Based on these encouraging data, we aim to establish a microscopy-based method to analyze and distinguish mitochondria in primary HSCs and progenitors *in vitro*. First, we will stain mitochondria by mitotracker and TMRM in freshly isolated, fixed HSC/MPPs and analyze them by confocal microscopy at high magnification. One read-out that we plan to use is the mitochondrial morphology that can be indicative of the mitochondrial activation status of stem cells (3). Mitochondria with low metabolic activity can thus be expected to have a punctated morphology, whereas highly metabolic mitochondria tend to fuse and form a network-like structure (4). We will develop image analysis tools to quantify individual

mitochondria in single cells in order to extract information on the stemness. Furthermore, a measurement of the fluorescence intensity of the TMRM staining should provide a good read-out of mitochondrial activity. Finally, we will analyze mitochondrial morphology (by Mitotracker) and activity (by TMRM) in live single cells by fluorescent (confocal) microscopy, which could yield a potent *in vitro* read-out on the stem versus progenitor cell state.

Assessment of metabolic marker changes during in vitro culture

We will apply the above-mentioned read-outs to monitor how individual HSCs change their metabolic phenotype during *in vitro* culture conditions, inducing known self-renewal versus differentiation divisions, as well as under hypoxic culture conditions (1-5%, using premixed gas with defined oxygen composition). As the native HSC niche is an extremely hypoxic microenvironment that forces cells into low metabolism, we expect hypoxic conditions to contribute to the maintenance of the stem cell state *in vitro* as well. Oxygen tension will be measured using an oxygen-sensitive ruthenium probe (FOXY-slide, Ocean Optics, Inc.).

Conclusions and Relevance for 3R

HSC transplantations, currently the only functional means to identify and characterize mammalian HSCs, are radical and expensive procedures used in countless labs all over the world. Therefore, the identification of novel *in vitro* markers that are predictive of the functional HSC state would be highly significant. Among other things, it could open up new avenues to rapidly identify stem cells under conditions of *in vitro* expansion.

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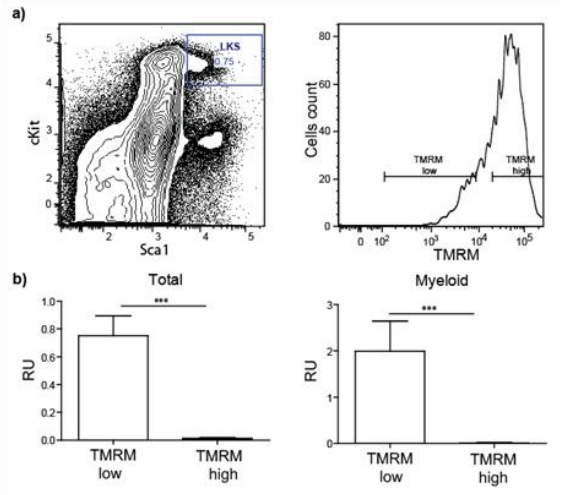

Figure 1: Transplantation of sorted stem/progenitor cells based on mitochondria activity. a) LKS are sorted in two populations based on their TMRM intensity (low and high). b) Total blood and myeloid compartment analysis of lethally irradiated transplanted mice demonstrate that only cells TMRM low (low mitochondrial activity) engraft successfully.

3R-Project 133-12

Development of an in vitro system to grow and investigate vascular endothelial cells under physiological flow conditions

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Keywords: *endothelia*; *coagulation; transplantation*; *ischemia*; *reduction; replacement*; *drug screening*

Duration: 1 year **Project Completion:** 2013

Background and Aim

Activation and damage of vascular endothelial cells is an important mechanism in the pathophysiology of several diseases and clinical entities. In particular, it plays an important role in cardiovascular diseases. The activation of endothelial cells is essential in transplantation also, because they are the donor cells that make first contact with the recipient's immune system.

It is relatively easy to isolate and culture endothelial cells, at least from large blood vessels, but currently available *in vitro* systems offer only limited scope to replace animal experiments since the endothelial surface layer, the so-called glycocalyx, looks different when building up under normal, static culture conditions as compared to the *in vivo* situation with pulsatile blood flow [1],[2]. Available systems for endothelial cell culture under flow either provide only two-dimensional culture of the cells, for example on the bottom of micro channels, or three-dimensional culture in opaque, tubular carriers and technically demanding setups.

We want to develop a new, simple *in vitro* model to grow and investigate endothelial cells using physiological conditions of pressure and flow.

Method and Results

in progress (present status)

The new system will be based on application of nanotechnology to reach optimal conditions for attachment and growth of the cells. The use of transparent carriers will allow the observation of interactions between the cultured endothelium and blood cells using normal intravital microscopy. So far, endothelial cell adherence and growth on different nanostructure patterns has been tested (Fig. 1), and a method to coat glass tubes with defined nanostructures has been developed (Fig. 2).

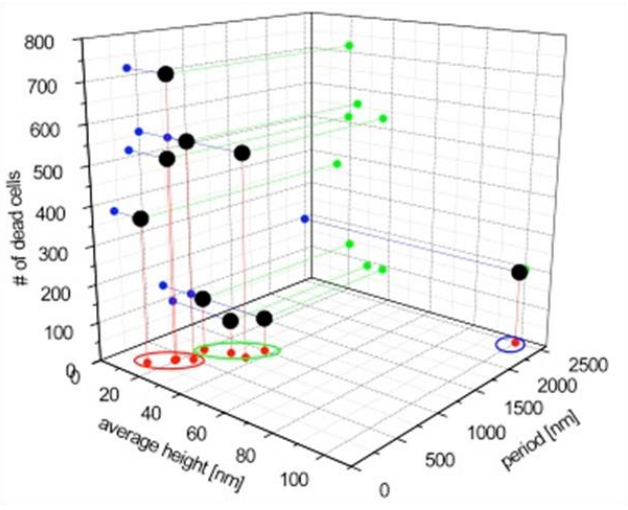

Figure 1: Comparison of 9 nano features coated on flat cover slips. When the number of dead cells was compared, it was lowest for nano surfaces of average height (20-40 nm) and average period (500-1000 nm; circled in green).

Figure 2: Scanning electron microscope that shows coating of nano surfaces on the inside of glass tubes. Scale = 3000 nm.

Conclusions and Relevance for 3R

Currently available, preliminary data suggest that the setup of the model is feasible. Once established, we hope that the number of animal experiments in which the investigation of endothelial function plays an important role can be reduced thanks to the novel *in vitro* system. This is particularly true for experiments on ischemia/reperfusion injury as well as transplantation, which usually are quite traumatic for the animals.

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3R-Project 134-12

Cardiovascular simulator with autoregulation

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Keywords: *medical devices*; *heart valves; in vitro testing*; *baroreflex; frank-starling; hemodynamics; simulator*; *reduction; refinement*

Duration: 2 years **Project Completion:** 2014

Background and Aim
The approval of new cardiovascular devices for clinical use depends upon data that are gleaned from *in vitro* and *in vivo*. Although the latter can never be entirely dispensed with, high-level *in vitro* simulators can partially replace them, thereby permitting a reduction in the number of experiments with living animals[1]. Simulators incorporating mammalian responses such as the regulation of vascular resistance[2], heart rate and stroke volume[3] are a requisite for testing of devices that interfere directly with the hemodynamics of the cardiovascular system. Devices that treat failing anatomic structures such as vessel dissections, septal defects or compromised heart valves, need to geometrically simulate the defect anatomy and the approach pathway.

Method and Results
Two simulators were developed to fulfill the requirements mentioned above. The first simulator was based on lumped-parameter modeling of the cardiovascular system, whereby properties of the circulation that are normally geometrically distributed throughout the body are lumped in one discrete component. For example, the hydraulic resistance that is built up in all arterioles and capillaries is implemented as one single resistance element. The unique feature of this simulator is that it is hybrid: part is implemented in software (Matlab/Simulink, The Math Works Inc.) and part as a fluid circuit, both interacting with one another in real time[4]. The most complex features and all of the autoregulation mechanisms are implemented via differential equations in the software part. The hydraulic part consists of two pressurized fluid tanks, between which a medical device can be inserted. Each tank can generate an active pressure waveform which is prescribed in real time by the software simulation, and in turn the boundary conditions of the software simulation are prescribed by the volume changes which are continuously measured in the fluid tanks. This model is characterized by great flexibility because the tanks can simulate any part of the cardiovascular system that is implemented in the software: ventricles, atria, pulmonary artery, veins, etc. Hence, this simulator is valuable for a broad array of cardiovascular devices.

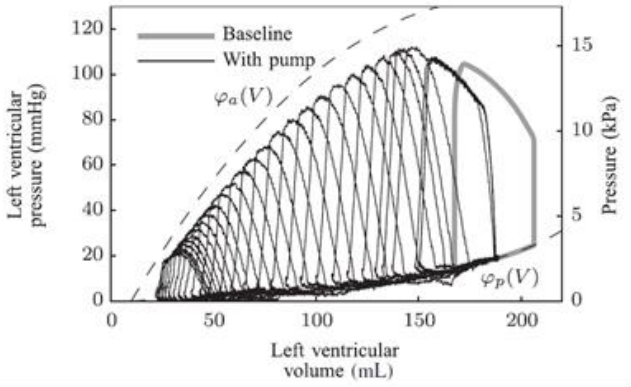

Figure 1: Sequential Pressure-Volume loops of the Left Ventricle demonstrating physiologic response to preload changes in the hybrid simulator

The second simulator focused on the geometry of anatomically-relevant structures. It consists of a simplified fluid circuit with lumped parameter elements implemented in hardware (compliance chamber, vascular resistor, and venous reservoir). A computer-controlled linear motor is suited with a custom pump head, simulating the systolic and the diastolic behavior of the heart with programmed volume waveforms. The anatomy of large veins, atria and valve planes are derived from CT scans and manufactured using rapid

prototyping techniques. Valved ports are installed at locations that are typically used for catheter access or for minimally invasive heart surgery. Frames for mounting septa or cadaveric heart valves are readily exchangeable without the need for specialized tools. Hence, the procedures can be repeated on fresh tissue without delays.

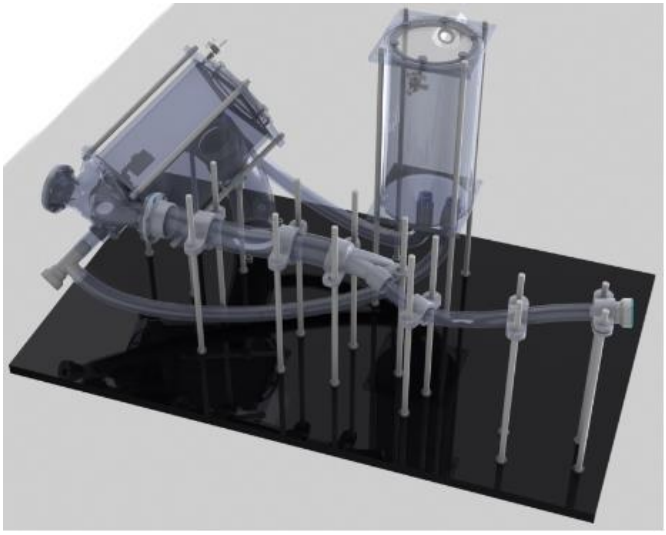

Figure 2: The hydraulic part of the second simulator

Conclusions and Relevance for 3R

The models have already been used for several studies[5,6]. The advantages have clearly been observed: repeatability, visibility, and a well-controlled environment, yielding data with low standard deviation and higher statistical significance. These simulators contribute to the 3R-principles in several respects: (i) they have been used to execute complex cardiovascular research studies without having to sacrifice animals. (ii) they have been used to prepare for animal studies so the equipment and protocol could be fine-tuned and less animals were needed to complete a successful animal trial. (iii) they have been used for training clinicians and testing device prototypes, thereby obviating the need for animal models.

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3R-Project 135-13

In-vitro engineering of a human cell-based threedimensional dynamic model of atherosclerosis

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Keywords: *cardiology; cardiovascular*; *atherosclerosis*

Duration: 3 years Project Completion: 2017

Background and Aim
Atherosclerosis is a chronic inflammatory disorder and underlies most cardiovascular diseases (Getz *et al.*, 2012). According to the *US National Center for Health Statistics(USNCHS)* it is a leading cause of death in the Western world. Not surprising therefore, that research on atherosclerosis is becoming of increasing importance and more and more research resources are being invested in this particular field. Alongside this development, the use of animal models of the disease has increased during the past few decades. Owing largely to the relative ease with which genetic manipulations can be made in mice to the relatively short span of time over which atherosclerosis develops in this species, murine models are most extensively used at present. However, the development of atherosclerosis in mice differs from humans (Whitman, 2004). Consequently, larger animals have been implemented as models. These include rats, hamsters, guinea pigs, rabbits, birds, carnivors, dogs, swine, and various non-human primates (Getz *et al.*, 2012). Although these models resemble more closely the human phenotype, substantial species-specific differences exist. Even when using dogs and non-human primates the data are not directly translatable to humans. Differences particularly in the lipoprotein profiles have been identified (Yin *et al.*, 2012). The bioengineering of a human cell-based dynamic-artery model of atherosclerosis *in-vitro* could potentially help to reduce and replace many of the *in-vivo* experiments and afford an opportunity of performing experiments with human cells.

Method and Results
In progress (present status)

After the isolation of human vascular cells and the manufacture of PGA-P4HB-composite matrices, the bioengineered constructs will be fabricated using dynamic, pulsatile-flow bioreactor systems. After a quality assessment of the bioengineered arteries using Evans-Blue-based assays and the performance of biomechanical uniaxial tensile testings, LDL and monocytes will be isolated from human EDTA-treated blood, fluourescently-labeled and introduced into the circulation loop. Next, the formation of atherosclerotic lesions will be analyzed using immunohistochemistry, confocal microcoscopy, histology (after staining with Masson-Trichrome, Sudan Red or Red-Oil-O) and grating interferometry. Finally, the creation of transgenic human cell-based bioengineered grafts will be investigated after the deletion of specific genes in vascular cells / monocytes using the *CompoZr® Zinc Finger Nuclease Technology* (transgenic disease modeling platform).

Conclusions and Relevance for 3R
The bioengineering of a human cell-based dynamic-artery model of atherosclerosis could potentially hell to reduce and replace many of the in-vivo experiments that are currently performed with animals with animals such as mice, rabbits, pigs and non-human primates, and afford an opportunity of conducting investigations with human cells. Instead of executing (non-representative) experiments with animals, researchers could use the functional experimental human cell-based (representative) artery model to study the development of atherosclerosis and to investigate therapeutic options for its treatment.

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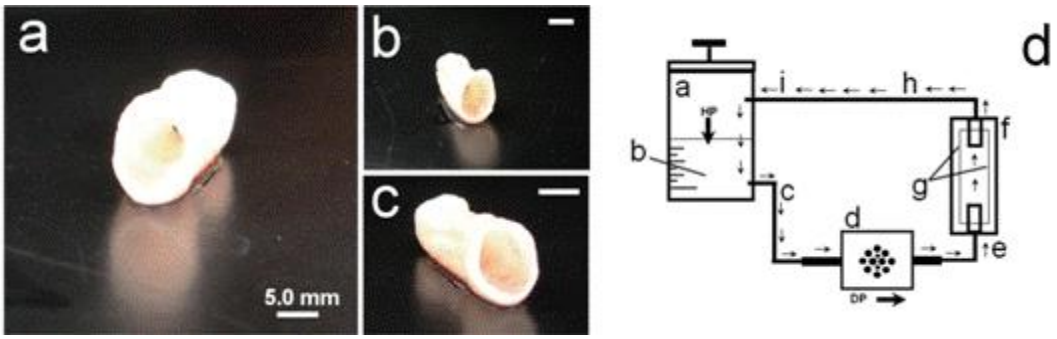

Figure 1: Tissue-engineered vascular grafts

(TEVG; a-c) after 2.5 weeks of *in-vitro* conditioning in a dynamic-flow bioreactor system (d). The basic set-up of the TEVG-system consists of a medium reservoir (A) filled with AFC-cell culture medium (B; HP = hydrostatic pressure). As a result of the pumping pressure, medium is sucked into the system (DP=dynamic-pressure generator) and via a connection tubing (E), is pressed into a container (F) containing the seeded TEVG-scaffold matrix (G). Next, the medium flows via further tubes (H-I) back to the reservoir and is replenished with nutrients. [Extracted from: Weber B., *Tissue Eng Regen Med* 2013]

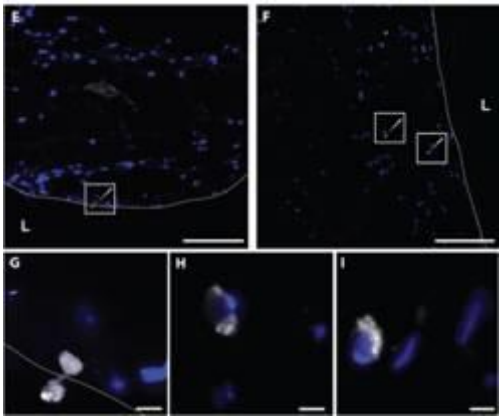

Figure 2: Monocyte transmigration in 3D artery-equivalents.

Monocyte adhesion and migration in the tissue were analyzed by the microscopic inspection of cryosections after pre-treatment with LDL´s. Microscopic observations revealed the adhesion of monocytes and their migration through the endothelium (dashed line) (E, G) as well as their subsequent accumulation in the tissue (F, H-I). Bars represent 200 µm (A-C, E-F) and 20 µm (G-I). [Extracted from: Robert J., Weber B., *PLOS One* 2013]

3R-Project 136-13

Development of an *in-vitro* potency assay for the *Clostridium chauvoei* vaccines: Replacement of the guinea-pig-challenge potency test

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Keywords: *vaccination*; *blackleg disease*; *efficacy test*

Duration: 3 years **Project Completion:** 2017

Background and Aim
The quality of vaccines for animals is of ultimate importance for their welfare and health, as well as for the sustainability of livestock production. Hence, each batch of vaccines must be subjected to a strict quality control to ensure the safety and the efficacy of the product as a whole. The required quality-control tests are described in international regulatory manuals, such as the *European Pharmacopoeia*.
Blackleg, a severe and lethal infectious disease of cattle and sheep, is caused by the bacterium *Clostridiumchauvoei*. Due to the fulminate development of blackleg, therapy is not possible. However, cattle and sheep can be successfully immunized against the disease using vaccines that contain *C. chauvoei* antigens. In spite of the efficacy of current blackleg vaccines, which are based on bacterin-containing and toxoided culture-supernatants there is no knowledge as to which antigens in current formulations are protective. From the beginning there has been uncertainty as to whether the supernatants of *C. chauvoei* cultures contain a sufficiently lethal dose of a toxin to account for its pathogenicity. This same uncertainty extends to whether the protective antigens reside in the culture-supernatant or within the cells themselves. Consequently, commercial blackleg vaccines are comprised of chemically-toxoided supernatants and inactivated bacteria. Currently, the potency of vaccine batches is monitored by a challenge model in guinea pigs (*European Pharmacopoeia* 7.7, p. 5333). In this test, the vaccine under assessment is administered to guinea pigs, which, together with an unvaccinated group of animals, are challenged with a virulent strain of *C. chauvoei*, 42 days after the first vaccination. For the test to be valid, all of the control animals must die within three days, and for the vaccine to pass the test, at least 90% of the vaccinated guinea pigs must survive for minimally five days after the challenge. During this procedure, the animals suffer great distress. All *C. chauvoei*-containing vaccines that are used in Europe, the USA, Canada and most other countries around the world have to pass this or a similar test. In a single European production plant of animal vaccines, approximately 1500 guinea pigs are used for this purpose annually. Worldwide, it is estimated that tens of thousands of guinea pigs are subjected to this challenge test every year.

Method and Results
Recently, we have discovered a novel protein toxin (Frey *et al.,* 2012), for which we have coined the term Clostridium-chauvoei-toxin A (CctA). The corresponding gene, *cctA*, has been detected in each of the seven strains of *C. chauvoei* used in our study, that have been isolated from cattle with blackleg over the past 50 years, and which have stemmed from various countries worldwide. The gene *cctA* is not present in any other pathogenic *Clostridium* species. CctA is highly toxic for various bovine cell-lines and is strongly haemolytic as shown in figure 1, which shows *Clostridium chauvoei* colonies on a medium containing erythrocytes. This standard diagnostic test shows the erythrocytes that are fully lysed (visualized by the halo) around the tiny colonies of the pathogen.
A rabbit polyclonal antiserum raised against recombinant CctA fully neutralizes the cytotoxic and haemolytic activities of supernatants from cultures of various *C. chauvoei* strains, thereby suggesting that CctA is the major virulence factor. Using the vaccine-challenge potency test, we have demonstrated that the immunization of guinea pigs with CctA in the form of a recombinant fusion protein protects the animals against challenge with virulent *C. chauvoei*. These experiments indicate that CctA is the main protective antigen in blackleg vaccines.
A central part of the project is to irrevocably confirm that the CctA-antigen confers - to a major extent - protective immunity against blackleg. To this end, we have shown the uniformity of the CctA toxin in strains that are spread worldwide. This is important as vaccine producers use different strains, mostly old isolates

and sometimes a cocktail of several strains, since the relevant antigens for the vaccines were not known when the vaccines were licensed. Moreover, due to the extreme costs for licencing new vaccines and the low prize cattle vaccines can be put on the market, vaccine producers do not intend to change their current vaccine production lines. Novel data that were obtained in the framework of this 3R project now clarify that the CctA toxin of all of 20 *C. chauvoei* strains collected from 4 continents and over a period of 60 years contain the identical CctA amino acid sequence and hence the identical toxin. Furthermore, only minor differences were found in general in these *C. chauvoei* strains including fimbriae, which were previously reported to be variable among strains and play a role in protective immunity. Hence, the CctA based approach for an ELISA assay is warranted. Subsequently, a recombinant protein containing the major antigenic domain but that is not toxic (for biosafety reasons) has been expressed from an *E. coli* expression strain that is used as standard in biotechnology. The antigen can be purified to very high degree and is currently analysed to be able to detect the serological response in vaccinated cattle.
In a parallel approach, cattle have been vaccinated with a commercial vaccine that is licensed in Switzerland according to standard veterinary procedures and sequential serum has been taken before and after vaccination. Currently the sera are tested immunologically with the recombinant CctA-derived peptide. If successful the data will be published and diagnostic companies will be contacted to implement the test at an industrial sustainable scale.

Conclusions and Relevance for 3R

The recombinant CctA-antigen will be produced using our in-house expression vector pETHIS1. However, we propose to induce the expression only of the major antigenic segment of CctA rather than that of the whole molecule. The reason for so doing is, that we wish to develop a safe laboratory test for the measurement of anti-CctA antibodies in vaccinated animals using an indirect enzyme-linked immunosorbent assay (ELISA).
The final aim of the project is to develop a method to replace the guinea-pig release test, which will be presented to the *European Partnership for Alternative Approaches to Animal Testing* (EPAA). The EPAA, in cooperation with the Biological Standardization Program of the European Department for the Quality of Medicines (EDQM), would undertake the validation and concordance testing of the newly-developed *in-vitro* test. On the basis of the results from a comparative study with the standard vaccine release protocol, the alternative method will then be presented for approval to the pertinent authorities.

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Rychener L., InAlbon S., Steven Djordjevic, S.P., Roy Chowdhury P., Rosangela Estel Ziech R.E., Castagna de Vargas A., Frey J. and Falquet L. (2017) *Clostridium chauvoei*, an evolutionary dead-end pathogen. Frontiers in Microbiology. 8 (1054): 1-13.

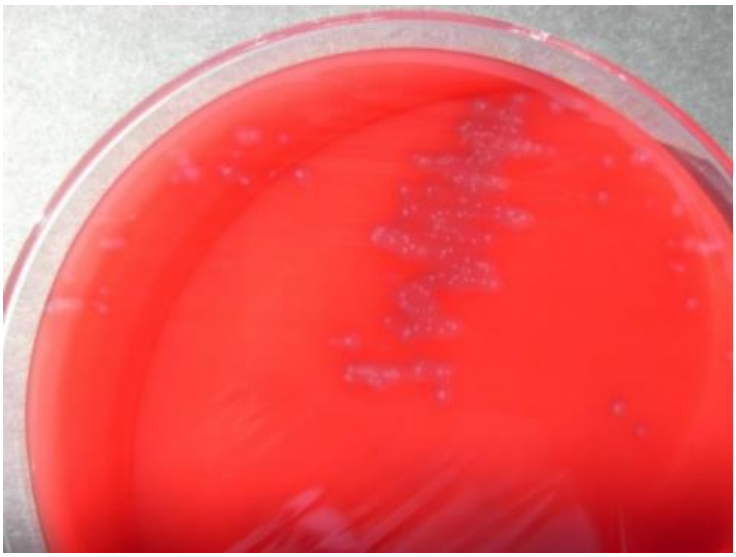

Figure 1: Colonies of *Clostridium chauvoei*, grown on blood-agar medium. The corona around the bacterial colonies is due to the hemolytic activity of the Clostridium chauvoei toxin that lyses the sheep erythrocytes in the growth medium

3R-Project 137-13

Validation of a new human *in-vitro* model of microglia

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Keywords: *brain*; *immunology; neurology*; *validation*

Duration: 1 year **Project Completion:** 2015

Background and Aim
Brain diseases are a burdensome problem for modern societies. Not surprisingly, therefore, substantial resources are invested in neuroscience research, which depends heavily on animal models. In this respect, microglia have assumed an important role. Animals are sacrificed for brain excision and the collection of cells of *in-vitro* studies. Animals are also used for *in-vivo* experiments. Depending on the aim of the study and on the nature of the procedure adopted, the *in-vivo* animal experiments involve different levels of invasiveness and suffering, which may include injuries to the brain and the spinal cord, if microglial response to trauma is being investigated, as well as exposure to toxic and infectious agents. Most of these experiments are conducted with human diseases in mind, even though animal microglia behave in many respects differently to human ones. The need to improve human *in-vitro* models of microglia is thus pressing.

Microglia are the unique resident immune cells of the brain. They play a crucial role in most brain diseases, being implicated in repair responses to injury, degeneration and infection-induced inflammatory reactivity. Being resident cells of the brain, they have to be isolated from fresh cerebral tissue for research purposes; only a few human microglial lines are available and for restricted applications only. We have developed a new human *in-vitro* model of microglia, which are derived from monocytes circulating in peripheral blood (Etemad *et al.* 2012). However, we now need to ascertain whether the properties of the human monocyte-derived microglia correspond to those of the brain-derived ones, which is a precondition for the validity of the *in-vitro* model.

Hypotheses:

The properties of human monocyte-derived microglia correspond to those of their brain-derived counterparts.

Human monocyte-derived microglia can be used to replace the brain-derived ones of animals for the *in-vitro* investigation of human cerebral diseases in which these cells play a key role.

Aims:

To compare the morphologies, phenotypes and functional activities of human monocyte- and brain-derived microglia.

To characterize the property-spectra of human monocyte- and brain-derived microglia with a view to establishing a gold-standard of this cell-type for research into human cerebral affections, taking Alzheimer's disease as a pathological model and infection with the Japanese encephalitis virus as an infectious one.

Method and Results
In progress (present status)

Human microglia:
Human monocyte-derived microglia will be generated *in-vitro* using a published protocol (Etemad *et al.* 2012). Human brain-derived microglia will be isolated from 10 cadavers, not later than 8 hours post-mortem. According to published data, the brain-derived microglia should still be viable within this time-frame (Melief *et al.*, 2012). In order to ascertain whether topographic differences in the properties of the brain-derived microglia exist, tissue blocks with a volume of 1-5 cm^3 will be excised from the frontal, parietal, temporal and occipital lobes of the hemispheres, as well as from the cerebellum, thalamus, mesencephalon, pons and myelencephalon. The microglia will be isolated and cultured and then subjected to a comparative analysis. Monocyte- and brain-derived microglia will be cultured under the same conditions.

Comparative analysis of the properties of monocyte-and brain-derived microglia:
Various morphological and functional parameters, as well as viability, will be evaluated by flow cytometry (Etemad *et al.*, 2012, *Prabhakaranet al.*, 2012, Melief *et al.*, 2012). The phagocytic activity of the microglia and their capacity to stimulate T-lymphocytes will also be assessed.

Influence of beta amyloid on human microglia:
There is an abundance of evidence indicating that microglia are responsive to treatment with beta amyloid, and for this reason, these cells have been implicated in the development of Alzheimer's disease (Gentleman, 2013). In preliminary experiments, we have shown that the exposure of monocyte-derived microglia to beta amyloid induces significant changes in the expression pattern of chemokine receptor (Filgueira *et al.*: unpublished data). Hence, both monocyte- and brain-derived microglial populations will be exposed to beta amyloid, after which, their phenotypic characteristics and cytokine-secretion profile will be compared. We anticipate that the two microglial populations will respond similarly to treatment with beta amyloid.

Rates of infection with, and the influence of the Japanese encephalitis virus (JEV) on human microglia:
Microglia play an essential role in the inflammatory response of the brain to infections. In preliminary experiments, we have shown that human monocyte-derived microglia can be infected with JEV (Filgueira *et al.*: unpublished data). This virus is known to induce changes in the expression patterns of diverse surface markers, including the receptors for chemokines. We will thus compare the rates of infection of monocyte- and brain-derived microglia with the JEV, as well as changes in the cytokine-secretion profiles that are thereby induced.

Conclusions and Relevance for 3R
It is expected that by validating our new *in-vitro* human model of monocyte-derived microglia, and by applying it to two important areas of brain research, namely Alzheimer's disease and viral infection, the prototype will gain wide acceptance in the implicated research community and will be used to replace animal experiments. In consequence, we anticipate a dramatic reduction in the consumption of animals for research in the field of neuroscience.

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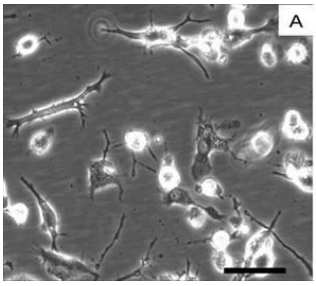

Figure 1: Human monocyte-derived microglia in culture.

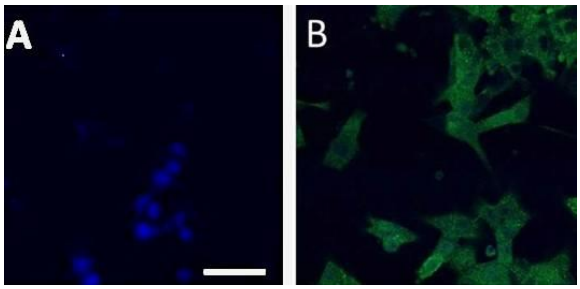

Figure 2: Expression of Iba1, a marker specific for microglia, by human monocyte-derived microglia. A) Staining control (blue nuclei stained with DAPI). B) Iba1 expressing cells are shown in green. The cells were treated with a monoclonal mouse anti-Iba1 antibody, and a secondary donkey anti-mouse antibody labelled with AlexaFlour 488.

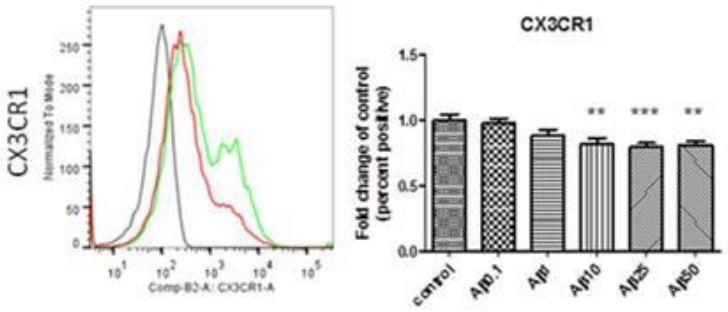

Figure 3: Influence of beta amyloid on human monocyte-derived microglia morphology. Left panel: light microscopy images of cells in culture before and after 48 hours of treatment with none or 25microgramm/ml of beta amyloid. Note the increased numbers of round shaped cells after 48 hours of treatment with beta amyloid indicating activation of microglia. Right panel: Quantification of activation of microglia after 0, 1, 8, 48 and 72 hours, after treatment with beta amyloid (0, 0.1, 1 and 25 microgramm7ml). There was a significant activation of the cells after 48 hours and treatment with 25microgramm of beta amyloid.

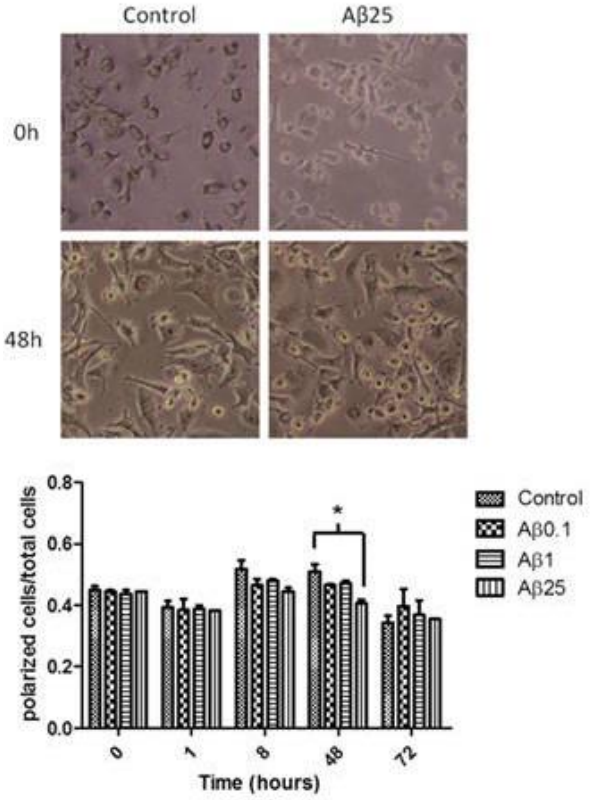

Figure 4: Down-regulation of expression of the chemokine receptor CX3CR1 in human monocyte-derived microglia after treatment with beta amyloid. Left panel: Representative histogram of flow cytometry measurements (black is staining control, green is CX3CR1 expression by control cells, red is expression of CX3CR1 after treatment with beta amyloid). Right panel: Quantitative analysis of CX3CR1 expression in cells treated with increasing concentrations of beta amyloid (0, 0.1, 1, 10, 25 and 50 microgramm/ml). n=4, ** is $p<0.01$, *** is $p<0.005$.

3R-Project 138-13

Optimization of the nerve-cell-mimicking liposome assay as an in-vitro alternative for the detection of *Clostridium-botulinum neurotoxins* and for a validation of their presence in complex sample materials

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Keywords: *toxicology*; *toxicity testing: neurotoxins; validation*

Duration: 3 years **Project Completion:** 2016

Background and Aim

The *Clostridium-botulinum* neurotoxins (BoNTs) are the most poisonous naturally-occurring protein toxins known to mankind, the toxicity range in humans being as low as 0.3 – 2 ng/kg when administered intravenously [1, 2]. The neurotoxins are mainly produced by the anaerobic, spore-forming, Gram-positive bacteria *Clostridium botulinum*, which is ubiquitously in earthy and aquatic environments [3]. Botulism is defined as a gradually escalating paralysis of the skeletal muscles, which ultimately leads to respiratory failure [4]. All BoNTs consist of two individual subunits, an ≈ 100-kDa heavy chain (HC) and an ≈ 50-kDa light chain (LC), which are linked by a disulphide bond (Figure 1). The HC is responsible for recognition – binding to specific receptors on the neurolemma of motor neurons – internalization by these nerve cells via receptor-mediated endocytosis, and translocation of the LC across the endosomal membrane through the HC-channel [5-8]. The LC, which is a zinc-endoprotease, specifically cleaves the soluble N-ethylmaleimide-sensitive-factor-activating protein receptor (SNARE), thereby resulting in an inhibition of neurotransmitter release and a consequent paralysis of the affected muscle [9]. If applied in small quantities, the toxin causes a locally-confined paralysis, which is beneficial in the treatment of various diseases and affections such as different forms of dystonia, hyperhidrosis, strabismus, chronic pain and headaches. Currently, however, it is most widely used in aesthetic surgery for the smoothing of wrinkles and frown lines [10]. Since these toxins are approved by the Food and Drug Administration (FDA), the safety of the patients has to be guaranteed. Consequently, it is crucial that the toxic activity of all batches of pharmaceutical BoNT-preparations is consistent. Currently, the potency of biologically-active BoNT in different batches is monitored using the murine LD_{50}-assay. In this assay, different dilutions of preparations containing BoNT are injected intraperitoneally into mice and symptoms are observed for several hours up to 4 days. The mice manifest a characteristic wasp-like abdomen with narrowed waist, which is the result of the increased respiratory effort that is necessitated by the paralysis of the diaphragm; respiratory failure and death follow [11]. Due to the enormous demand for pharmaceutical products containing BoNT, it is estimated that in the USA and Europe, more than 600`000 mice are annually sacrificed for these batch tests [12, 13].

Over the years, different functional, immunological and spectrometric assays or combinations thereof have been developed for the detection of BoNTs [14]. To replace the current murine LD_{50}-assay, a new test would have to assess all three essential functions of BoNTs, namely the binding of the toxin to specific receptors, the translocation of the LC, and the LC-mediated cleavage of distinct SNARE-proteins. However, most *in-vitro* assays are capable of detecting only one of these key biological activities [14-16]. Only cell-based assays, using, for example, rat spinal-cord cells or embryonic stem-cell-derived neurons, or *ex-vivo* ones, such as the hemidiaphragm assay, would be suitable as potential alternatives [14].

We have developed a new concept using nerve-cell-mimicking liposomes (Figure 2, A & B) [17]. Functionalized liposomes with integrated receptor – which permit the HC-binding of BoNTs – and an encapsulated substrate – bearing the specific cleavage site for the LC – are used (WIPO Patent Application WO/2013/011055).

Initially, BoNT of the serotype A (BoNT/A) binds via its HC_C to the presented receptors on the liposome surface (Figure 2B, left). When the pH of the surrounding medium is lowered, the HC_N translocates the LC into the liposomal lumen, wherein it exerts its endoproteinase activity by cleaving the encapsulated peptide substrate with specific cleavage sites and a quenched fluorophore pair (Figure 2B, middle). Upon cleavage, the fluorophore pair is unquenched (Figure 2B, right) and its fluorescent signal can be detected, thus permitting a quantification of the actual biological activity of BoNT/A in the system. With the use of well-defined components, the liposomes may be produced under reproducible conditions.

Against this background, we now wish to further test the concept of nerve-cell-mimicking liposomes. The main goal is to increase the sensitivity and the robustness of the assay for BoNT A. Subsequently, we would like to optimize the assay for a broader application to other serotypes and more complex sample materials.

Method and Results

In progress (present status)

To date, reporter assays have been established for the detection of BoNT/A and BoNT/B, even under liposome-compatible conditions. Fully-assembled liposomes (FALs) for the detection of BoNT/A and BoNT/B have been successfully produced using lipid compositions similar to those found in mammalian nerve cells. FALs contain high amounts of the peptide-reporter molecules, and the respective receptors are integrated into the liposomal membrane. Currently, experiments are in progress which aim to enhance the sensitivity and the robustness of the assay for the detection of BoNT/A.

Conclusions and Relevance for 3R

During the past decade, new sensitive methods for the detection of BoNT-activity have been developed. However many of these methodologies suffer from the drawback of being incapable of systematically monitoring each of the three sequential activities that are requisite for the toxin`s functional competence. Consequently, the murine LD_{50}-assay is still considered to be the gold standard for the detection of BoNTs in complex sample materials. Our liposome assay holds the potential to replace the murine bioassay. It can detect all three of the essential toxin activities of BoNTs (binding, translocation, cleavage) and can be conducted within 2 to 4 hours. Once the liposome assay has been shown to be of sufficient robustness and sensitivity to detect BoNT A, it could be used to replace the murine LD_{50}-assay in the batch control of pharmaceutical products containing this serotype, thereby resulting in the sparing of up to 600`000 mice per year. Furthermore, if the assay proves to be sufficiently robust when applied to complex sample materials (e.g., food products, blood, faeces), it could be employed to detect BoNTs in a clinical environment.

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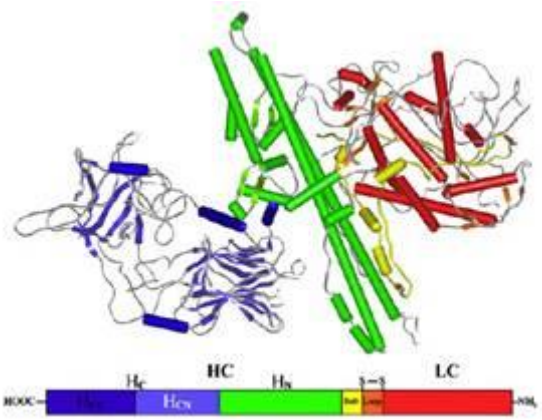

Figure 1: Schematic representation of the four-domain structure of the single-chain 150-kDa clostridial neurotoxins (bottom) and the corresponding crystal structure of BoNT/B [5].

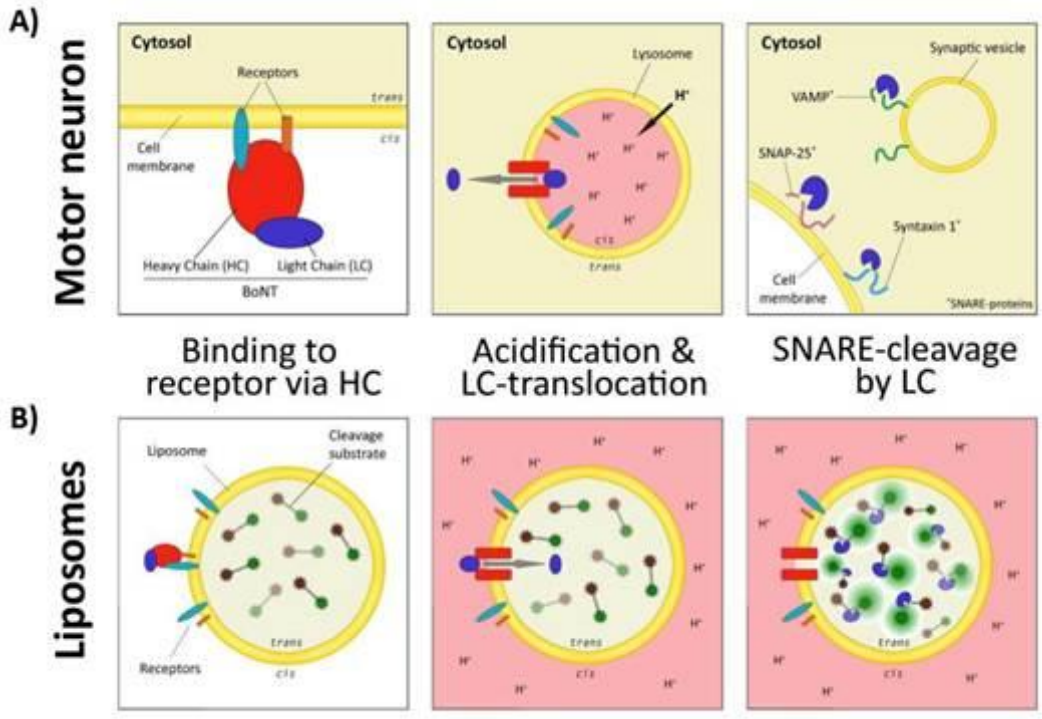

Figure 2: Schematic representation of the three steps of BoNT toxic activity in motor neurons (A) and functionalized liposomes (B) [17]

3R-Project 139-14

An *in-vitro* microvascular model of the endothelial barrier

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Keywords: *cardiovascular; endothelia*; *angiogenesis*; *angiogenesis*; *cell cultures: intact tissue; tissue engineering*

Duration: 2 years **Project Completion:** 2017

Background and Aim
Pericytes are an important component of the endothelial barrier in capillaries and other microvessels. They are vascular mural cells which reside in the basement membrane together with endothelial cells. Their spatial distribution, abundance and relationship with endothelial cells reflect the function of individual tissues. For example in organs that are characterized by high gaseous and metabolic exchange rates, the distribution of pericytes is such that diffusion is minimally hindered [1]. Interestingly, during the past few years, it has become evident that perivascular cells may also represent a physiological reservoir of adult mesenchymal stem cells (MSCs) [2]. MSCs have the potential to differentiate into adipogenic, osteogenic and chondrogenic lineages, and are therefore widely used for the regenerative engineering of musculoskeletal tissues. However, most of these studies have drawn on MSCs that have been expanded in monolayer cultures, since suitable models and techniques for achieving this in a more physiologically relevant three-dimensional environment are not available. At the same time, the culture of MSCs in monolayers is known to exert a great influence on their phenotypic and functional parameters [3].

The aim of this project is to establish an *in-vitro* model for the study of perivascular cells in a physiologically relevant context.

Method and Results
The project aims to establish a model system embodying the most important parameters that cells perceive in their physiological environment: cell-cell interactions, the extracellular matrix, paracrine factors and mechanical forces, such as flow and shear, which are particularly important in regulating the phenotype and behaviour of vascular cells. Although microfluidic systems hold the potential to closely mimic the cellular microenvironment at spatial and temporal levels, they are usually comprised of endothelialized microchannels; only a few include also a perivascular compartment. Hence, cell-cell interactions between vascular and perivascular cells have been mostly studied in static three-dimensional co-culturing systems [4]. In a recent study conducted by the group of Abraham Stroock, both approaches were combined for the first time [5; 6]. In this model, perfused microvessels are embedded within a collagenous hydrogel. Endothelial cells are applied to the circulating medium and are able to adhere to and align with the microchannels during the first few days of culturing. Using this system, the microvessels could be successfully cultured for up to 2 weeks, during which time they were remodeled into units with an elliptical cross-section and underwent angiogenesis. In this project, we will employ a similar system, in which a hydrogel will be used to imitate the perivascular compartment containing pericytes and perfused endothelial-cell-lined microchannels (Figure 1).

In the first part of the project, we will focus on the development of the microfluidic chamber. Here, we will test different vessel configurations (Figure 2A, B) and hydrogels, and optimize the rate of perfusion to accord with physiological parameters. Thereafter, cell-seeding and imaging protocols will be established. Fluorescence labelling will be used to track the movements of the cells during the phase of barrier-assembly. Finally, the model will be validated with respect to barrier function and cell-cell interactions. For this purpose, the microchannels will be perfused with fluorescent molecules, such as FITC-labelled dextran; the diffusion of the dye will be followed by live fluorescence microscopy. Immunofluorescence staining will be applied to image the proteins that are involved in the communication between pericytes and endothelial cells and to detect matrix components of the basement membrane. The migration of the cells towards different stimuli will be studied by introducing a gradient of chemokines (Figure 2C).

Conclusions and Relevance for 3R

In this project an *in-vitro* model of the endothelial barrier will be established. The system will embody the parameters that are of major importance in the three-dimensional environment of endothelial cells and pericytes. As such, it will afford a new insight into the phenotypic and functional characteristics of pericytes, which are believed to represent a physiological reservoir of MSCs. To date, most studies involving MSCs have relied on data that have been gleaned from monolayer-cultured cells, which has often hindered a translation of *in-vitro* findings into preclinically and clinically relevant information. Owing to the lack of suitable *in-vitro* models at the present time, data with more relevance to the physiological situation can be obtained only from *in-vivo* experiments. Our model may also be useful in the wider fields of angiogenesis, carcinogenesis and drug delivery. Hence, its development could help to numerically reduce animal experimentation across several research fields.

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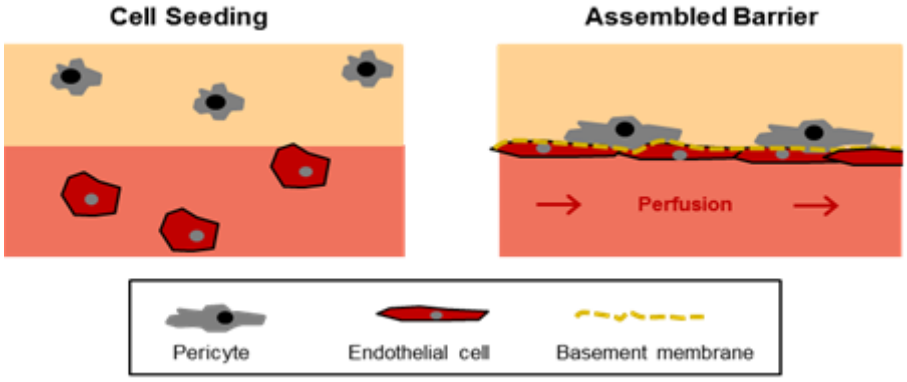

Figure 1: *In-vitro* model of the endothelial barrier. The model is comprised of two compartments (i) a hydrogel with encapsulated pericytes mimicking the perivascular tissue (beige) and (ii) a perfused microchannel containing endothelial cells (light red). In the assembled barrier the endothelial cells cover the microchannel, produce components of the basement membrane, and promote the migration of pericytes towards the endothelial layer.

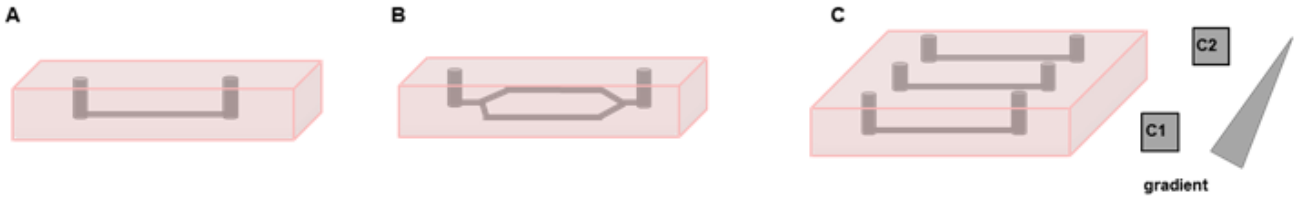

Figure 2: Various vessel configurations in the microfluidic system. A, B: Examples of microvessel geometries and designs; C: Parallel channels can be used to generate gradients of chemokines within the hydrogel.

3R-Project 140-14

Hydrocephalus simulator for testing of active ventriculoperitoneal shunts

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Keywords: *medical devices*; *cns, brain disorders*

Duration: 2 years **Project Completion:** 2016

Background and Aim

Hydrocephalus is a pathological condition that is characterized by an active distension of the ventricular system, which is caused by an impaired flow through the cerebrospinal fluid (CSF)-pathways [1]. Since CSF consists mainly of almost incompressible water, its accumulation causes an enlargement of the ventricles and, more importantly, can lead in the absence of invasive therapy to a degeneration of the brain parenchyma. Hydrocephalus is typically treated by diverting the excessive CSF from one of the two lateral ventricles via a shunt to other bodily compartments, such as the peritoneum (Figure 1). Although shunts are not a novel development, the typical outcome of this treatment is far from satisfactory. The deficiency is being addressed by the development of new, sophisticated active shunting systems in which the draining range can be adjusted to the actual need of the patient [2]. However, before these devices can be implanted in patients, extensive testing and validation are required.

Current shunt-testing platforms are too simplistic to assess these new devices, in so far as they are incapable of replicating the interdependence between the shunt, the CSF-system and the intra-peritoneal dynamics [3]. The deficiency will render necessary the undertaking of animal trials at all stages of the development process. With this project, we aim to provide a testing platform that can replace the preliminary animal studies. It reveals the fluidic dynamic functionality of a shunt in a realistic and accurate environment and reproducibly simulates the patient's response to the device.

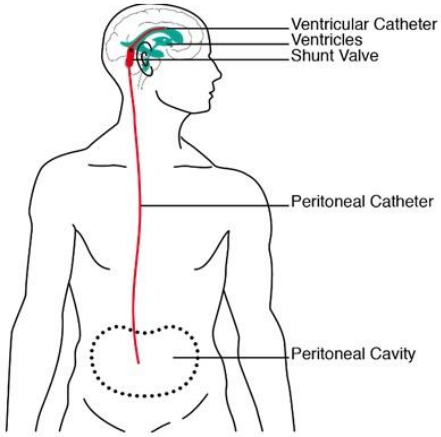

Figure 1: Sketch of a ventriculoperitoneal shunt as it is positioned in a patient. Cerebrospinal fluid is drained from one of the lateral ventricles into the peritoneum. Source: Schmid Daners, 2012 [4]

Method and Results

The simulator is based on the hardware-in-the-loop principle: the component to be tested is integrated into an artificial setup, with the usual environment being simulated in parallel. This was implemented by:

- using a posture mechanism to modify the location and orientation of the tested shunts according to a predefined test scenario;

- applying simulated intracranial pressure (ICP) and intraperitoneal pressure (IPP) to the proximal and the distal catheter ends using two highly dynamic pressure interfaces;
- measuring the resulting drainage rate through the tested shunt; and
- calculating ICP and IPP by simulating the patient's relevant pathophysiology as a function of posture and measured drainage in real-time.

A realistic testing environment is ensured by placing the shunt in a water bath and heating shunt and test medium to 37 ± 1 °C using a surrounding incubator. A lumped parameter model is used to simulate the patient's relevant pathophysiology. This mathematical model includes the influences of the measured drainage rate, posture, cardiac-induced pulsations, and viscoelastic effects.

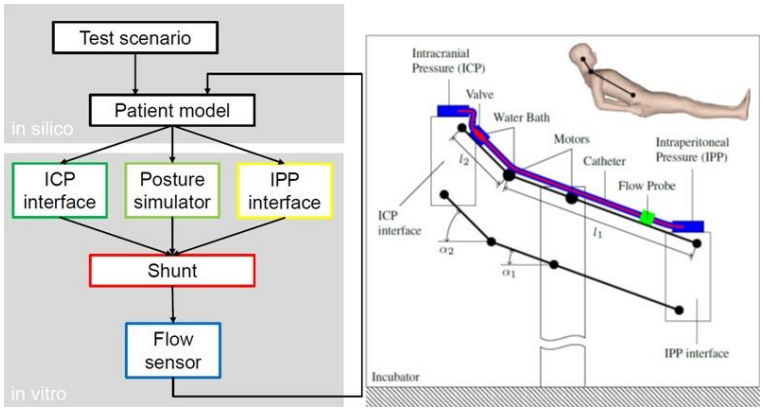

Figure 2: Left panel: Flow-diagram of the proposed shunt-testing platform, depicting the signal flow-paths between the *in-silico* patient simulator and the *in-vitro* shunt-testing hardware.

Right panel: Schematic drawing showing the posture mechanism of the hardware-in-the-loop (HIL) testing platform in semirecumbent position. Different postures are simulated by adapting the angles

In two studies [5, 6], we have shown that the HIL testing platform can be used: to accurately analyze and quantify the dynamic interaction between shunt and patient; to test and evaluate anti-siphon devices in a reproducible yet realistic environment; and as a development platform for actively controlled valves.

Conclusions and Relevance for 3R

The developed *in-vitro* shunt-testing platform will render obsolete most animal trials during the first stages of the development process of future active CSF shunts. Its accessibility to the taking of measurements, as well as the repeatability and the reproducibility of the *in-vitro* tests, helps to define the questions that still need to be answered more precisely in animal models. But these experiments with animal models will be undertaken only when satisfactory results appertaining to the various functionalities have been achieved in the *in-vitro* simulations. Using this approach, the otherwise inevitable animal trials can be markedly reduced in number.

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3R-Project 141-14

In-vitro alternatives to *in-vivo* bioconcentration-testing in fish: restricted to rainbow trout or broadly applicable ?

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Keywords: *carp; rainbow trout*; *bioaccumulation*; *pbt-assessment*; *bioaccumulation; in-vitro metabolism assay*

Duration: 2 years **Project Completion:** 2015

Background and Aim

Worldwide programmes appertaining to the regulation of chemicals call for an assessment of their risks to mankind and the environment in three respects: Persistence, Bioaccumulation and Toxicity (PBT). In aquatic organisms, the bioaccumulation of chemicals is assessed using a standardized *in-vivo* bioconcentration-test in fish (OECD Test Guideline 305). The OECD-305 test is implemented to determine the bioconcentration factor (BCF), which represents the ratio between the concentration in the fish and its concentration in the animal's aquatic environment (under steady-state conditions). The test involves exposing many fish (> 100) to the targeted chemical for long periods of time (> 50 day), and is a costly undertaking.

As an alternative to the *in-vivo* test in fish, an *in-silico* approach can be implemented to predict the BCF from the lipophilicity of the chemical (Arnot and Gobas 2006, Nichols *et al*. 2007). Although this approach works well for lipophilic chemicals that undergo no biotransformation in the organism, it overestimates the BCF-values of metabolized xenobiotics (Lombardo *et al*. 2014). Such overestimates could be corrected using *in-vitro* biotransformation assays that are able to identify the metabolized compounds. By combining the *in-silico* methods with the *in-vitro* metabolism-assay in an Integrated Testing Strategy (ITS), it should be possible to greatly reduce if not to replace altogether *in-vivo* BCF-testing in fish (de Wolf *et al*. 2007, Weisbrod *et al.* 2009, Lombardo *et al*. 2014). However, as yet the *in-vitro* methodologies have neither been validated nor implemented.

Previous studies, which have also been funded by the 3R-Research Foundation, have succeeded in standardizing an *in-vitro* metabolism assay using the isolated hepatocytes of rainbow trout (Fay *et al*. 2014). In addition, they revealed that the *in-vivo* BCF-values of chemicals in fish can be reasonably well predicted from the *in-vitro* metabolic data. The available *in-vitro* methodology has been developed exclusively for rainbow trout (*Oncorhynchus mykiss*), whereas *in-vivo* BCF-testing relies on the use of a broad array of cold- and warm-water species of fish. The nature of the species that is used for testing purposes can depend on the geographic region. In Northern European countries, rainbow trout are preferred, whereas in Asian ones, carp are favoured. Hence, an *in-vitro* assay that is relevant for only one of the many species that are employed for *in-vivo* testing would severely restrict the applicability and acceptance of the alternative assay. The proposed project will overcome this limitation by demonstrating that the *in-vitro* methodology that has been developed for the cold-water species – the rainbow trout – can be instrumented also for the warm-water species, the common carp (*Cyprinus carpio*), thereby proving that its use is not confined to one specific species of fish. More specifically, the objectives of the project are

- To ascertain that the *in-vitro* methodology that has been developed for the hepatocytes of rainbow trout is relevant also for another species of fish (the carp) that is used in the OECD-305 test;

- To ascertain whether species differences in the metabolism of xenobiotics that exist *in-vivo* are reflected by the *in-vitro* hepatocyte assay.

In addition, it will be ascertained whether the *in-vitro* hepatocyte-assay is useful not only in determining metabolic rates, but also in identifying metabolite patterns.

Methods and Results

In a first step, a standardized protocol for the in vitro metabolism assay with carp hepatocytes was developed. It included the implementation of the methodology for isolation of carp hepatocyte isolation (Segner et al. 1993, Vogt and Segner 1997), and the adaptation of the assay protocol. In a second step, metabolism of selected xenobiotics was comparatively tested in both trout and carp hepatocytes. The in-vitro metabolic rate values were extrapolated to the intact fish in order to learn whether the in-vitro measurements are able to predict species differences in the in-vivo metabolism of xenobiotics. The results of the experiments showed that the in vitro metabolism protocol which has been developed originally for rainbow trout can be successfully transferred to non-salmonid species like carp.
The currently established protocol of the in vitro-metabolism assay using fish hepatocytes assay is intended to predict in vivo-metabolism rates of xenobiotics; it is not intended to identify the metabolites arising from biotransformation of the parent compound. For biocide regulatory testing, however, metabolite data are required. Therefore, the question arised whether the in-vitro metabolism assay may provide an efficient tool for metabolite identification. It is well known that metabolite spectra can differ strongly among fish species, and this can have major implications for chemical toxicity. Thus, we examined the suitability of the in- vitro assay with fish hepatocytes to provide information on species-specific metabolite patterns. The results clearly demonstrated that this is the case: differences of metabolite spectra existed between isolated trout and carp hepatocytes, and, importantly, the in-vitro differences well reflected the in-vivo differences between the two species.

Conclusions and Relevance for 3R

The project provided two deliverables. First, it highlighted that interspecies differences of xenobiotic metabolic rates as they exist in-vivo, can be predicted from the respective in-vitro systems. Interspecies scaling of in-vitro metabolism data has been found to be a critical issue in human toxicology, and this question for fish must be addressed in fish as well. Here, the results of the present project represent an important step forward as they point to the transferability of the in vitro assay across species. This information is critical with respect to the acceptance of the in vitro hepatocyte assay for reduction or replacement of the in-vivo bioaccumulation test with fish.
Second, the project demonstrated the utility of the in vitro hepatocyte assay to inform on species differences in the metabolite pattern. This information is of relevance for biocide regulations and could open an avenue to introduce in vitro approaches also in that part of chemical regulations.

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3R-Project 142-14

Validation of human stem-cell pluripotency using a bioreactor-based culturing system instead of a murine model to effect the development of embryoid bodies into teratomas

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Keywords: *embryoid body; stem cells; teratoma*; *cell cultures: bioreactor; immunodeficient mice*; *replacement*

Duration: 2 years **Project Completion:** 2016

Background and Aim
Stem cells play a key role in clinical as well as in experimental research. Pluripotent stem cells possess the capacity to elaborate each of the three germ layers (endoderm, mesoderm and ectoderm). However in contrast to totipotent cells, they are unable to “build” an entire organism. Induced pluripotent stem cells (IPS) have been generated by a reprogramming of somatic cells. Before these can be used experimentally, their stem-cell properties (“stemness”) must be validated by confirming their capacity to differentiate into cells of the three germ layers. To date, the gold standard for so doing has been an *in-vivo* assay, in which stem cells are engrafted into immunodeficient mice for the development of teratomas containing components of all three germ layers. The teratomas thus formed are routinely analyzed immunohistochemically for the various derivatives of the three germ layers. The site of implantation, the strain of mouse, and the post-grafting growth period, are amongst the variables that influence the formation of teratomas. To avoid the use of living mice, we wish to establish an *in-vitro* system to mimic the *in-vivo* formation of teratomas. With this end in view, we are using a commercially-available bioreactor (Fig. 1) for the three-dimensional culturing of collagen-embedded human embryoid bodies (EBs).

Figure 1: Bioreactor system

Method and Results
EBs that are derived from two different human embryonic stem-cell lines and one IPS-line are embedded within a collagenous scaffold (Ultrafoam®). These constructs are then cultured for up to five weeks in either a commercially-available bioreactor (Cellec Biotek AG, Basel), in which the culture medium circulates, or statically in multiwell plates. As

a positive control, EBs are implanted subcutaneously in immunodeficient mice for 8 weeks. The tissues from each of the experimental groups are harvested and the vitality of the cells is assessed. The capacity of the cells to differentiate into the three germ layers is evaluated immunohistochemically and by the polymerase-chain reaction, using selected markers for each tissue type. In addition, the supernatants of the cultures are collected upon each change of medium for an analysis of metabolic activity.

To date, we have already established that, after a three-week period of culturing in the bioreactor, each of the two stem-cell lines developed into teratoma-like tissue-masses, as evidenced immunohistochemically by the expression of selected markers for the three germ layers. The collagen-embedded EBs hat were cultured statically in multiwell plates did not develop into teratoma-like structures, and immunohistochemistry for markers of the three germ layers revealed no evidence of their expression. We have also established the growth time for the differentiation of EBs into teratomas to be more rapid in the bioreactor-based culturing system than in living mice (three to five weeks instead of eight weeks).

For the bioreactor-based culturing system to serve as a viable alternative to the murine model, two additional factors must be investigated and satisfied:

1. An estimation of the cost-benefit ratio between the bioreactor-based *in-vitro*- and the murine-model-based *in-vivo* systems.
2. Comparison of gene expression characteristics of the teratoma formed in the bioreactor with those of teratoma grown conventionally in immunodeficient mice.

Conclusions and Relevance for 3R

The use of stem cells in experimental and clinical research is increasing explosively, and for this reason, a proof of their pluripotency is essential. To date, experiments with living mice are indispensable for this purpose. We wish to replace the conventional *in-vivo* approach with a bioreactor-based culturing system, which is easier to handle and more controllable. The *in-vitro* approach will be further refined and validated with a view to establishing a surrogate for the *in-vivo* teratoma-formation test that is currently implemented to evaluate the pluripotency of novel stem-cell lines.

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3R-Project 143-15

An advanced *in-vitro* model of pulmonary inflammation based on a novel lung-on-chip technology

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Keywords: *lung*; *lung inflammation; mechanical stress; sepsis*; *chip*; *reduction; replacement*

Duration: 1 year **Project Completion:** 2016

Background and Aim
Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are important causes of morbidity and mortality in critically-ill patients. These life-threatening diseases are characterized by an accumulation of fluid in the alveolar sacs that ultimately prevents the diffusion of oxygen into the lung microvasculature. As an *in-vivo* model, lipopolysaccharide (LPS)-induced ALI is frequently used to study the molecular mechanisms that underlie, and potential therapies for inflammation-associated lung injury. However, since the experimental protocols are not standardized, inter-study comparisons are difficult. In addition, septic mice often suffer a high degree of distress during the experimental procedures. The development of an *in-vitro* system to accurately simulate the pathophysiological environment would, in serving as an alternative to the LPS-induced ALI-model *in-vivo*, help to cut down on the number of experiments with living animals.

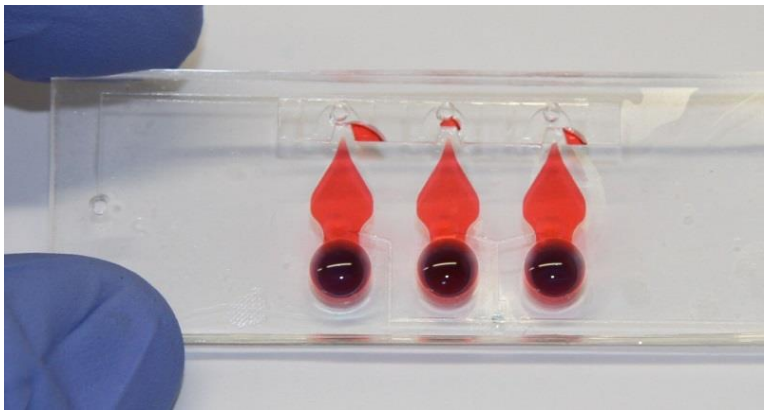

Figure 1: Lung-on-chip with three alveolar barriers. The thin, porous and flexible membrane can be cyclically stretched to mimic respiratory movements.

Method and Results
This project aims to establish an *in-vitro* model of pulmonary inflammation as an alternative to currently used *in-vivo* models of ALI. The *in-vitro* model is based on a recently developed lung-on-chip technology (Fig.1), which closely mimics the thin air-blood barrier as well as the cyclic mechanical stress that is imposed upon pulmonary tissue by respiration, and thus reproduces the lung parenchyma environment in an unprecedented way. On the chip, co-cultured primary murine lung epithelial and endothelial cells will be exposed to LPS, a potent microbial pro-inflammatory factor, which is known to induce sepsis and ALI. Key parameters of inflammation will be assessed in the *in-vitro* lung-on-chip model and the data directly compared to the responses that are elicited in the murine system.

Conclusions and Relevance for 3R
Apart from its major potential to *replace* animal studies, which are associated with severe distress, we expect that this novel technology will also permit a *reduction* in the overall number of studies with living mice. In particular, since a single chip contains several model alveoli, we anticipate that the data generated using each of these will substitute those that are gleaned from several individual research animals. Moreover, given the dimensions of the chip, sufficient primary lung

endothelial and epithelial cells to seed several chips can be harvested from a limited number of donor animals, thereby promoting further reductions in mice usage.

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3R-Project 144-15

Development of *in-vitro* three-dimensional multi-cellular culture models to study the role of heterotypic cellular interactions in colorectal cancer invasion

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Keywords: *colorectal cancer; tumour microenvironment*; *heterotypic interactions*; *reduction; replacement*

Duration: 2 years **Project Completion:** 2017

Background and Aim
Colorectal cancer (CRC) is the third most frequent cause of mortality due to cancer in Europe. Local invasion and distant metastasis are the critical steps that negatively impact patient survival. Although cells of the tumour microenvironment (TME) are known to promote invasion and metastasis, the mechanisms underlying these processes are only partially understood. An understanding of how the cells from the TME promote invasion in CRC is crucial for the development of novel anti-invasive therapies.
The study of heterotypic cellular interactions *in vivo* is difficult, owing to constraints in accessing the tissue and in modulating specific cells or intercellular interactions. *In-vitro* models mimicking the TME would be invaluable in studying the role of individual cells of the TME and their interactions with CRC-cells. We have developed an *in vitro* three-dimensional (3D) model to study the interactions between fibroblasts and CRC-cells. This model is now well suited to study the interactions between cancer cells and others, such as endothelial and immune/inflammatory cells, from the TME. In this project, we aim to improve this 3D *in vitro* culture model by introducing additional cell types of the TME, and by studying their interactions, with a view to improve our understanding of their role in promoting cancer cells invasion as the first step in the metastatic process. Finally, we will validate the *in-vivo* observations, in order to set on a firm footing a robust alternative to study the heterotypic cellular interactions in the TME.

Method and Results
We have already developed a 3D *in vitro* co-culture model consisting of fibroblasts and CRC-cells. To improve this model, endothelial cells (Ea.hy296) will be embedded together with or without human fibroblasts in a 3D-Matrigel-matrix to generate blood-vessel-like structures. Several CRC-cell lines with different degrees of invasiveness, such as SW620, HCT116 and HT29, will be included in the matrix in the form of spheroids to better mimic a tumorous nodule. All cells will be fluo-labelled with mCherry, Azurite or GFP to track them specifically. Co-cultures will be monitored for up to two weeks using time-lapse fluorescence microscopy, with a view to ascertain how the TME-cells induce CRC-cells invasion and progression. 3D cultures will then be treated with cytokines, growth factors, blocking antibodies or pharmacological inhibitors of signalling to determine the key elements that are implicated in CRC-cells invasion. Monocytes will then be added to complete the model and to study their role in this context.
To recommend the use of these heterotypic models as an *in vitro* surrogate of the TME *in vivo*, it is essential to demonstrate that the *in vitro* effects of several stromal cells on tumoral ones can be reproduced *in vivo*. To this end, colon-cancer cells will be injected subcutaneously or orthotopically into the caecum of immune compromised mice in the absence or presence of fibroblasts or endothelial cells. Progression of the tumour will be monitored on the basis of the luciferase activity of the CRC-cells. Preliminary results are really promising and show that our 3D *in vitro* model correctly mimic the *in vivo* interaction between cells of the tumour microenvironment and cancer cells.

Conclusions and Relevance for 3R
Tumour-host interaction is now emerging to be a critical event in tumoral growth and development. However, a study of the TME is a complex undertaking, since many cells and factors act simultaneously. A differential evaluation of the underlying mechanisms *in vivo* is thereby rendered extremely difficult.
The development of our *in vitro* assay recreates a simplified and well-controlled TME. This model could promote progression in this field, reduce costs, and minimize animal use. An improvement in our understanding of how tumoral and host cells interact afford opportunities for elucidating the molecules and mechanisms that are implicated in the invasive process underlying metastasis.

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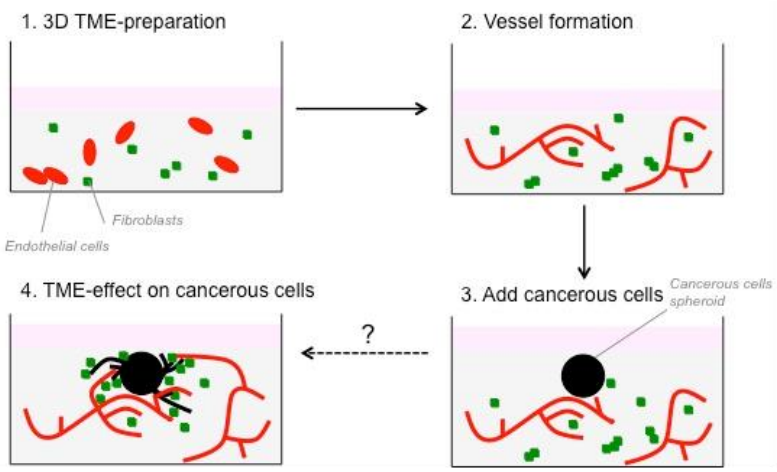

Figure 1: Representation of the 3D-model strategy

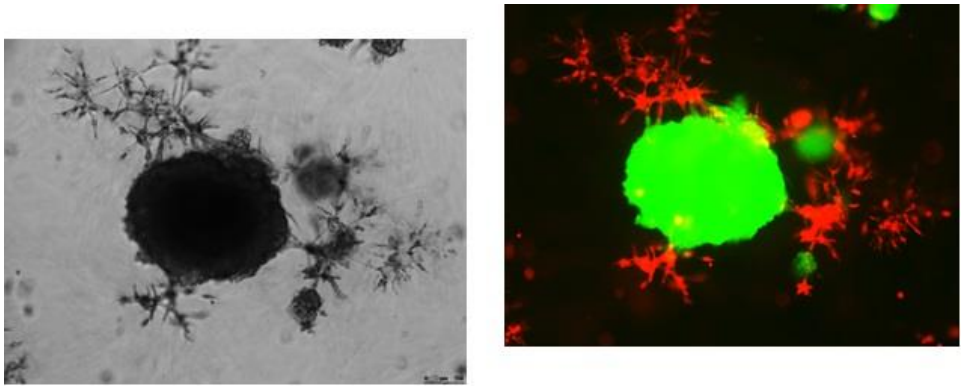

Figure 2: Brightfield and fluorescent images of 7-day-old 3D co-culture assay with GFP-expressing SW620 cancer cells and mCherry-expressing Fibroblasts. Magnification: x10.

3R-Project 145-15

Combining computational modelling with *in-vitro* cellular responses in order to predict chemical impact on fish growth

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Keywords: *fish*; *fish cell line*; *chemical risk assessment*; *toxicokinetic and toxicodynamic modelling*; *replacement*

Duration: 2 years **Project Completion:** 2017

Background and Aim

We propose to develop an animal-free alternative to experiments with living fish to quantify the impact of chemicals on their early growth. Specifically, this project is based on the hypothesis that it should be possible to correlate the *in-vitro* cellular responses to various concentrations of these chemicals with their effects on the growth of different fish species over time. In particular, we postulate that by combining mathematical modelling with *in-vitro* cellular responses, it should be possible to assess the retardation of growth in juvenile fish without having recourse to living animals. We base our method on the assumption that the same chemical concentration in the internal organs of fish and in cultured cells would have the same impact on cell survival and cell population growth. In a former proof-of-principle investigation, cyproconazole and propiconazole were used as the reference chemicals, and the rainbow-trout gill cell-line, RTgill-W1, as a relevant *in-vitro* model (Figure 1; Stadnicka-Michalak *et al*., 2015). We now wish to further strengthen our approach by testing a wider variety of chemicals with the following specific aims:

Prediction of the internal concentrations in fish to determine those that are needed for the *in-vitro* experiments.

Measurement and prediction of the chemical toxicity in the *in-vitro* experiments using the RTgill-W1 cell-line.

Scaling up of the *in-vitro* cellular responses to the chemical impact on fish.

Extrapolation of the prediction to different fish species.

Validation of the approach on the basis of independent measurements (no additional experiments with fish are needed).

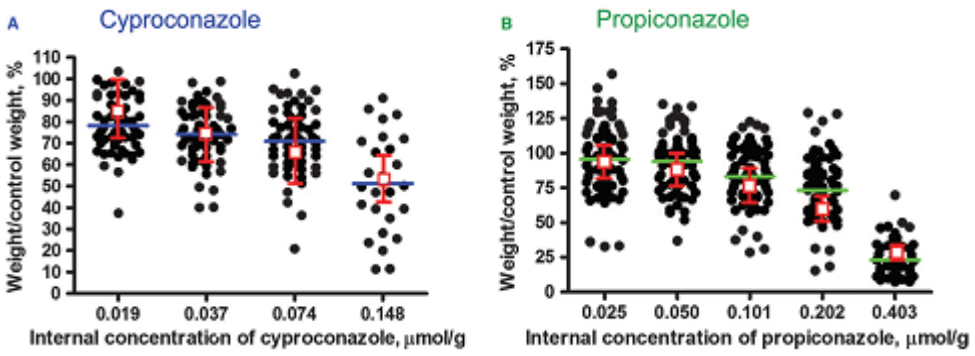

Figure 1: Proof-of-principle data revealing that a combination of RTgill-W1-cell-line responses and mathematical modelling can be used to predict reductions in fish growth. The black dots represent the measured reduction in fish weight, with each symbol representing one fish ("—" mean). The red squares represent the predictions that are based upon *in-vitro* cell-population growth data (including model uncertainties). For further details, see Stadnicka-Michalak *et al*., 2015.

Method and Results

The project will be carried out in four main steps. No animals will be needed in any of the performed experiments. The chemicals to be used in this project will be selected on the basis of their mechanisms of action on fish growth and their properties (e.g. volatility and hydrophobicity). Raw experimental fish-growth data will be gleaned from literature searches or obtained directly from companies/regulators).

STEP I: Prediction of the internal concentrations of the chemicals in fish using the Physiologically-Based Toxicokinetic (PBTK) model (Stadnicka *et al*., 2012): The PBTK-model will be applied to predict the chemical concentrations in various fish organs.

STEP II: Correlation of the chemical concentrations in fish with those in the *in-vitro* experiments: *In-vitro* experiments will be designed such that the concentrations in the cells should be the same as those in the tissues that are implicated in fish. The concentrations that are derived for various organs using the PBTK-model are taken as proxy for those that are needed in the cells in the *in-vitro* toxicity experiments (reverse toxicokinetics).

STEP III: Measurement and prediction of the chemical toxicity to fish cells: Fish growth depends on many factors, amongst which cell survival and proliferation play a dominant role. Thus, in this step, we will focus on measuring cell survival and proliferation after exposure to chemicals at the concentrations that will be established in STEP II.

STEP IV: Scaling up of the *in-vitro* cellular responses to the level of the whole organism: The cellular end-points ─ measured in STEP III ─ determine cell-population growth, which will be computationally correlated with the chemical impact on fish growth. We assume the total mass of all fish cells to be equivalent to fish weight. Hence the impact of cell-population growth on fish growth can be described by the von Bertalanffy growth model.

Conclusions and Relevance for 3R

Hundreds of thousands of juvenile fish are used annually to assess the influence of chemicals on their growth. To reduce this number, we propose a method which, for the first time, can quantitatively predict the impact of chemicals on fish growth from *in-vitro* data. This promising step towards alternatives to toxicity testing in fish is simple, cheap and rapid, and requires the use only of *in-vitro* data for model calibrations.

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3R-Project 146-15

Validation of a novel cell-based approach to study thyroidal physiology: Reduction and/or replacement of experiments with rodents

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Keywords: *braf; follicle; goiter; stem cells; thyroxin*; *replacement*

Duration: 3 years **Project Completion:** 2019

Background and Aim

Hormones that are secreted by the thyroidal gland are necessary for embryonic development and for a proper functioning of the adult organism. Since the organ in mice is exceedingly small, weighing no more than 2mg, research on the thyroid has been traditionally conducted on rats, with the exception of genetic manipulations. However, in recent years, knockout (KO) murine models have been implemented and, owing to the size limitation, in necessarily large numbers per experimental group (*n*=8-12). Unfortunately, conventional (two-dimensional) thyrocytic lines of murine, rat or human origin are not representative models of the *in-vivo* situation, the reason thereof being that the specific functions of the organ depend upon the formation of anatomic units, namely, follicles, which are thyrocyte-lined spherical structures. Recently, protocols have been published that permit the generation of mature thyroidal follicles from murine and human embryonic stem (ES) cells (1-3). These protocols rely on the engineering of ES-cell lines that express transgenes encoding the transcription factors NKX2.1 and PAX8, which are central to thyrocytic specification (4). For a complete differentiation of the ES-cells into functional thyrocytes and the formation of follicles, an induction of NKX2.1- and PAX8-expression, followed by exposure to specific differentiation-promoting factors [such as activin and the thyroid-stimulating hormone (TSH)] and culturing in a three-dimensional (3D)-matrix, are requisite. The functionality of the resulting follicles is attested by their ability to capture and organify iodine for the synthesis of thyroidal hormones (1, 2). Furthermore, after transplantation into mice lacking a thyroid, such follicles can form functional thyroidal tissue *in vivo* and rescue the animals from otherwise lethal hypothyroidism (1).

Thus far, these protocols have been used to model the specification and differentiation of thyroidal cells, and to validate the importance of specific genetic and humoral factors in this process. However, the utility of the new cellular system as an alternative to animal models for the study of thyroidal physiology has not yet been put to the test. As a proof-of-concept, we will investigate whether the functional thyroidal follicles that are derived from murine ES (mES)-cells can be used to simulate the effects that are observed in murine KO or knock-in (KI) models of genes that are central to specific relevant pathways in thyroidal physiology, growth and/or oncogenesis, such as the Keap1/Nrf2 antioxidant-response pathway (5) and the BRAF-branch of the MAPK-pathway. The main goals are: (i) to ascertain whether a pharmacologically-mediated up- or down-regulation of a pathway of interest in thyroidal follicles that are differentiated from wild-type (for the specific pathway) endodermally-matured ES-cells can recapitulate the effects that are observed in the corresponding murine KO-models (genetic up- or down-regulation of the same pathway); and (ii) to ascertain whether thyroidal follicles that are differentiated from endodermally-matured ES-cells that have been genetically engineered before or after differentiation to up- or down-regulate a pathway of interest can recapitulate the observed effects in the corresponding murine KO or transgenic models of the same pathway (cellular KO/transgenic versus animal KO/transgenic).

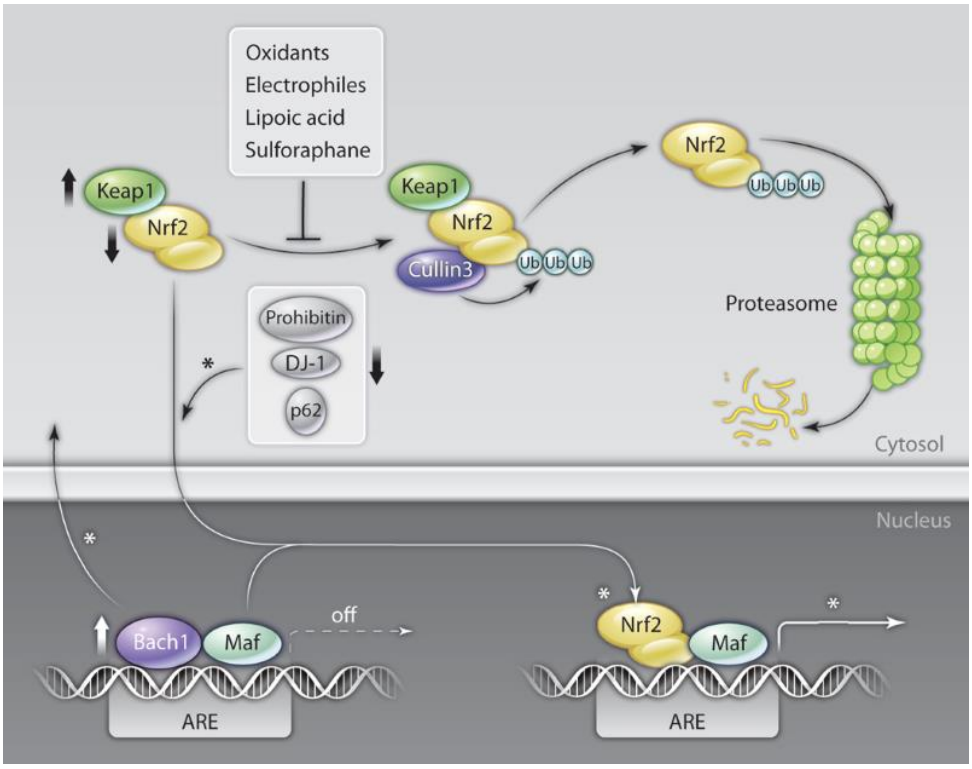

Figure 1: Scheme of the Keap1/Nrf2 antioxidant-response pathway. Under basal conditions, Nrf2 is targeted by Keap1 in the cytoplasm for Cullin3-mediated ubiquitination (Ub) and proteasomal degradation. Oxidative stress or specific compounds block the Keap1-mediated inhibition of Nrf2, thereby promoting an accumulation of the transcription factor in the nucleus and an induction of the transcription of its target genes by binding to antioxidant-response elements in their regulatory sequences.

Method and Results

Aim 1. *Hypothesis: Thyroid-specific murine embryonic stem cells (ts-mES) can recapitulate the thyroid-specific phenotypes that are observed in the corresponding murine KO-models under basal and stressful conditions.*We will combine the recently published 3D-follicle protocols with CRISPR/Cas9 gene-targeting or with lentivirus-mediated shRNA-expression to generate 3D-cultures of differentiated follicular cells with functional gains or losses in the Keap1/Nrf2 antioxidant-response system. We will characterize these follicles under basal conditions as well as under stressful ones (iodine treatment) and/or after a pharmacological manipulation of Nrf2-signalling (using the Nrf2-activator, CDDO, and the Nrf2-inhibitor, brusatol). Assayed parameters will include: (i) proper follicular organization; (ii) thyroid-specific expression of antioxidants at the gene and protein levels; and (iii) functional properties regarding iodide trapping, iodide organification and thyroid-hormone synthesis.These experiments will permit us to directly compare and contrast the molecular and biochemical phenotypes of mES-derived thyroidal follicles with those of thyroid-specific Nrf2- or Keap1-KO-mice (using PAX8-Cre-driven recombination), which we are creating and analyzing in parallel as part of an SNF-funded project. We expect to be able to model *in vitro*, either partially or fully, the *in-vivo* murine phenotypes.

Aim 2. *Hypothesis: Thyroid-specific murine embryonic stem cells (ts-mES) can model experimental goitrogenesis and can recapitulate the thyroid-specific phenotypes that are observed in the corresponding murine models.* To maximize the utility of the *in-vitro* model from a 3R-perspective, we will attempt to establish an *in-vitro* model of goitrogenesis using ts-mES-cells, and will compare the data thereby generated with the results of experimental goitrogenesis in our KO-models and in wild-type mice. To this end, ts-mES-cells will be induced to form thyroidal follicles in 3D-cultures and then subjected to different goitrogenic experimental conditions (low-iodine media, treatment with high doses of recombinant TSH, and/or anti-thyroid drugs in various combinations). The impact of genetic manipulation of the Keap1/Nrf2-pathway will also be assessed under these conditions.

Aim 3. *Hypothesis: Thyroid-specific murine embryonic stem cells (ts-mES) that constitutively or conditionally express BRAF-V600E can model BRAF-V600E-mediated thyroidal oncogenesis and can recapitulate the phenotypes that are observed in the corresponding murine models.* A papillary carcinoma is the most common form of thyroidal malignancy. In ~50% of cases, the tumours harbour activating mutations in *BRAF*, a serine/threonine kinase that transduces

regulatory signals via the MAPK-cascade. The BRAF-V600E-mutation is the most common genetic aberration (>90%). We will explore whether the functional thyroidal follicles that are obtained from mES-cells that have been genetically engineered to conditionally express BRAF-V600E using a tetracycline-inducible system can reproduce most of the effects that are observed in the corresponding murine models *in vivo*. Specific measures will include: (i) BRAF-activity; ii) escape from proliferative arrest; (iii) morphological and architectural changes in the thyroidal follicles and the lumenal compartment; (iv) loss of cellular polarity; (v) signs of thyroidal dedifferentiation; (vi) changes that are consistent with an epithelial-to-mesenchymal transition; and (vii) genomic instability.

Conclusions and Relevance for 3R

We will characterize various molecular and morphological parameters in thyroidal follicles that have been derived from mES-cells under normal conditions and under the influence of different pharmacologically- and genetically-induced perturbations that are relevant to thyroidal physiology and pathologies (iodine deficiency or excess, goitrogenesis, oncogenesis). We will then compare the profiles of the mES-derived thyroidal follicles with data that have been gleaned from mice of the corresponding genotypes in the context of our ongoing SNF-funded project (31003A-153062-1) as well as with literature findings. These comparisons will permit us to directly validate (or refute) the utility of the new *in-vitro* model as a proxy, a screening assay and/or a complete replacement method for *in-vivo* studies on thyroidal physiology using KO-mice. If validated as an alternative method, the use of ES-cell-derived follicles will greatly reduce and largely replace experiments with wild-type and KO-mice in studies appertaining to thyroidal physiology. To promote the goals of 3R, all materials that are implemented and all knowledge that is gained will be made freely available to other investigators.

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