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ALTEX

ALTERNATIVES TO ANIMAL EXPERIMENTATION

Spektrum
AKADEMISCHER VERLAG

Presenting a special issue of ALTEX

This special issue of *ALTEX* is publicising what the 3R Research Foundation has achieved in the past and what projects are currently underway. This special issue is intended to improve awareness of the projects funded by the Foundation and to provide a training tool.

The first section includes updated descriptions of 20 projects from the past ten years. These projects, involving basic research, the development of new medicines and safety testing, were continued by the project heads after funding from the Foundation ceased. This sustainability can be seen as a great success of the Foundation's activities. The second part of the special issue concentrates on the 17 current projects, illustrating how 3R-relevant projects are being developed every day both in Switzerland and abroad. The contributions provide specialised knowledge that has already been acquired and that can and should be adopted and developed further.

Thanks to this special issue of *ALTEX*, the projects will be accessible in the international database, which means that an increasing number of people will become aware of them. Furthermore this issue will be used in compulsory training courses for animal experimentalists. The fact that the information exceeds the limits of one single research area may well give many young scientists new ideas relating to the 3R principle.

The present special issue provides a snapshot of 20 years of research efforts. The projects that are completed in the future will continue to be listed in the 3R-Info Bulletins, which are published three times a year. Current activities can be accessed on the Foundation's website (www.forschung3r.ch). This internet site is the Foundation's most important tool for documenting and publicising research results.

Peter Maier
Prof. Dr. sc. nat. ETH
Scientific Adviser
Chairman of the Evaluation Committee
3R Research Foundation

The 3R Research Foundation Switzerland

The 3R Research Foundation was set up twenty years ago by the Parliamentary Group for Animal Experimentation Questions. The aim of this group was to bring together the federal authorities, people concerned about animal protection and the pharmaceutical industry to discuss how animal experimentation could be reduced, refined and replaced. The fate of laboratory animals was to be improved without affecting research within the Swiss pharmaceutical industry.

The 3R concept developed by the British scientists Russell and Burch in 1959 was the obvious tool to achieve this aim. Russell and Burch were of the opinion that first-class scientific work inevitably went hand in hand with humane treatment of laboratory animals. Badly planned experiments and incomplete statistics were bound to result in unnecessary suffering on the part of laboratory animals and an unnecessarily high number of animals being sacrificed. The British scientists described their 3R concept – reduction, refinement and replacement of laboratory animals in the life sciences – *in The Principles of Humane Experimental Technique*.

Research into the development of alternative methods is directly connected to the aim of the research. The development of the actual method is rarely the subject of a published article and requires extra backing. For this reason it was essential that the federal authorities and the pharmaceutical industry provide research funds that could be awarded to projects which complied with the 3R principle through the 3R Research Foundation. The Foundation has strict requirements for such projects. They have to meet scientific criteria and be clearly relevant to the 3R principle! Each application is evaluated by a group of experts which includes specialists from the pharmaceutical industry and research as well as people concerned about animal protection.

For the past 20 years the collaboration between the pharmaceutical industry and the federal authorities on the funding side, as well as between specialists from industry, academia and animal protection, has proven its worth and led to results that are relevant to the 3R principle.

Dr. Hugo Wick
President of the 3R Research Foundation

PROJECTS IN PROGRESS

Skin Sensitization: Understanding the *In Vivo* Situation for the Development of Reliable *In Vitro* Test Approaches

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Summary
Due to increasing public concern and the adoption of the 7th Amendment to the Cosmetics Directive, the development of in vitro models for predicting the sensitizing potential of chemicals is receiving widespread interest. This overview describes some of our current research projects exploiting known molecular and cellular events occurring during the acquisition of skin sensitization. Once combined in a test battery, these different in vitro approaches are expected to provide reliable methods for the detection of contact allergens.

Keywords: cosmetics, contact allergens, skin sensitizing potential,

Background Information

Allergic contact dermatitis

Allergic contact dermatitis (ACD) is a delayed-type hypersensitivity reaction induced by small reactive chemicals (haptens). Currently, the sensitizing potential of chemicals is usually identified on the basis of animal studies, such as the local lymph node assay (LLNA). There is, however, an increasing public concern regarding the use of animal testing for the screening of new chemicals and further to the adoption of the 7th Amendment to the Cosmetics Directive in Europe, animal testing of ingredients used in cosmetics will be subjected to severe restrictions by 2009 and tightened by 2013. The development of in vitro models for predicting the sensitizing potential of new chemicals is therefore receiving widespread interest.

In vitro sensitization tests need to resume the complex interactions of a chemical with the different compartments of the immune system (Fig. 1): The chemical must penetrate the skin and react with endogenous proteins. Some chemicals, termed prohaptens, require activation through skin metabolism in order to become haptens capable of binding to skin proteins. Haptenated self-proteins are internalized and processed by immature dendritic cells (DC) that become activated. The activated DCs start to migrate from the epidermis into the draining lymph node, complete maturation and present fragments of the haptenated self-proteins to T-helper cells, resulting in an antigen-specific immune response.

Since the in vitro method for skin penetration is already accepted (OECD guideline 428), our current line of research concentrates on two major aspects of the skin sensitization process amenable to in vitro approaches: protein binding and DC activation. We are thus developing assays for the measurement of the protein binding properties of chemicals and further refining cell based assays for the evaluation of the DC activation potential of chemicals. These two lines of research should provide novel in vitro test systems and endpoints for the development of an in vitro test battery for assessing the sensitization potential of chemicals.

Assays for detecting protein reactive chemicals

A prerequisite for the occurrence of both antibody- and cell-mediated allergic reactions against chemicals is the activation of chemical-specific T lymphocytes. The vast majority of chemical-specific T cells do not recognize the chemical itself but a conjugate of the chemical with a protein fragment (a peptide) presented in class I or class II MHC molecules on the surface of antigen-presenting cells, such as Langerhans' cells. The chemicals can act as an hapten (i.e. it binds covalently to the side chains of amino acids) or as a pro-hapten (which is metabolically or chemically converted to protein-reactive species).

Procter & Gamble, in collaboration with COLIPA (The European Cosmetic Toiletry and Perfumery Association) has therefore initiated research projects to determine the correlation between sensitization potential and chemical reactivity. Chemicals representing allergens and non-allergens have been evaluated in a peptide reactivity assay using two synthetic peptides containing either a single cysteine or lysine as reaction target. After reaction with the test chemical, the samples are analyzed by HPLC to monitor the depletion of unreacted pep-

http://3r-training.tierversuch.ch/en/module_3r/sensitization_in-vitro/acd

tides. The data showed that by using a prediction model based on a classification tree approach, peptide reactivity measurement demonstrates a good association between chemical reactivity and allergenic potency (Gerberick et al., 2007). Generally, non-allergens and weak allergens demonstrated minimal to low peptide reactivity, whereas moderate to extremely potent allergens displayed moderate to high peptide reactivity.

As a complementary approach (with the support of COLIPA), we are also developing and evaluating an immunological detection of the cysteine- or lysine-side-chain modification using specific monoclonal antibodies in an ELISA-like format. If successful this immunological approach may provide an alternative and user-friendly system for the detection of protein reactive chemicals. Altogether, these approaches, integrated in an *in vitro* test battery for skin sensitization, should provide a rapid, simple and cost effective screening method for new chemicals.

Analysis of the *in vitro* activation of dendritic-like cells (DC)

The next biological step in the sensitization process is the internalization and processing of the haptenated self-proteins by immature DCs. During this process DCs mature to an activated state and up-regulate the expression of a set of cell surface markers (e.g. CD83 or CD86), secrete various cytokines such as IL-1ß and down-regulate proteins involved in antigen uptake such as aquaporins. DCs, whose central role during the induction process of skin sensitization is well documented, were perceived as an obvious opportunity for developing *in vitro* approaches for detecting potential sensitizers. Recent advances in the *in vitro* generation of immature DCs and the availability of various cell lines with DC-like phenotypes have led to the development of many *in vitro* protocols for measuring the activation of DC-like cells upon exposure to chemicals.

We have developed and published (Aeby et al., 2004) an *in vitro* test protocol based on human peripheral blood monocytes derived DCs that are exposed for 3 to 30 hours to the test chemicals. DC maturation is evaluated by flow cytometric measurement of the percentage of CD86 positive cells and quantitative measurement of the mRNA expression of interleukin-1ß, interleukin-8 and aquaporin P3 using real time PCR.

This new approach has been used successfully to analyze the sensitizing properties of many chemicals and we are using it for a detailed analysis of the sensitizing properties of p-phenylenediamine (PPD) through its oxidation products and possible inhibition by acetylation in the skin (publication in preparation). We conclude that the described *in vitro* test system allows a refined analysis of the sensitizing properties of chemicals and will further improve product safety.

To identify further DC genes that are modulated by exposure to allergens, the effect of exposure to a contact allergen was also examined at the transcriptional level using Affymetrix GeneChip®. This analysis revealed 173 genes that are significantly modulated (Gildea et al., 2006). It is hoped that some of the identified transcript changes will be suitable for further improving our *in vitro* DC activation assays.

DC activation test using the U937 myeloid cell line

Major drawbacks of PBMDCs are their complex and expensive preparation procedures and their inherent donor-to-donor variability. As a possible alternative, human myeloid leukemia cell

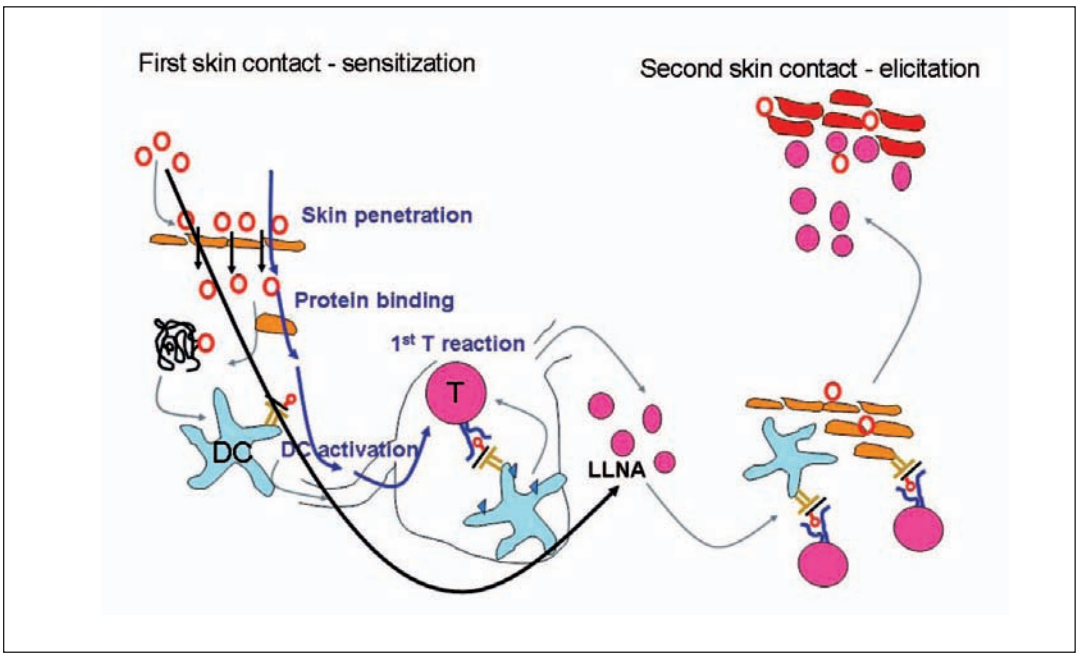

Fig. 1: Interactions of a chemical with the different compartments of the immune system.

lines represent good candidates as DC surrogate. Therefore, in collaboration with COLIPA we have developed and evaluated an *in vitro* test system using the human myeloid cell line U937 for the detection of contact allergens (Python et al., 2007). Briefly, cells are seeded in 12-well plates and treated with the test chemicals for 24 h, 48 h and 72 h. The cells are analyzed by flow cytometry for CD86 surface expression and cell viability. In parallel, IL-1ß and IL-8 gene expressions are measured by quantitative real-time RT-PCR. The biological response for each tested chemical is evaluated by considering modulation of the three selected activation markers (CD86, IL-1ß and IL-8) at each time period. Our results suggest that a chemical inducing a significant up regulation of the expression of at least two markers might be considered sensitizing. In that first phase, the described test system (U937 activation test) was able to correctly classify 15 out of 16 tested chemicals.

Collaboration with external partners

Due to increasing public and political concerns regarding the use of animal tests for the screening of new chemicals, international co-operations (academic and industrial) are being setup in order to promote and speed up the development of *in vitro* test systems for toxicological endpoints. In our effort to develop an *in vitro* sensitization test, we are closely collaborating with:

A) The European Cosmetic Toiletry and Perfumery Association (COLIPA)

The COLIPA Skin Tolerance Project Team is involved in a range of research projects exploiting our current understanding of the molecular and cellular events occurring during the acquisition of skin sensitization.

Research projects reflecting many aspects of the complex interactions of a chemical with the different compartments of the immune system are being supported: These approaches range from aspects of chemistry/peptide binding/skin metabolism, through evaluation of intracellular signaling pathways induced by allergens, to allergen induced changes in dendritic/Langerhans cells measured at genomic and protein level. Knowledge gained from this research will be used to support the development and pre-validation of novel *in vitro* approaches for the identification and characterization of skin sensitizing chemicals. (Aeby et al., 2006)

B) European Union Framework Programme 6 “Sens-it-iv”

We are also involved in a large research consortium entitled “Novel Testing Strategies for *In Vitro* Assessment of Allergens” (Acronym: Sens-it-iv) sponsored by the European Union Framework Program 6. This project has 28 participating laboratories and its overall goal is the development of *in vitro* alternatives to animal tests for the risk assessment of potential skin and respiratory sensitizers. We, as a Sens-it-iv contractor, are participating in the identification and evaluation of relevant sensitization markers using PBMDCs and the human cell lines MUTZ-3, THP-1 and U937. Our work package involves exposing these cells to known sensitizers under defined culture conditions and total RNA purification for genomic profiling at the Microarray Resource Centre (MARC) of Lund University (part of the Sens-it-iv consortium), using Affimetrix gene arrays. The research is currently progressing and results obtained from the different cellular sources will be compared.

Once validated and combined in a test battery, these different *in vitro* approaches are expected to provide reliable and biologically relevant methods for the detection of contact allergens and will significantly reduce our reliance on animal tests.

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Improvement of Pain Therapy in Laboratory Mice

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Summary
Pain of minor grades is difficult to detect in laboratory mice, because mice hide signs of injury and suffering. Low-grade post-operative pain was detected by continuous measurements of heart beat rate and variability, as well as nesting behaviours. Pain relief regimens with non-steroidal anti inflammatory drugs were shown to be effective.

Keywords: mouse, ethology, refinement, analgesia, telemetry, pain

Background Information

Pain and animal experimentation

In 1985, the U.S. Interagency Research Animal Committee (IRAC) advised that "unless the contrary is established, investigators should consider that procedures that cause pain or distress in human beings may cause pain or distress in animals" (www.aclam.org/pub_pain_distress.html). Accordingly, the humane use of research animals requires adequate veterinary medical care during animal experimentations and includes the prevention or the alleviation of pain associated with procedural or surgical protocols (clinical pain). Guidelines on the recognition of pain are accepted by researchers but often not so easy to put into practice, especially with mice, the most frequently used laboratory animals. Why?

Mice live in constant fear of falling prey to their enemies, therefore they are prone to show as few signs of disease, suffering or weakness as possible (Stasiak et al., 2003; Peterson, 2004, Van Sluyters and Obernier, 2004). Accordingly, during animal experiments, or even when a person is simply present in the room, the mouse will hide almost all signs of light or middle-grade pain. Additionally, endogenous stress-induced analgesic systems that are well developed in mice affects their responses to pain and therefore complicate the recognition of the actual degree of pain (Hohmann et al., 2005).

Decreased food intake or loss of body weight are retrospective indicators of pain or stress and therefore not suitable per se. In consequence, there are no reliable indicators to detect low and middle-grade clinical pain in the mouse. The appearance of clinical signs (e.g. changes in posture, fur or eyes) often represents severe pain appearing sometimes at a nearly moribund state. Severe pain is unforeseen, affects the outcome of a study and terminated by euthanasia. Another consequence of the dilemma is that even a successful analgesic treatment cannot be monitored well and uncertainty remains about the efficacy of many analgesic regimens. Accordingly, more research is necessary to improve the recognition (Flecknell et al., 2005) and justification of pain in laboratory mice.

Pain assessment – different types of pain

Pain assessment in laboratory mice includes the determination of symptoms and indicators of pain and correlating them to the type of pain (e.g. acute vs. chronic pain). Contemporary ways for measuring acute pain are well described for reactions (e.g. withdrawal time, pressure range) upon defined, short, stimuli in analgesiometric tests (e.g. tail flick, hot plate). These tests are frequently used for substances and/or analgesics dosage testing, because the read-out is quantified according to defined endpoints.

Acute clinical pain is a different situation which occurs e.g. after surgical interventions carried out in many biomedical animal experimentations, after artificially induced inflammatory reactions (e.g. antibody production, arthritis) or experimental infections for immunological research. Clinical pain, especially in mice, is difficult to estimate because its quality and duration is influenced by an number of variables, e.g. anaesthesia, the surgeon's expertise, individual sensitivity, metabolic disorders, etc. In addition, in genetically modified mouse lines the impact of their phenotype on the animal's well-being is often not known. The consequences are that i) recognising clinical pain of a moderate degree in mice is based on assumptions and ii) the establishment of a protocol for pain relief and controlling the effectiveness can easily be guesswork.

www.forschung3r.ch/en/projects/pr_71_00.html

New approach for the assessment of clinical pain

Clinical pain is an undesirable variable in research projects. Stressors can disrupt biological functions which might be critical not only to the animal's welfare but also to the read-out of the experiment. Accordingly one might conclude that in mice, every suspicious manipulation (as little as it might be) must be treated by a standardised pain therapy. But dosages and time course of pain therapy may be poorly adapted to the animal's needs. The goal of the present study was to develop an approach which is suitable to recognise mild to middle grades of pain during an experiment. In the present study we used NSAIDs as analgesics. The use of opioids would induce physiological and behavioural changes which hamper the assessment of pain by an altered behaviour.

Behavioural approach: Nesting

From daily experience, we couldn't see any changes in the appearance and behavior of the mice after laparatomy performed during embryo transfer and vasectomy with and without analgesics. Consequently, it was impossible to prove whether the pain killers that we administered routinely were effective or even necessary to relieve pain. However, when we observed the animals in 12-h-intervals and took drafts of their cages' appearance, e.g. condition of their nest, we found a clear difference in the animals' nest building activities. The untreated mice destroyed their nests after the operation and needed 3 days to rebuild a proper nest and structure their cage area (Fig. 1). The mice that received pain killers damaged their nests negligibly during the first hours after the operation and came out with well established nests within the first day. This damage of nest building behavior was not found in animals which were subjected to anesthesia only. Accordingly it was concluded that the altered behavior can be correlated to the degree of post-operative pain and could be used as a setting that provoke mice to exhibit pain-related behavior. The final goal is to establish tests that are feasible under routine laboratory conditions and could be integrated in specific pain scales and scoring systems used by investigators.

Physiological approach: Heart beat rate and variability

In order to detect a sensitive physiological marker for the immediate recognition of pain, we established a telemetry system, in which we used vasectomy as model for mild to moderate post-operative pain. Telemetric transmitters were implanted in adult, male NMRI mice. After 6 weeks of recovery, they were subjected to vasectomy followed by the application of two different analgesic regimens (NSAIDs). One group received no analgesics. Post-operatively, none of the animals exhibited overt clinical signs of pain. Locomotor activity remained stable in all groups, which confirms the absence of intolerable pain. Body core temperature showed negligible increase (below 0.5°C), suggesting that post-surgical inflammation was of no influence. Nevertheless, after vasectomy in the group with no NSAIDs, the heart beat rate and variability measures increased, particularly during the daylight phase, and food consumption and body weight curves decreased by 20% respectively 2-3%. These changes were not seen in the two groups that received high doses of NSAIDs for pain therapy (Arras et al., 2005) or in control groups subjected to anesthesia and analgesics/vehicle administration only. We concluded that heart beat rate is a pain-related symptom which allows to detect in real-time low to middle-grade post-surgical pain and accordingly the effectiveness of the analgesic regimen in mice.

Molecular approach in the future: Pain-associated gene regulation?

Using the telemetric measurements, we were able to define time points at which the mice experienced pain. Accordingly the expression of genes involved in pain processing should be altered when compared to tissues from animals without pain. We designed a low-density microarray with over 100 selected genes involved in pain and distress. Preliminary results from hybridisations with material obtained after vasectomy indicate changes which correlate with pain management.

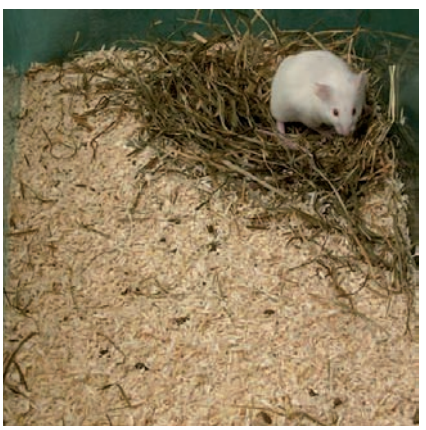

Fig. 1: Mice, 12 hours post-operatively, in their home cage.
A well-preserved nest and clear territory structure was found frequently, if post-operative pain was alleviated (left). Damaged nests and several dents, which point to diverse resting places were mainly observed if pain relief was accidentally omitted (right).

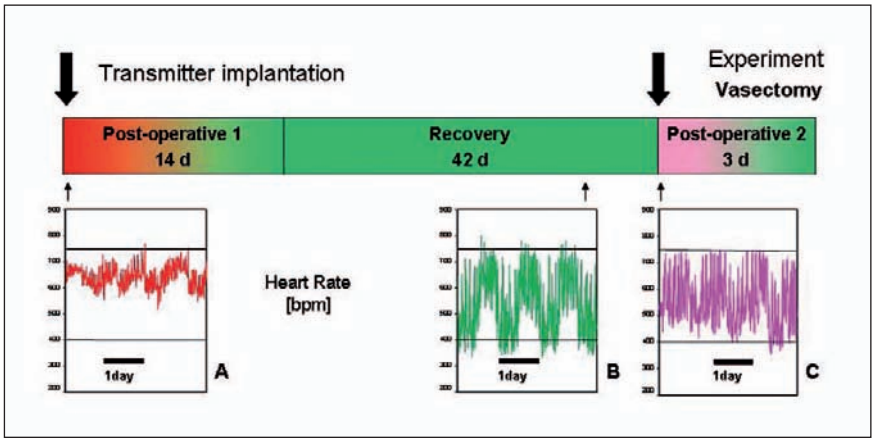

Fig. 2: Experimental schedule and real-time heart rate curves over 3 day periods.
(a) Elevated heart beat values (up to 650 bpm) over the whole diurnal rhythm after transmitter implantation despite NSAID analgesic. (b) Following a higher dose, almost normalised cardiac rhythm and normal circadian fluctuation were measured similar to recovery phase. (c) A slight increase of heart rate during the daylight (resting) phase after vasectomy without analgesics indicates mild pain, which can be reduced completely with NSAIDs.

Benefit for mice, men and science

The combined behavioural, physiological and molecular approach in mice will provide tools for the experimenter to recognise moderate and mild clinical pain. Ideally he/she will be enabled to judge the degree of pain more precisely and to apply and control the effects of clearly targeted pain relief protocols. As a result, less side effects will be expected from pain-related physiological changes in the animal (e.g. increased stress hormone levels, complicated wound healing) and analgesics-induced adverse effects (e.g. bleeding, behavioural aberrations) will be limited to the level necessary for effective pain relief.

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Exploring Natural Anticoagulation by Endothelial Cells: A novel *In Vitro* Model

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Summary
Acute damage and activation of endothelial cells (EC), the inner lining of blood vessels, is linked to release of heparan sulfate proteoglycans and exposure of a proinflammatory and procoagulant cell surface. To study the pathophysiology of EC activation and the effect of substances which protect EC in vitro we have developed a model with EC cultured on small beads, which are then incubated with whole, non-anticoagulated human blood.

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

Background Information

Endothelial cells in disease

Endothelial cells form the inner lining of blood vessels, the endothelium, which is the interface between circulating blood and surrounding tissue. Endothelial cells are involved in control of blood pressure, coagulation, atherosclerosis, angiogenesis and inflammation. Endothelial activation, leading to dysfunction and damage, has been demonstrated in several different clinical situations:
- Acute vascular rejection in allo- and xeno-transplantation: Endothelial cells are activated due to preformed antibodies and complement.
- Ischemia/reperfusion injury in surgery, transplantation or after myocardial and cerebral infarction: Changes on the endothelial cell surface during ischemia lead to complement activation upon reperfusion.
- Severe sepsis or septic shock: Vascular leakage and disseminated intravascular coagulation due to dysfunctions of the endothelium.

In vivo, endothelial cell activation is generally linked to both the complement system (a bio-chemical cascade of the immune system) and the blood coagulation cascade. By activation-induced shedding of the endothelial surface layer of heparan sulfate proteoglycans, dendritic cells in the blood may undergo maturation and transform into efficient antigen presenting cells. Endothelial cell damage is therefore linked to activation of innate immune defense mechanisms as well as to blood clotting. So far, studying interactions between the endothelium on one hand and the coagulation system, the complement pathways, antibodies, and other players of the innate immune system on the other hand inevitably necessitated the use of in vivo models. This is because conventional ex vivo or in vitro systems can only operate with serum, plasma or anti-coagulated whole blood. With the coagulation system and/or the critical interaction with blood cells "out of bounds", the results derived from such in vitro experiments paint an incomplete picture. The challenge, therefore, was to establish an in vitro model for endothelial cell activation and damage under conditions comparable to in vivo.

The endothelium's natural anticoagulant properties

The resting endothelium effectively maintains an anti-inflammatory and anti-coagulant intravascular environment. This state is mainly upheld by the endothelial surface layer, the glycocalyx, which is composed of glycolipids, proteoglycans and associated glycosaminoglycans. The association of soluble factors from the blood with the glycocalyx secures help to maintain an anti-coagulatory state of the endothelium *in vivo*.

www.forschung3r.ch/en/projects/pr_81_02.html

Preservation of anticoagulant properties *in vitro*

In conventional flatbed culture systems, work with whole, non-anticoagulated blood leads to coagulation as the endothelial surface per blood volume does not suffice to ensure that the endogenous anticoagulant state can be maintained. Accordingly, the interaction between blood cells and derived natural factors and interactions with endothelial cells cannot be studied in conventional cell culture systems. However, replacing flatbed cultures through cultivation of endothelial cells on microcarrier beads increases the surface-to-blood volume ratio twenty-fold to reach that of small arteries and veins (16 cm^2 endothelial cell

surface per ml of blood). This endothelial surface suffices to ensure "natural" anticoagulation in whole blood, rendering the use of anticoagulants unnecessary.

Set-up of whole blood *in vitro* assay

The system is based on endothelial cells (as an example, porcine aortic endothelial cells, PAEC) which are previously expanded *in vitro* in conventional flatbed culture systems. Once a sufficient cell number is available, the cells are transferred to spinner flasks and mixed with collagen-coated Biosilon microcarrier beads (5 ml, equal to 800 cm^2, Nalge Nunc International) and cultured until the cells form a confluent monolayer on the beads. Washed beads are then be incubated with non-anticoagulated, freshly withdrawn whole human blood at a bead-to-blood volume ratio of 1:4. Samples of the bead-blood mixture are taken at regular intervals during the experiment. The beads can be analyzed by immunofluorescence for various cell activation markers, cell integrity etc., while the plasma is used to analyze the coagulation/inflammatory status. In this system potential "endothelial cell protectants", i.e. substances which preserve endothelial integrity and prevent activation of the endothelial cells, can be pre-incubated with the cells in the flask or co-incubated with the whole blood, and their effects assessed.

Search for "endothelial cell protectants"

Conventionally, (pre-)screening of substances with respect to their effects as endothelial protectants needed to be performed *in vivo* in animal models of e.g. ischemia/reperfusion injury, transplantation, or shock. All of them induce a high discomfort to the animals. The novel *in vitro* EC culture system allows for the screening of such substances without the use of animal models. Compounds identified may be candidates for later application *in vivo*.

Preventing activation of cascade systems

One strategy in tackling the problem of endothelial damage is to prevent activation of the cascade systems, mainly complement and coagulation. In collaboration with academic partners in the field of bioorganic chemistry our laboratory has been developing soluble complement/coagulation inhibitors and analyzing their potential role as "endothelial cell protectants". As an example low molecular weight dextran sulfate (DXS), a sulfated polysaccharide which inhibits complement activation in human serum (Laumonier et al., 2003), prevents hyperacute rejection of pig lungs xenoperfused with human blood and significantly reduces the extent of tissue damage in a porcine model of acute myocardial infarction (Fiorante et al., 2001;

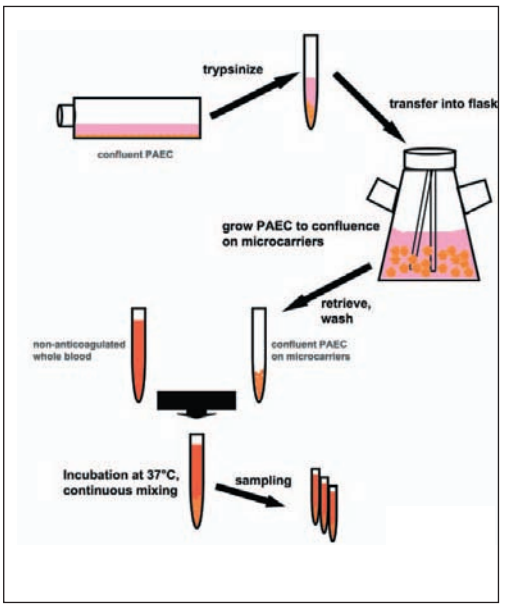

Fig. 1: Schematic representation of the test principle.
Porcine aortic endothelial cells (PAEC, used here as an example of endothelial cells in general) are expanded in conventional flatbed cell culture and then grown to confluence on microcarriers. PAEC-coated microcarriers can then be incubated with whole, non-anticoagulated blood, exploiting the natural anticoagulant properties of the endothelium.

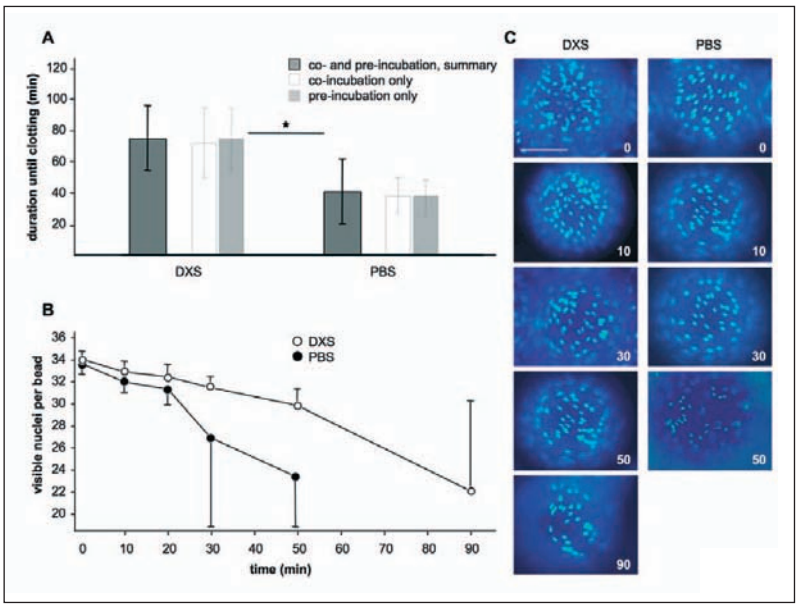

Fig. 2: Effect of the endothelial cell protectant DXS.
(A) Duration of experiments in minutes until occurrence of clotting. Shown are averages and standard deviations for experiments with DXS and PBS controls. *P < 0.05 for DXS vs. PBS. (B) Amounts of nuclei per bead for DXS- and PBS experiments at incubation time, as counted on the surface visible in panel C. (C) Representative images of single beads with DAPI staining nucleiat baseline (0 min), after 10, 30, 50 and 90 min of incubation with human blood. Scale bar represents 100 µm (Banz et al, 2005a).

Banz et al., 2005b). DXS was also tested *in vitro* in our EC-bead system (Banz et al., 2005a) (see below). Several other, fully synthetic substances have been developed and tested for their anti-coagulatory and anti-complement effects using conventional *in vitro* systems. Further testing is now required to see which of these substances act as EC protectants and have the potential for clinical application. This information can now be acquired in the described *in vitro* system before choosing the most promising agents and reverting to *in vivo* animal models for confirmation.

Mimicking xenotransplantation *in vitro*

The addition of non-anticoagulated human blood to beads coated with porcine aortic endothelial cells was used to mimic a xenotransplantation setting in our *in vitro* model. Human blood contains naturally occurring antibodies which bind to galactosyl antigens on porcine cells and activate the complement cascade. The subsequent activation of the endothelial cells leads to a loss of their anticoagulant surface layer of heparan sulfate proteoglycans and therefore initiates the coagulation cascade. *In vivo* this is the hallmark of hyperacute rejection and a major reason for the failure of pig-to-human xenotransplantation. In our *in vitro* system a loss of the PAEC from the polystyrene beads, complement deposition on the beads and a loss of von Willebrand factor expression on the remaining PAEC on the beads were observed. In addition, clotting of the human blood occurred within a few minutes. Time until clotting can be used as an indicator of endothelial cell activation and damage. Addition of our "prototype" endothelial cell protectant DXS significantly prolonged time until clotting as shown in Figure 2a and reduced cell loss from the beads (Fig. 2b, 2c).

3R benefit and limitations

The described *in vitro* model is currently suitable for the analysis of acute endothelial cell damage like antibody- and complement-mediated (hyper)acute vascular rejection or for studying certain aspects of ischemia/reperfusion injury. Most probably due to contact with air, blood clotting occurs within about 90 minutes even with the use of DXS as an endothelial cell protectant. Situations in which endothelial damage takes several hours or days to occur can therefore not be mimicked with the current model. In addition, flow conditions and shear stress which may play an important role *in vivo* are not present in the model. Also the phenomenon of vascular leakage, in which the endothelial cells stay intact but loose their barrier function due to intracellular gap formation, cannot be mimicked. Nevertheless, the possibility to do a first screening with substances aimed at protecting endothelial cells from ischemia/reperfusion injury or antibody- and complement-mediated attack, offered by our new *in vitro* model, has the potential to significantly reduce the number of animals derived from animal models. Furthermore, mechanistic studies can be performed much easier *in vitro*, which in addition reduces the use of animals in the respective areas of research.

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Non-Invasive Methods: Investigation of Airways Diseases by MRI in Rats

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Summary
Current techniques to evaluate the efficacy of potential treatments for airways diseases in small animal models are generally invasive and terminal. In this contribution we illustrate the usefulness of magnetic resonance imaging (MRI) to obtain anatomical and functional information of the lung, with the scope of developing a non-invasive approach for the routine testing of drugs in rat models of airways diseases. With MRI, the disease progression can be followed in the same animal. Thus, a significant reduction in the number of animals used for experimentation is achieved, as well as minimal interference with their well-being and physiological status. In addition, MRI has the potential to shorten the duration of the observation period after disease onset since the technique is able to detect changes before these are reflected in invasively determined parameters of inflammation.

Keywords: reduction, refinement, rat, lung, asthma, drug screening, magnetic resonance imaging (MRI), inflammation, non-invasive

Background Information

Rodent models of airways diseases

To study specific aspects of human respiratory diseases and its treatment it is necessary to measure the induced symptoms and the impairment of lung function in living animals. For example, key features of asthmatic inflammation (airway hyperresponsiveness, eosinophilic inflammation together with an increase in activated T cells in the airways) are induced in actively sensitized Brown Norway (BN) rats exposed to allergen (ovalbumin, OVA). Alternatively, an inflammation similar to that observed in chronic obstructive pulmonary disease (COPD) patients (neutrophilia and mucus cell metaplasia) can be elicited in rodents by the administration of endotoxin (lipopolysaccharide, LPS). Emphysema, one of the most critical components of COPD, which results in the destruction of lung parenchymal tissue by a number of proteases (neutrophil elastase or matrix metalloproteinases), is induced in rats by the application of a single dose of porcine pancreatic elastase (PPE). This destruction leads to an enlargement of air spaces and to a loss of lung elasticity, ultimately impairing gas exchange. In all cases the symptoms resemble those seen in patients in the early onset of the disease.

The inflammatory status of the lung is routinely inferred from post mortem analyses of broncho-alveolar lavage (BAL) fluid. Occasionally, time consuming histological analysis is also performed. Lung function is assessed in terminal experiments where animals are treated with a muscle relaxant, tracheotomized and artificially ventilated. Airflow, transpulmonary pressure, and airway resistance are determined for each respiratory cycle. Following these measurements animals are killed by an overdose of anesthetic. Clearly, the invasive character of these procedures precludes repeated assessments in the same animal. Therefore the flexibility of MRI was explored by Nicolau Beckmann and his colleagues to obtain anatomical and functional information of the rat lung, with the scope of developing a non-invasive means of analysis.

MRI: A powerful tool

Based on the use of magnetic fields and radiofrequency, MRI basically maps the distribution of hydrogen nuclei (protons) from water and fat in a region of the body. MRI is primarily a clinical diagnostic tool, however, in the past ten years, significant developments have been achieved in imaging small animals as well.

Because of physical characteristics, the living lung is one of the most challenging organs to image by MRI. Using conventional acquisition techniques, the lung appears dark in the images (Fig. 1). In order to acquire signal from lung parenchyma, special techniques need to be used. A further challenge for lung MRI is that cardiac and respiratory movements may cause marked image artefacts. These problems are more evident in small rodents, because of the higher cardiac and respiratory rates.

For drug testing *in vivo*, it is important to keep the acquisition conditions as simple as possible so that repeated measurements interfering minimally with the physiology and the well-being of the animals can be carried out on a routine basis. Beckmann et al. developed an approach based on a conventional MRI tech-

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nique, which produces sharp images from the chest of a rat respiring spontaneously (Beckmann et al., 2001). The examination time is of approximately 25 min, and during this time the animal is kept anaesthetized by the same anesthetic gas used in the clinic.

Detection of structural and functional changes

With this approach, effects of inflammation (Beckmann et al., 2001), mucus secretion (Beckmann et al., 2002), airway and vascular remodeling (Beckmann et al., 2004; Tigani et al., 2007), and parenchymal destruction (Karmouty et al., 2006) can be assessed in the rat lung serving as important readouts for models covering a variety of respiratory diseases. The disease progression is followed in the same animal. Two examples illustrate the relevance of the information that can be obtained with MRI.

A characteristic feature of respiratory diseases such as asthma is edema in the airways due to an increased permeability of the lung microvasculature to plasma proteins. Assessment of the fluid leaking out from the microvascular circulation into the surrounding tissue is important for diagnostic purposes. In rats actively sensitized to ovalbumin (OVA) and challenged intra-tracheally (i.t.) with the antigen (OVA, 0.3 mg/kg), an intense, even, fluid signal is detected in the lungs 24 h after challenge (Fig. 2a). Despite the extensive presence of fluid in the lungs, no abnormal behavior of the rats is noticed. The MRI fluid signal that is observed for about 4-5 days, before it resolves spontaneously, significantly correlates with perivascular edema assessed by histology (Tigani et al., 2003).

This correlation provides the basis to address the effects of anti-inflammatory therapies in the allergen model. In one experimental paradigm the drugs are given in a therapeutic regimen 24 h after the challenge with OVA, a time point when an extensive MRI signal is present in the lungs. Treatment with budesonide, a corticosteroid approved for clinical use, accelerates the rate of resolution of the MRI signal (Fig. 2b). The decline in the edematous signal correlates significantly with the reduction in perivascular edema quantified by histology of the lungs. By contrast, BAL fluid markers of inflammation are not affected by budesonide. It seems, accordingly, that the early resolution of MRI edematous signals by the anti-inflammatory drugs does not

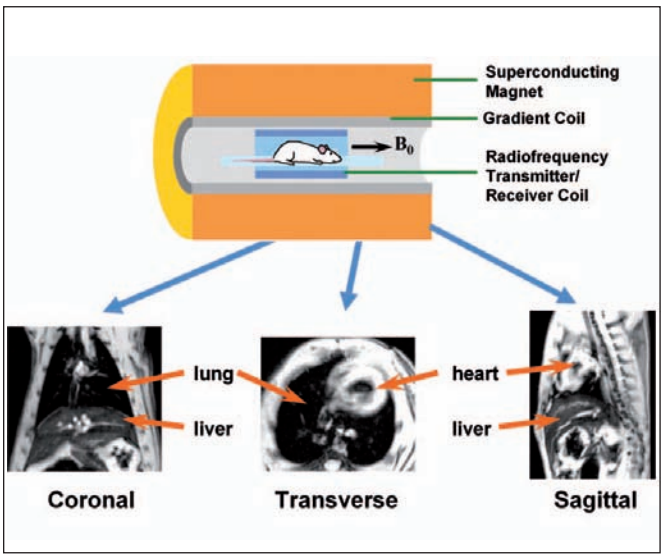

Fig. 1: MRI equipment for small rodent imaging.
Essential elements are a magnet (usually superconducting operating at high B0 field, e.g. 4.7 T) generating a homogeneous constant magnetic field, a gradient system capable of producing a position-dependent magnetic field when data acquisition takes place, and a coil which transmits and receives radio-frequency waves. An image is reconstructed from such waves and represents a weighted distribution of water and fat protons in the body. Images with an arbitrary orientation can be obtained using MRI, for instance coronal, transverse, or sagittal sections, as illustrated. Animals are kept under anaesthesia (e.g. with an anaesthetic gas administered through a mask) during the imaging session.

Fig. 2A

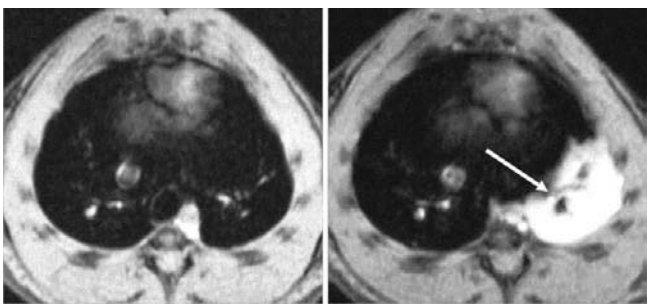

Fig. 2B

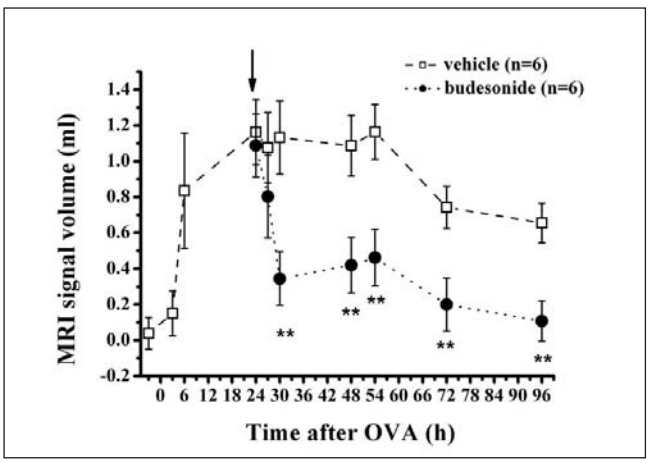

Fig. 2: Allergen challenge.
(A) Axial images through the chest of an actively sensitized BN rat, acquired before (left) and 24 h after intra-tracheal (i.t.) OVA instillation (right). Note that the lung parenchyma appears dark; however, following allergen, a prominent MRI fluid signal is seen (arrow). This signal is due to edema. The acquisition time for one image is of 1 min.
(B) Volume of fluid signals detected by MRI in the lungs for an OVA dose of 3 mg/kg (i.t.). Administration of the corticosteroid budesonide (1 mg/kg i.t.) 24 h after OVA (arrow) leads to an acceleration of the resolution of the MRI signals, suggesting a rapid effect of the compound. This effect is not detectable by conventional BAL fluid analysis. Data are expressed as means ± SEM (n=6 rats per group).

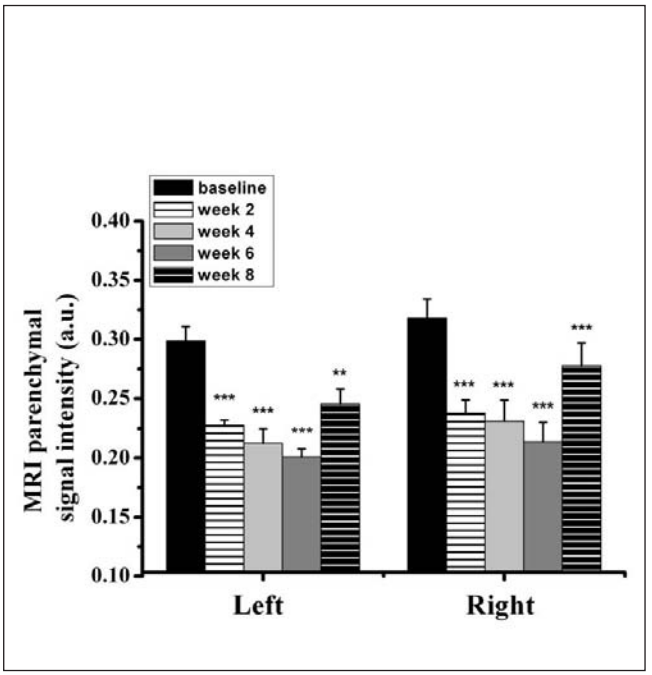

Fig. 3A

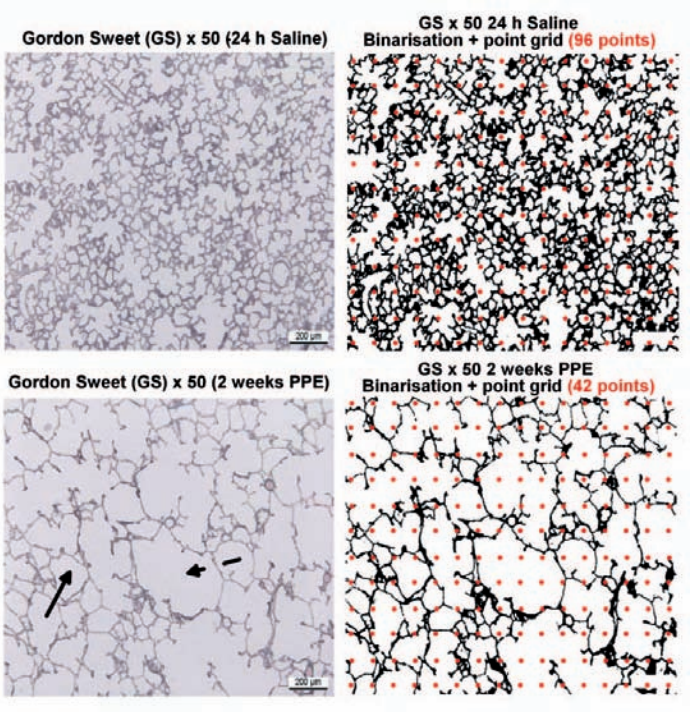

Fig. 3B

involve general suppression of the inflammatory response at least as monitored by BAL fluid analysis (Tigani et al., 2003).

A second example concerns the use of MRI in a rat model of emphysema (Quintana et al., 2006). For animals treated with PPE administered i.t., the parenchymal signal intensity was decreased in the first 6 weeks following PPE (Fig. 3a). Consistent with this, extensive enlargement of the alveoli was observed in alveoli rich sections of histological slices (Fig. 3b). A tendency towards recovery of the MRI signal intensity was apparent at week 8, which correlated with a reduction of the emphysematous damage assessed histologically by point morphometry. Related to this reduction in damage could be the fact that following PPE the elastin content initially decreases, but appreciable elastic fiber deposition, and granulation of the alveolar airspaces containing fibroblasts, endothelial cells and a provisional collagen matrix are observed weeks after injury. The significant negative correlation between the MRI signal intensity of lung parenchyma and the percentile alveolar content determined by histology (Fig. 3c) indicates that proton MRI is sensitive to non-invasively detect structural changes of the lung parenchyma related to the development of emphysema in this model.

Less discomfort, shorter experiments

With MRI one has the potential to shorten the overall experimental duration after injury onset since the technique is able to detect changes before these are reflected in parameters of inflammation present in BAL fluid. This results in less discomfort for the animals.

Less animals and more relevant data

The non-invasive MRI approach results in a significant reduction in the number of animals used for experimentation. Depending on the application, a reduction between 80 to 90% is estimated. Since repeated measurements are feasible, each animal can serve as its own control, thereby reducing the variability of the data. As acquisitions are performed on spontaneously breathing rats, interferences with their well-being and physiological status are minimized. MRI is able to provide data on rapid effects of anti-inflammatory compounds on established inflammation, an information that is not accessible to conventional, *post mortem* BAL fluid analysis. Thus, data that are more relevant to address therapeutic effects can be obtained using MRI. Anesthesia is the primary limiting factor of the approach. However, following an examination, rats recover from anesthesia within 10-15 minutes.

Overall, MRI provides a global picture of the disease status in the animal model. As this imaging technique is largely available in hospitals, there is potential to address translational aspects from the models in rats to the human situation.

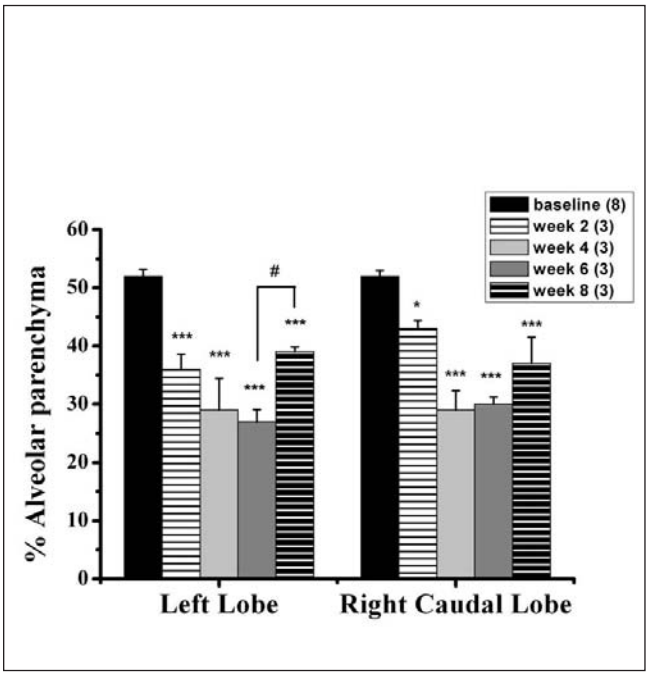

Fig. 3C

Fig. 3: Elastase challenge.
(A) Signal intensities (means ± SEM) from coronal slices corresponding to the lower right and lower left anatomical sides of the lung acquired prior to (baseline) and at 2, 4, 6 and 8 weeks after treatment with elastase. Significance levels *0.01<p<0.05, **0.001<p<0.01, ***p<0.001 refer to Anova comparisons (Bonferroni test vs. control) between the different time points and baseline values, for each region.
(B) Histological slices and corresponding binarization with a 192 point-grid used for analysis showing the alveolar area from the left lobe of rats administered with saline or with PPE. The black arrow shows the increased area of alveoli and the dotted arrow the increased terminal bronchi in the PPE-administered rat.
(C) Percent alveolar parenchyma (means ± SEM) assessed histologically from the left and the right caudal lobes from animals treated with PPE. Significance levels *0.01<p<0.05, and ***p<0.001 refer to Anova comparisons (Bonferroni test pair wise) between the different time points and baseline values, for each lobe. For the left lobe, a significant difference (#0.01<p<0.05) was found in the percentile alveolar area at 6 and 8 weeks. Remarkable is the resemblance between the curves representing the MRI signal intensity of parenchyma in the left and right sides, and the percentile alveolar area assessed in the left and in the right caudal lobes, reflected in a significant correlation (R=0.84, p<0.001) between both values. Histological samples corresponded to the same animals analyzed by MRI. This result suggests that the MRI signal reflects changes induced by PPE at the parenchymal level.

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Simulation of Stroke-related Damage in Cultured Human Nerve Cells

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Summary
We describe a novel cell culture protocol for the generation of neurons from a human teratocarcinoma cell line. These neurons were used to investigate hypoxic-ischaemic cell damage and for developing neuroprotective strategies. Cultures of human model neurons should eventually serve to reduce the number of experimental animals in cerebral stroke research.

Keywords: Excitotoxicity, glutamate, ischaemia, neural differentiation, neuroprotection, human, brain-cell cultures, organ-specific, reduction, replacement, pharmacological testing

Background Information

Why animal models for ischaemic brain damage?

Two rodent models are widely employed: the transient global forebrain ischaemia model, in which the entire blood supply to the brain is transiently interrupted (imitating cardiac arrest), and the focal cerebral ischaemia model, in which the proximal middle cerebral artery is occluded. In addition to the initial damage in the immediate vicinity of occluded blood vessels, cerebral ischaemia also results in a wave of delayed cell death that spreads to surrounding tissue, the so-called penumbra. Whereas neuronal death occurs rapidly in the ischaemic core, neurons remain viable for many hours in the surrounding penumbra, providing a period of possible therapeutic intervention ("time to treatment" window). After inducing transient ischaemia, the animal is allowed to recover for some time, then is sacrificed and the brain damage studied in detail. In such studies, the bioavailability of a potential drug, its pharmacokinetics, effects on blood pressure, body temperature and motor activity are also determined.

Excitotoxic cascade during stroke

Stroke is the third leading cause of death and an important cause of adult disability in industrialised countries. Most strokes are caused by an acute interruption of the brain's blood supply, which leads to tissue ischaemia in the particularly vulnerable central nervous system. To investigate the cellular mechanisms occurring in ischaemic brain damage, a variety of rodent models have been developed that mimic the pathogenic environment of nerve cells during stroke. Cell culture models are also finding increasing use (Honegger and Pardo, 2007).

Ischaemic neurons deprived of oxygen and glucose rapidly lose their energy currency ATP, their intracellular pH then drops, and they depolarize. Among other consequences, the neurotransmitter substance glutamate is released. The accumulation of the excitatory transmitter glutamate excessively stimulates glutamate receptors of neighbouring cells, inducing increases in intracellular calcium levels and the production of free radicals, which in concert orchestrate cell injury. This cellular disorder is called excitotoxic cascade. One approach to reducing brain damage following a stroke might involve the application of neuroprotective substances that inhibit various stages of this cascade.

Generation of human nerve cells in the petri dish

We started with a well-characterised teratocarcinoma cell line (Ntera-2, NT-2) derived from a human testicular cancer. Upon treatment with the morphogen retinoic acid, the NT-2 line can be induced to differentiate into postmitotic neurons (Pleasure et al., 1992). This protocol involves a 5-7 weeks exposure with the morphogen, followed by two replates, a 7-10 days lasting treatment with mitotic inhibtors, and selective trypsinisation steps for neuronal purification. Taken together, the differentiation of NT-2 precursor cells into neurons requires between 44 and 56 days of cell culturing work and this is also the main disadvantage of this differentiation method.

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Time for differentiation is reduced by half

The conventional differentiation protocol uses adhesive substrates on which the cells are grown as monolayers. We seeded NT-2 precursor cells onto bacteriological grade petri dishes upon which these cells do not adhere. Under these conditions the cells proliferated as clusters in the shape of free floating spheres (Fig. 1a). At later stages of the neuronal differentiation process, the cells were again cultured as a monolayer. Allowing the cells to proliferate as free-floating cell spheres cuts the total time needed to obtain high yields of purified NT-2 neurons to about 24 - 28 days (Paquet-Durand et al., 2003). The cells obtained show neuronal morphology, migrate to form ganglion-like cell conglomerates, and are immunoreactive to neuronal cytoskeletal markers (Fig. 1b). Some epitheloid cells were also present in the petri dishes, but staining for neuronal markers indicated that after final plating the cell cultures were composed of approximately 90-95% human nerve cells (Fig. 1b). Calcium imaging techniques showed that NT-2 neurons express glutamate receptors including the N-methyl-D-aspartate type (Paquet-Durand and Bicker, 2004).

Cultured nerve cells are sensitive to ischaemic injury

In cell cultures the cell densities are lower than in intact tissue. Conversely, the volume of the surrounding medium is considerably larger than the extracellular tissue volume. Thus, only traces of oxygen are sufficient for cell survival. To mimic anoxia in culture, the NT-2 neurons were kept under an atmosphere of 95% argon and 5% CO_2; to ensure complete anoxia, remaining traces of oxygen in the medium were removed with non-toxic concentrations of dithionite. Glutamate served as excitotoxin. Cell viability was assessed using the Alamar Blue assay, which measures metabolic activity. The reduction in the cells' viability over time increased with the duration of anoxia (Fig. 2) and increasing glutamate concentrations. Anoxia times of four hours resulted in the destruction of more than 80% of the cells. Control experiments with undifferentiated NT-2 teratocarcinoma cells showed no vulnerability to anoxia (Paquet-Durant and Bicker, 2004).

An important parameter that influences the vulnerability of the mature postmitotic NT-2 neurons is the time in culture (Fig. 3) following the differentiation process. Older NT-2 neuronal cultures are more vulnerable to ischaemic insult, reflecting ongoing maturation of neurochemical properties *in vitro*. The cell culture model can be used to assay for potential neuroprotective compounds. We have shown that low doses of diltiazem, a licensed drug that is commonly used to treat cardiovascular disorders, protects the human model neurons against ischaemic damage (Paquet-Durand et al., 2006).

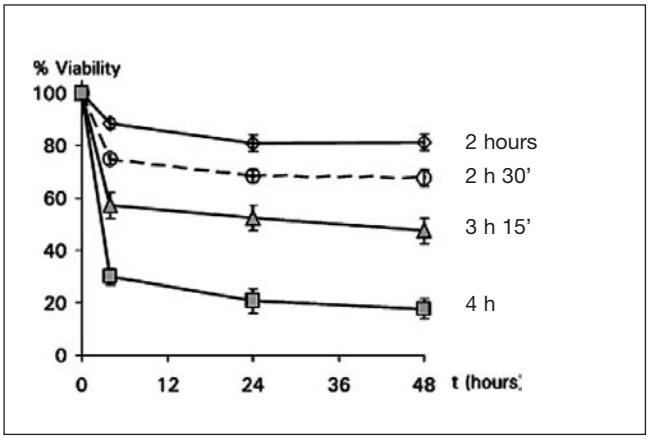

Fig. 2: Dependence of viability on duration of anoxia.
Cultured NT-2 neurons were subjected to anoxia in the presence of dithionite and 1mM glutamate. Duration of anoxia ranged from 2h to 4h. Viabilities were followed for 48 hours post anoxia treatment and plotted as percentage of internal control (100 %).

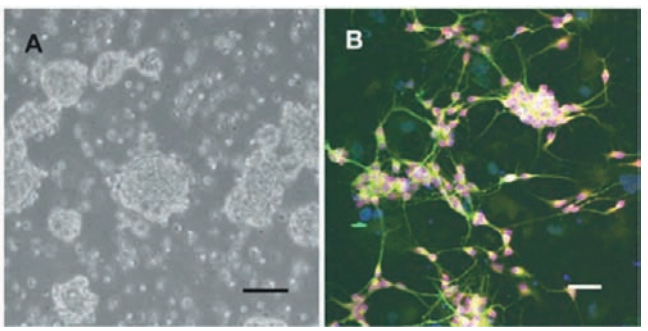

Fig. 1: Differentiation of NT-2 cells into neurons.
(A) Expansion of NT-2 precursors as free floating cell spheres. This technique resembles the sphere culture method used in embryonic stem cell differentiation. Scale 100 µm.
(B) Differentiated NT-2 neurons stained by immunofluorescence. Spindle shaped cell bodies send out neural processes.
Scale 50 µm

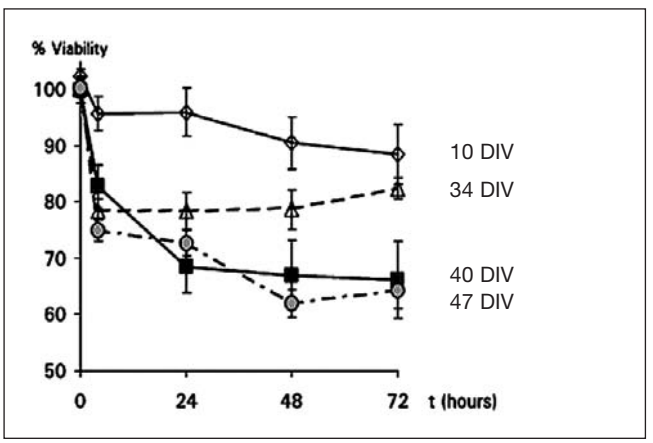

Fig. 3: Vulnerability of mature, post-mitotic NT-2 neurons depends on days *in vitro* (DIV) prior to anoxia treatment.
Times in culture ranged from 10 to 47 days. After 2h of anoxia, percentage of viabilities was followed for 72 h.

Reduction and replacement of laboratory animals

Stroke-related research often depends on animal models, because the interest is mainly focused on general brain damage after ischaemic lesions rather than on the effects on individual brain cells. The NT-2 cell culture system, in contrast, focuses more on the neuronal cell biology which has a number of advantages (Paquet-Durand and Bicker, 2007):

1) NT-2 neurons are derived from a human cell line, thus they are especially suitable for screening for neuroprotective drugs effective in the human brain.

2) The fairly homogeneous cellular composition of clonally derived neurons allows for large-scale cell-based assays.

3) Culturing NT-2 neurons in a monolayer readily allows electrophysiological recordings and optical imaging studies to investigate the excitotoxic cascade at a cellular level. Accordingly this cell culture system might

i) replace the use of primary cell cultures prepared from rodent embryos,

ii) reduce the number of laboratory animals needed in initial stages of drug screening and

iii) has the potential to replace laboratory animals in basic stroke research.

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Animalfree Screening of Biological Materials for Contamination by Rodent Viruses

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Summary
Pathogenic bacteria and viruses may be transmitted to laboratory rodents by contaminated biological materials potentially causing disease and confounding research results. Biological materials have historically being screened by using the mouse antibody production (MAP) test. We developed an alternative assay using polymerase chain reaction (PCR/real-time-PCR) technology to detect viral contamination directly in biological material. The assay was found to be of equal or greater sensitivity when compared to the MAP test.

Keywords: MAP test, real-time PCR, mouse, tumor

Background Information

Mouse antibody production test

Biological materials, such as serum, transplantable tumors, cultured cells and hybridoma lines that originate from or have been passaged through rodents may be inadvertently contaminated by rodent pathogens. If inoculated into rodents they may transmit these infectious agents to the graft recipient. The resulting, generally subclinical, infection may not only alter the outcome of the specific experiment, but may also be transmitted to other animals in the colony. It is therefore paramount that all biological materials be screened before they are inoculated into rodents. Traditionally this is carried out by means of the MAP test (Parker and Reynolds, 1968), which involves inoculating the test material into the relevant rodent species and serologically testing the inoculated animals 30 days later for antibodies specific to known viral pathogens.

DNA *in vitro* instead of antibodies *in vivo*

The mouse antibody production (MAP) test, traditionally used to screen biological material prior to their use *in vivo*, has a number of drawbacks. One is the fact that animals are required for the test. Since most of the pathogens that are being screened for do not cause clinical disease in immunocompetent adult animals, the pain and distress suffered by the test animals is usually minimal. However, some infectious agents (e.g. ectromelia virus) may cause disease or even death. These cannot be ruled out in advance since the types and concentrations of viruses present in the test material are unknown. In addition, the MAP test is fairly costly and slow (5-6 weeks). There are no reliable numbers, but it is estimated that 200-300 MAP tests are conducted annually in Germany alone. Previous attempts to replace the MAP test with an *in vitro* assay using virus isolation in cell culture were unsatisfactory: not all viruses could be detected reliably in culture. With the advent of the polymerase chain reaction (PCR), a new, highly sensitive and specific tool became available. In order to replace the MAP test, it was necessary to establish PCR assays to detect all the possible viral agents being screened for (Tab. 1), otherwise, a MAP test would still have been required to rule out the remaining pathogens.

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Development of PCR Assays

For each pathogen (Tab. 1), specific primers were designed based on published sequencing information. Primers were situated in conserved regions of the respective genomes to ensure that all known substrains of a virus could be detected. Where available, published PCR assays were used if they met the above criteria. All PCR assays were tested for their specificity and sensitivity using virus stock cultures at various dilutions. Virus stocks were obtained from ATCC and various collaborators (Bootz and Sieber, 2002; Bootz et al., 2003).

Semiquantitative analysis improves the interpretation

A TaqMan® DNA amplification system was provided by a generous grant from the Doerenkamp Zbinden Foundation. This system allowed us to follow the PCR reaction kinetics in real time: Each reaction well is equipped with optical fibers. Laser light transmitted through these fibers stimulates target molecules in the reaction mix to emit fluorescent light. These target molecules are present in an inactive form on the primers and are cleaved off, becoming active, when the primer anneals and starts the strand formation. Fluorescence is measured every 5 seconds; the light emitted is directly proportional to the amount of primer

Tab. 1: List of viruses detected by the IMPAT and limit of detection:

Viruses detected by IMPAT	Abrevations	Units	Initial stock virus conc./ml	Antibody Production in the MAP Test	Standard PCR	Real-Time PCR (TaqMan®)
Mouse Adenovirus	MAD	*	10 (5,5)	10 (-1)	10 (-2,5)	10 (-2,5)
Mouse Cytomegalovirus	MCMV	*	10 (5,2)	10 (1,2)	10 (1,2)	10 (-0,8)
Ectromelia Virus	Ectro	*	10 (3,5)	10 (-1,5)	10 (-2,5)	10 (-2,5)
K-Virus	K-Virus	+	2	10 (0)	10 (-5)	10 (-7)
Mouse Minute/Parvo Virus	MVM	*	10 (4,5)	10 (2)	10 (0,5)	10 (-2,5)
Polyoma Virus	Poly	*	10 (4,5)	10 (-0,5)	10 (-0,5)	10 (-4,5)
Lactate Dehydrogenase Elevating Virus	LDV	“	10 (10)	10 (2)	10 (-2)	10 (-2)
Mouse Hepatitis Virus 3	MHV	*	10 (3)	10 (-3)	10 (-3)	10 (-3)
Pneumonia Virus of Mice	PVM	+	32	10 (-2)	10 (-4)	10 (-4)
Reovirus 3	Reo	*	10 (5,5)	10 (1)	10 (1)	10 (0)
Sendai Virus	Sendai	*	10 (2,5)	10 (0,5)	10 (-1,5)	10 (-2,5)
Theiler’s Meningo-encephalitis Virus	TMEV	*	10 (5,5)	10 (1)	10 (1)	10 (0)
Lymphocytic Corio-meningitis Virus	LCMV	o	10 (6)	10 (0)	10 (0)	10 (0)
Epidemic Diarrhea Virus of Infant Mice	Rota	*	10 (3,5)	10 (1,5)	10 (-1,5)	10 (-1,5)

*=$TCID_{50}$=50% Tissue Culture Infectious Dose
“ =ID_{50}=Infectious Dose (50% of mice)
( ) = indices of dilution factors.
+=HA-units=Haemaglutinin units
o=PFU=Plaque Forming Units

used and therefore to the number of new amplicons. This method allows a semi-quantitative analysis of the results as the amplification progresses, i.e. in real time. The test was named Infectious Microbe PCR Amplification Test (IMPAT).

IMPAT, a replacement for the MAP-Test

In order to validate the IMPAT, it was compared with the MAP test. Samples of known concentration (tissue culture infective dose) were generated for each of the 14 viruses on the screening panel (Tab. 1). For each virus four 100-fold serial dilutions were made.

MAP test: Groups of 2 mice were inoculated with each virus at each of the dilutions. After 30 days the animals were euthanised and exsanguinated. The serum was tested for antibodies specific to the respective viruses using immunofluorescence assays (MicroBioS GmbH, CH-4153 Reinach).

IMPAT: To mimic routine conditions, the virus dilutions were not analysed directly by IMPAT, but rather used to inoculate a standard tumor cell suspension. The tumor cells were incubated with the virus samples for 1 hour (DNA viruses) or 5 minutes (RNA viruses) before DNA or RNA extraction. Nucleic acids isolated from the contaminated tumor material were analysed using standard PCR and the TaqMan® method.

Sensitivity: The three methods were equally sensitive in detecting two of the 14 viruses. One of these was mouse hepatitis virus, which was lethal in 7 of 8 mice inoculated. While no serology could be done the samples were considered positive. The molecular methods were more sensitive than the MAP-Test in detecting all the other pathogens. This was the case for six of the twelve remaining viruses when using the conventional PCR and for all 12 when using the TaqMan®. The fact that real-time PCR was in many cases more sensitive than the standard PCR was attributed to two reasons: 1) The TaqMan® methodology using fluorescence emission is considered to be more sensitive, and 2) a standard annealing temperature was used for the con-

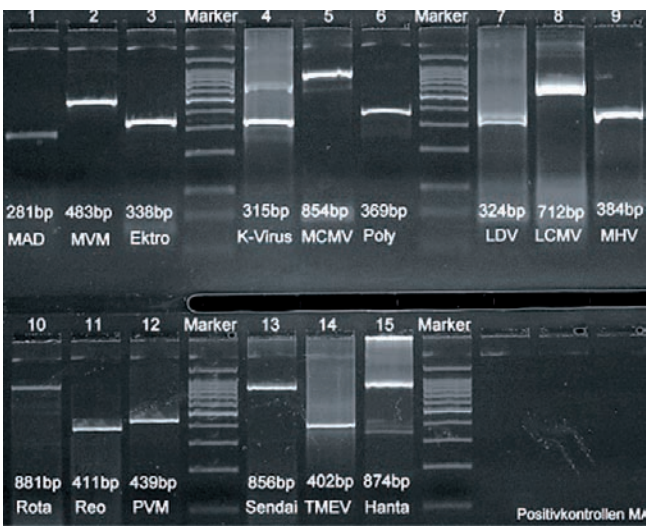

Fig. 1: Size of the PCR-Products in Clearose Gels from the different viruses tested.

ventional PCR assays, while for the TaqMan® real-time PCR the annealing temperature was optimised for each primer set.

Important advantages of the *in vitro* method

The specificity and the sensitivity of the IMPAT are at least equivalent and in most cases superior to those of the MAP test. In addition there are a number of other advantages over the *in vitro* method:

- No animals are required (replacement)
- Turn-around time is reduced to 1-2 days (MAP test 5-6 weeks)
- Independent of the individual immune response of the mouse (smaller variability)
- No animals inoculated with unknown infectious agents need be housed in the animal facility (no risk to the animal facility or the investigators)

The main difference between the MAP test and IMPAT is that the MAP test detects only the immune response of a mouse to infectious virus particles whereas IMPAT detects the presence of nucleic acids from infectious but also from non-infectious, inactivated virus. Here IMPAT has a further clear advantage, since detection of inactivated virus is a warning sign that a contamination had occurred at some time and such material should be excluded due to the increased risk associated with it.

Negligible drawback

The only drawback of IMPAT is its high specificity. While serological methods are based on the immune reaction to multiple proteins, IMPAT detects only a very specific sequence of the viral genome. This carries the risk that it may not detect a mutant variant of the pathogen. We have tried to keep this risk to a minimum by carefully choosing the primers in regions of the genome that are highly conserved. The IMPAT is now offered as a routine service by most commercial diagnostic laboratories.

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Identification of Neurotoxic Chemicals in Cell Cultures

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Summary
In order to identify the neurotoxic potential of drugs or chemicals in vitro, a combination of different in vitro cell culture tests is required. Depending on the type and use of a given test compound, a sequential exposure of freshly isolated and cultured hepatocytes and chicken brain cells is suitable. In order to find out more about the stability of liver-derived metabolites, co-cultures are appropriate. In order to determine how metabolites enter the brain, a combination of an in vitro system which mimics the blood-brain barrier and choroid plexus is proposed.

Keywords: brain, chicken, liver, rat, neurotoxicity, xenobiotics, co-culture, organ-specific cell culture, reduction, replacement

Background Information

Cellular neurotoxicity model systems

Model systems are chosen because of similarities to humans but are defined by their limitations in mimicking the original. Estimating the neurotoxicity of chemicals or drugs must be based on the fact that a compound which has entered the body passes through the liver and reaches the brain cells via the blood supply. When this situation is translated into an in vitro approach the set-up includes a sequential exposure of cells from liver and from brain tissue. The specific choice of system is defined by the type of test compound and its use.

Ideally the cell system should be based on human cells with organ-specific functions. Genetic differences between humans and laboratory animals (in most cases rodents) introduce a degree of uncertainty when toxicity data are extrapolated from animal experiments to humans. The use of human cell lines of neuroblastoma cells would therefore be an alternative for neurotoxicity tests. The genetic amenability of these neoplastic cells is not defined, however, and the functional capacity is not comparable to that of normal human brain cells. Primary cells from human nervous tissue would therefore be the best choice. But human tissue as source is limited because of ethical and legal considerations. Adult human tissue can only be obtained occasionally and if it can be obtained each sample will come from donors of different ages, different sexes and from a different brain area. Furthermore, the survival of adult nerve cells is reduced, especially in the case of dissociated cells as required for initial cultures. This would not be the case with isolated primary fetal brain cells which, in humans, can only be obtained only from therapeutic abortions. An alternative that is still being developed involves cells from embryonic human stem cell lines (Schrattenholz and Klemm, 2007). An already established and reliable alternative is nerve cell cultures obtained from embryonic chicks; a number of research groups have demonstrated a similar sensitivity to a variety of drugs in such embryonic chicks and humans (e.g. Bruinink et al., 1998).

Assessment of neurotoxicity

The identification of a potentially neurotoxic activity (damage to nervous tissue cells) of chemicals and drugs is an important task within the framework of the assessment of the toxicity of chemicals. Current guidelines recommend the use of animal tests because of the possibility that a compound might be converted by xenobiotic metabolism to metabolites that are neurotoxic. Current risk assessment guidelines for neurotoxic organophosphorus compounds (OPs) are based on the *in vivo* hen model, owing to its high sensitivity. Initially it was thought that OP toxicity could be estimated by measuring its effect through cholinesterase inhibition in a cell-free system and the consequent cholinergic hyperstimulation. Recent data suggest that the OP-induced neurotoxic effects are achieved by an interaction with cellular processes that are unique to brain development, however (Slotkin, 2004). This implies that OP toxicity can only be correctly measured using living nerve cells.

Conversion of non-toxic chemicals into neurotoxic metabolites

A chemical may exert its toxicity directly or indirectly, following metabolism in the liver. Stable metabolites can be released from liver cells into the blood stream and transported to the brain

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after crossing the blood-brain barrier. This pathway has been well established for some OPs, but cannot be reproduced in conventional human or animal brain tumour (neuroblastoma) cell cultures. Accordingly cell culture systems based on one single organ fail to identify "indirectly acting" neurotoxic compounds (compounds that need bioactivation) (Bruinink, 2008). The metabolic activation or detoxification of chemicals can be clearly detected in a variety of established cell culture systems from different species. Accordingly the possible interactions between organs require a combination of representative cell types from the organs involved. Hepatocyte cultures or co-cultures of different cell types from the liver are suitable for detecting liver-specific toxicity (Milosevic et al., 1999), while in order to detect neurotoxicty the liver cell cultures have to be combined with brain cell cultures and the blood-liver-brain pathway has to be taken into account.

Co-cultures *versus* sequential cultures

The goal of the project was to assess the possibility of identifying chemicals which are neurotoxic only after bioactivation by the liver, using a method based on cell culture. Co-cultures or sequential cultures were established between primary rat hepatocytes (Ohno et al., 1995) and chicken brain cells isolated from total brain of Tetra SL embryonic chicks at stage 29 (Bruinink et al., 1998). The hepatocytes (Fig. 1a) represent the liver, the embryonic brain cells (Fig. 1b) the ultimate target. Stable hepatic metabolites in the supernatant have a high chance of reaching central (e.g. brain) and peripheral nervous tissue via the blood circulation. The test compound can be added either directly to the co-cultures (Fig. 2) or initially to hepatocyte cultures, after which the supernatant is transferred to the brain cells (Fig. 2). A comparison between the two results provides information on the stability of the hepatocyte-derived metabolites.

Brain cell toxicity was assessed by measuring the bioreductive capacity (conversion of the dye MTT), the lysososmal activity (neutral red uptake) and the brain-specific acetylcholinesterase (AchE) activity of the cells. A reduction of the latter activity may be provoked by a direct effect on AChE or indirectly by impairing cellular processes of the nerve cells (Slotkin, 2004).

Hepatocytes release neurotoxic metabolites

Two chemicals were compared: cyclophosphamide (CP), known to be converted in the liver to a metabolite which is cytotoxic for extrahepatic tissues but without a specific brain toxicity, and isofenphos (Bayer AG), a representative organophosphate known to be neurotoxic in humans after bioactivation.

Brain cells were exposed directly, in co-cultures or with the supernatant collected from exposed hepatocytes (exposure time

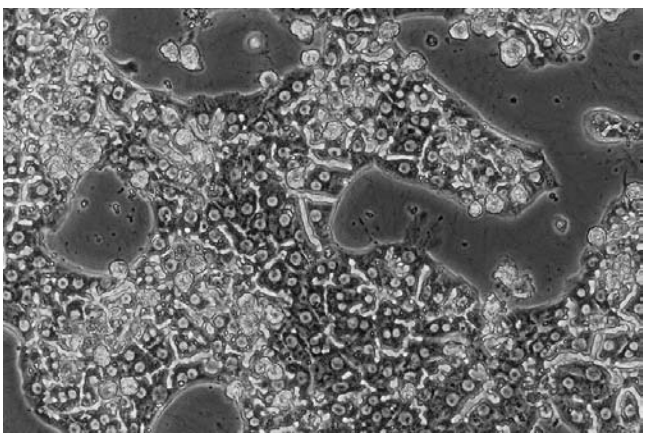

Fig. 1a: Rat hepatocytes (4-day culture)

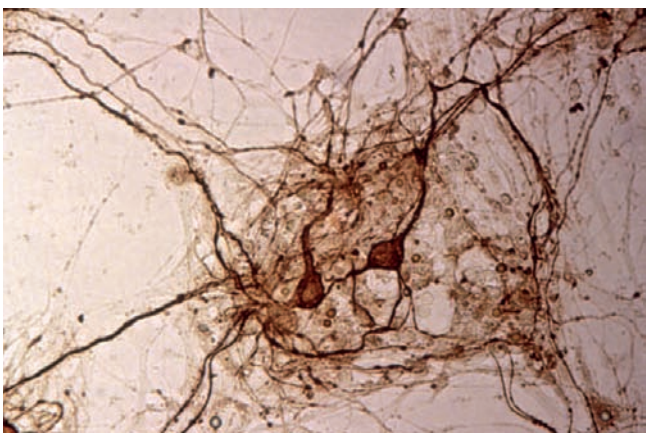

Fig. 1b: Chicken brain cells (8-day culture)

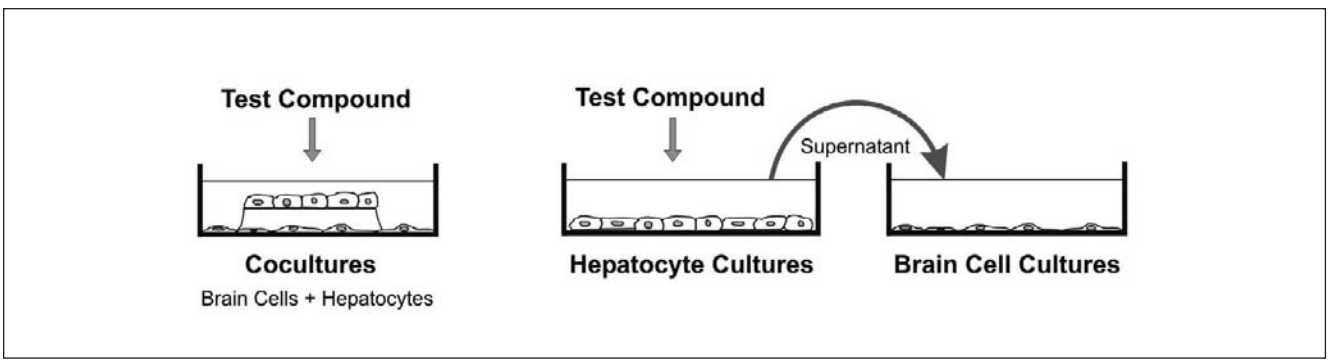

Fig. 2: Exposure of brain cells to liver-cell-mediated metabolites in co-cultures or via a supernatant (sequential cultures).

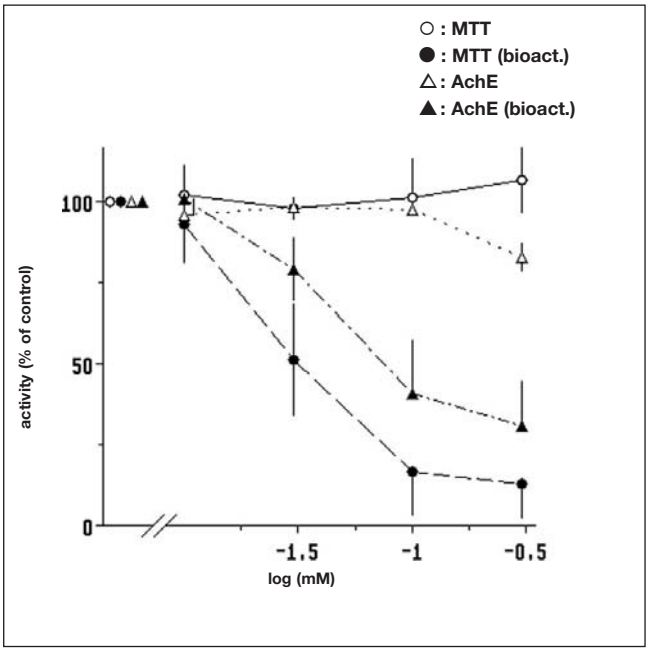

Fig. 3: Dose-response curves with cyclophosphamide: brain cells are exposed directly (△○) or to the supernatant (▲●) from hepatocyte cultures treated with CP.

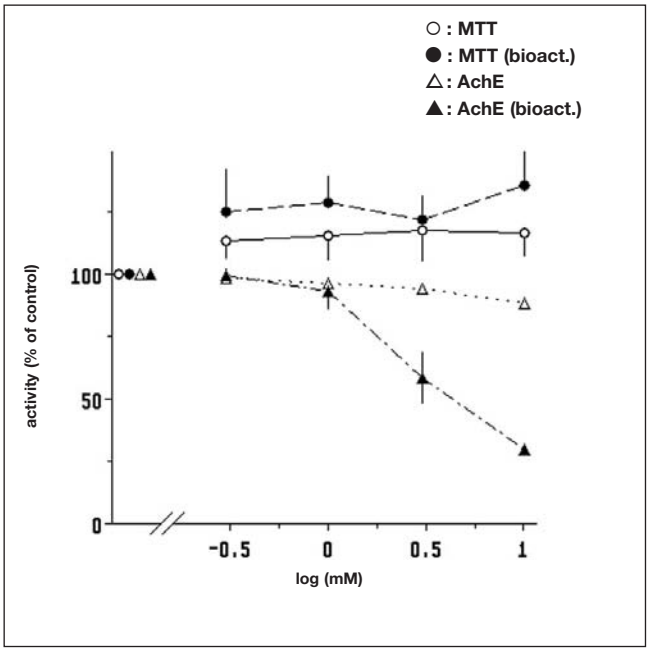

Fig. 4: Dose-response curve with isofenphos; brain cells show inhibition of AchE only after exposure to the supernatant (▲) from hepatocyte cultures treated with isofenphos at doses without reduced cell activity (MTT).

6-24 hours). Cultured chicken brain cells died only after CP had been in contact with hepatocytes (supernatant or co-cultures) (Fig. 3). This indicates that the metabolic competence of the hepatocytes is preserved and comparable to the situation in the living organism. Furthermore it indicates that the intermediates might have a sufficiently long half-life to reach the brain.

Brain-cell-specific functions can be impaired without cell death

As an overall rule, organ-specific toxicity should not be based on basal toxicity data. In most cases organ-specific toxicity is due to failure of proper functions. This should be mirrored in a corresponding choice of read-outs. In the range of concentrations used here, isofenphos inhibited AchE activity without affecting cell activity (MTT conversion), again only after contact with hepatocytes (Fig. 4). This inhibition (in this case the cholinergic nerves) prior to an overall cytotoxicity contrasts with the response to CP metabolites (Bruinink et al., 2002). Accordingly the present experimental model allows a distinction to be made between the induction of general cytotoxicity (MTT activity) and specific neurotoxic activity (inhibition of AChE activity without cytotoxicity) by chemicals and drugs.

Potential of co-cultures and sequential cultures

Co-cultures involving different cell types from the same tissue (e.g. nerve cells and glial cells or hepatocytes and Kupffer cells) allow intercellular interactions to be identified (including intercellular signalling) which take place within an organ and might affect cellular toxicity or the pharmacological behaviour of drugs. Individual cell populations can be pretreated with modulators of xenobiotic metabolism before being used in co-cultures. Such an approach enables specific aspects of pharmacological pathways to be investigated (Milosevic al., 1999).

Co-cultures of cells from different organs, e.g. the addition of hepatocytes to cultures of chicken brain cells or other extrahepatic tissue, provides a metabolic activation system comparable to that of the liver. Sequential testing of the cytotoxicity of the supernatant from hepatocytes treated with the parent compounds is a way of measuring the stability and half-life of the reactive metabolites under investigation. Combining the different set-ups helps to elucidate interactions which may be responsible for divergent results in toxicity and pharmacological tests *in vitro versus in vivo*.

Concerning actual exposure of the brain

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 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.

In conclusion, the *in vitro* set-up can be adjusted depending on the requirements for safety assessment. Even the most simple sequential test involving liver hepatocytes and chicken brain cells might be reliable enough to replace the hen test as proposed by an OECD guideline.

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Permanent Fish Cell Cultures as Important Tools in Ecotoxicology

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Summary
Permanent fish cell cultures such as hepatoma cells (PLHC-1) and gonadal cells (RTG-2) have successfully been used for acute toxicity assessment of a variety of environmental chemicals such as organotins, substituted phenols and pharmaceuticals. Cytotoxicity significantly correlates with physico-chemical properties such as lipophilicity of the compounds (log Dow). A significant correlation of in vitro with in vivo acute toxicity in fish (organotins, substituted phenols) and zooplankton Daphnia magna (pharmaceuticals) was found. This indicates the usefulness of fish cell lines for screening and toxicity assessment of chemicals within REACH. Furthermore, a transfection system based on PLHC-1 cells, was developed for the determination of estrogenicity of chemicals and environmental samples.

Keywords: fish cell line, ecotoxicity, REACH, estrogenic activity

Background Information

Environmental assessment of potentially hazardous chemicals

Numerous anthropogenic chemicals from household, industry and agriculture enter aquatic systems directly or via wastewaters. Residues in wastewaters consisting mainly of partially and highly persistent organic chemicals may harm fish, a factor that may contribute to population declines as currently observed in Switzerland. Among the prominent chemicals suspected to be involved are endocrine active chemicals. However, residues of veterinary and human pharmaceutical do also occur in significant concentrations in wastewater contaminated rivers and lakes. For the protection of aquatic systems, in particular of fish, new chemicals should undergo ecotoxicological testing before marketed. The environmental safety assessment of chemicals and pharmaceutical ingredients requires acute, and in many cases, chronic ecotoxicity tests.

In vitro systems such as fish cell lines have become of growing importance in ecotoxicology in the last decade.

The aim of this project was the assessment of the ecotoxicity of chemicals and pharmaceuticals including some mixtures using different in vitro cell culture methods.

The pending implementation of the European chemicals regulation REACH will lead to significant additional animal testing, in particular with fish. This project is aimed at reducing and replacing fish tests and demonstrates the advantages of in vitro tests wich maylead to better acceptance, because there is still a gap in full regulatory acceptance of cell culture systems.

In vitro systems in ecotoxicology

The development of *in vitro* assays in ecotoxicology is needed for scientific, animal welfare, and economical reasons. About 100,000 chemicals (including some 1,000 pesticides) are currently being used, and more than 1,000 enter the market every year. Their ecotoxicological properties should be assessed prior to release into the environment. *In vitro* systems could play a valuable role in such assessments, but they are only recently gaining recognition from governmental bodies and industry. Within the framework of REACH, cell culture systems gain further importance. Toxic effects are often species-specific, and consequently, toxicity towards fish can only be assessed in fish-specific systems. By using permanent fish cell lines, our goals were (i) to develop novel *in vitro* assays for the assessment of environmental toxicity to fish, thereby reducing and replacing animal testing, and (ii) to demonstrate the general usefulness of this approach in basic and applied ecotoxicology and as a consequence, for application as a screening tool and at the first step in the ecotoxicological assessment within the framework of REACH. After development of basic methods and application for different groups of compounds, we have used this approach recently for further development and analysis of a series of pharmaceuticals present in aquatic systems (Caminada et al., 2006).

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Fish cell line PLHC-1

The permanent fish cell line PLHC-1, derived from a hepatocellular carcinoma in the topminnow *Poeciliopis lucida*, has several advantages over currently used fibroblast-like cells. Firstly they have metabolic activities, necessary to study the metabolism of environmental chemicals acting *via* metabolic activation. Secondly PLHC-1 are easy to cultivate. Thirdly they contain an aryl hydrocarbon receptor and possess inducible and stable cytochrome P450 (CYP) enzymes, which are important for metabolism and detoxification of environmental chemicals. This cell culture system is ideally suited to assess the acute and chronic toxicity of chemicals, pollutants and environmental probes, and to derive structure-activity relationships within chemical classes.

Correlation of *in vitro* and *in vivo* toxicity

The cytotoxicity of more than 50 important environmental chemicals with various modes of action, including organotin (organic tin) compounds (Brüschweiler et al., 1995), chloro- and nitrophenols, sulfonic acids, and hormone-disrupting estrogenic chemicals (alkylphenols) have been assessed (Fent and Hunn, 1996; Brüschweiler et al., 1996). Inhibition of neutral red (NR) uptake into cells based on the accumulation of neutral red in lysosomes of viable cells and the tetrazolium salt reduction (MTT) assay based on the mitochondrial metabolic function were found to be most suitable tests, allowing rapid and reliable assessment. We have recently shown that these assays are very useful for the determination of cytotoxicity of 34 pharmaceuticals to which fish may potentially be exposed in wastewater-contaminated aquatic systems (Caminada et al., 2006).

An excellent quantitative correlation was found between the two assays (Caminada et al., 2006, Brüschweiler et al., 1995). Organotin compounds were the most toxic, followed by higher substituted phenols including estrogenic nonylphenol, lower substituted phenols, and sulfonic acids. The *in vitro* results showed a trend similar to the *in vivo* acute toxicity in fish. Hence, acute fish toxicity of chemicals acting via different modes of action can be estimated *in vitro*. Recently, we have also demonstrated that cytotoxicity was perfectly correlated with the lipophilicity of pharmaceuticals, defined by their log Dow in case of generally acting or narcotic compounds (Caminada et al., 2006). In addition, the cytotoxicity of pharmaceuticals having a general mode of action was clearly correlated with *in vivo* acute toxicity in the zooplankton organism *Daphnia magna*, for which acute data were available. PLHC-1 cells are therefore a promising tool in the toxicity screening and in the evaluation of chemicals prone to contaminate aquatic systems.

Detection of hormone-disrupting chemicals

Considerable public and scientific concern has arisen over chemicals that act on hormone systems, because of their negative effects on reproduction. *In vitro* systems to determine the estrogenic activity of chemicals (e.g. permanent fish cell culture systems) are urgently needed. We have developed a transfection assay based on transient transfection of PLHC-1 cells using plamids containing an estrogen receptor and a reporter gene (Fent 2001) for assessing estrogenic chemicals and estrogenic activity in environmental samples (Ackermann et al., 2002). Estrogenic chemicals that act via binding to the estrogenic receptor showed clear dose-response curves.

Relevance regarding the 3Rs

Fish cell culture systems using permanent hepatoma (PLHC-1) or RTG-2 gonadal cells (Caminada et al., 2006) are a promising tool for basic and applied research, and for routine ecotoxicology tests, namely for screening purposes, but also as a basic step in the assessment of chemicals within REACH. Our studies

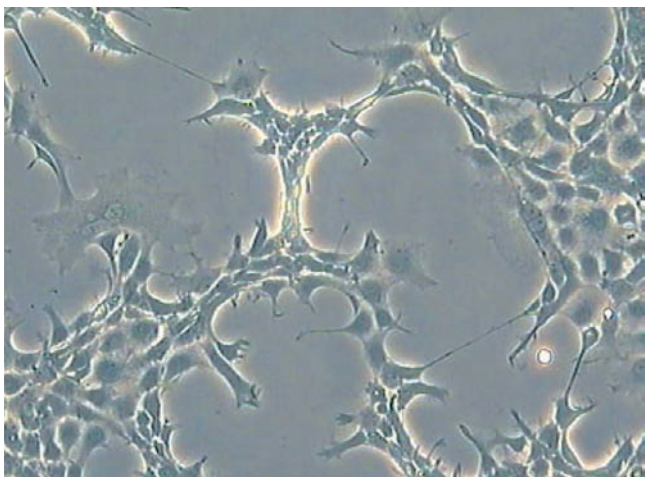

Fig. 1: Permanent fish hepatoma cells PLHC-1.

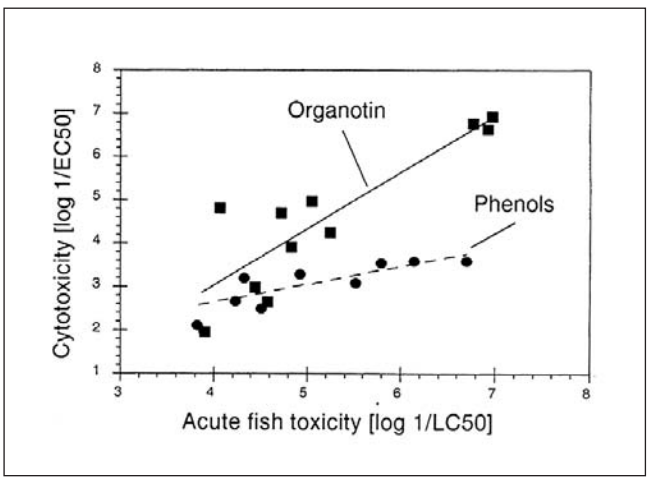

Fig. 2: Relationship between *in vitro* cytotoxicity and acute *in vivo* toxicity in fish demonstrated with two compound groups.

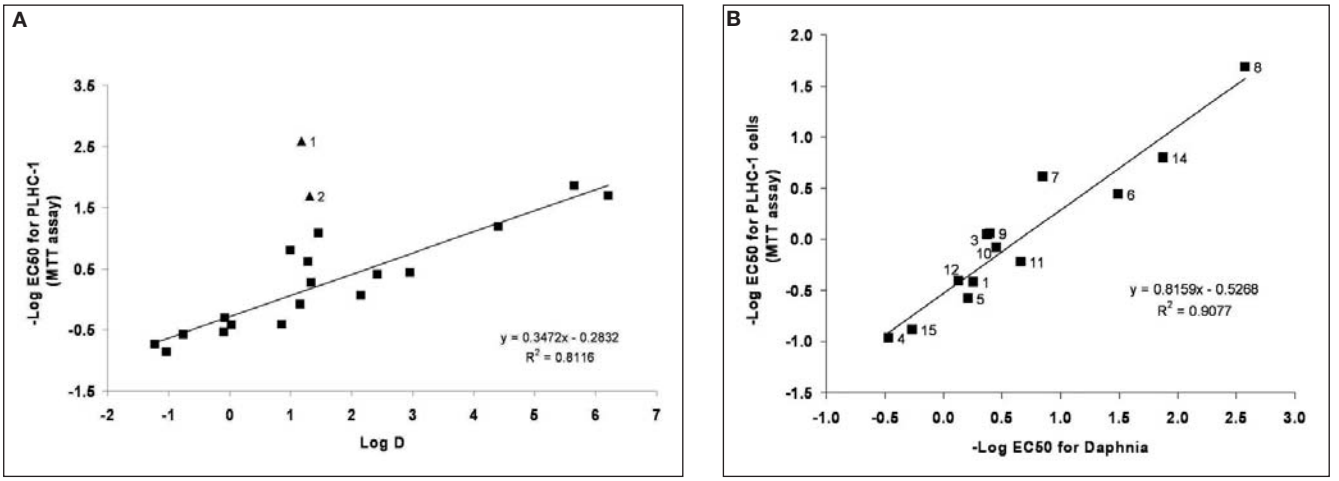

Fig. 3: A) Significant correlation between cytotoxicity of pharmaceuticals acting by a general (narcotic) mode of action and physico-chemical properties of the compounds.
B) Significant correlation between cytotoxicity and *in vivo* acute toxicity in *Daphnia magna*.

clearly demonstrate their usefulness for ecotoxicological research, rapid initial ecotoxicity screening and for environmental risk assessment of chemicals and environmental samples for cytotoxicity and estrogenicity. Cytotoxicity was correlated both with physico-chemical properties and *in vivo* acute toxicity. We are convinced that this helps in a reduction of animal testing in aquatic ecotoxicology.

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Generation of Parasite Cysts in Cultured Cells Instead of Living Animals

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Summary
In order to develop any effective means of prevention or therapy against Neospora caninum infection, all three life cycle stages of this parasite have to be taken into account. In this project, the in vitro culture of N. caninum bradyzoites containing tissue cysts has been developed, thus allowing to the initiation of stage conversion without the use of animal experimentation. This enables researchers to study novel aspects of stage differentiation, to investigate novel diagnostic antigens, and explore the usefulness of potentially interesting drugs and drug targets not only in tachyzoites but also in bradyzoites.

Keywords: parasites, neosporosis, bradyzoites, Vero cells

Background Information

Neospora caninum and neosporosis

Neospora caninum (Apicomplexa: Eimeriina: Sarcocystidae) is a Toxoplasma-like parasite that was first identified in 1984 in dogs with encephalomyelitis and myositis. It was later also reported in various species of livestock, especially cattle. In the USA, the EU, and many other countries worldwide, neosporosis is the leading diagnosed cause of abortion in cattle, and thus of major economic impact. One possible route of transmission of N. caninum is through the oral uptake of sporozoite-containing oocysts which are shed in the feces of infected dogs (= definitive host). Sporozoites then enter and traverse the intestinal epithelium, infect macrophages and lymphocytes which leads to dissemination throughout the body, and transform to the rapidly proliferating and disease-causing tachyzoite stage. Subsequently, the host immune response and possibly other factors trigger stage conversion of the parasite to the slowly proliferating N. caninum bradyzoites. Bradyzoites encapsulate themselves within intracellular cysts surrounded by a solid cyst wall. Oral ingestion of tissue cysts by dogs, e.g. through infected meat, will again lead to infection and subsequent oocyst shedding. Bradyzoites can survive within a latently infected but immuno-competent intermediate host for many years without causing any clinical symptoms. However, reactivation of bradyzoites during immuno-suppression (e.g. during pregnancy) leads to severe neosporosis, namely abortion or birth of weak offspring, in cattle.

Dog-cattle- and tachyzoite-bradyzoite stages

Two different intracellular stages of *N. caninum* occur in tissues of both final (dog) and intermediate (e.g. cattle) hosts: the actively proliferating and disease-causing tachyzoite stage has been found in many different tissues and cell types, can be vertically transmitted from mother to the fetus, and accounts responsible for acute disease. The slowly proliferating and tissue cyst-forming bradyzoite stage has so far been found only in the central nervous system and muscle tissue (Fig. 1). A third stage, the sporozoite-containing oocyst, is formed within the intestinal tissue of dogs and possibly other canids, is placed into the environment by fecal shedding, and is responsible for horizontal transmission from dogs to other animals (Fig. 1). Until recently, in vitro culture of *N. caninum* was limited to the tachyzoite stage, but now tissue culture procedures have been developed to achieve tachzyoite-to-bradyzoite stage conversion, and thus tissue cyst formation, *in vitro*.

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N. caninum bradyzoites represent highly relevant targets for intervention

Bradyzoites are of particular epidemiological relevance for two reasons (reviewed in Hemphill et al., 2004; 2006): First, if the infected host becomes partially immuno-compromised, as occurs during pregnancy, bradyzoites are reactivated, which leads to bradyzoite-to-tachyzoite stage conversion, and tachyzoites will infect placental and fetal tissue, resulting in abortion or severely impaired offspring. Secondly, in dogs that consume meat infected with *N. caninum* bradyzoites, the parasite is likely to undergo sexual reproduction within the intestine. This produces oocysts that are excreted in the faeces and can contaminate soil, water and animal fodder. Thus, strategies to prevent neosporosis must also take into account the importance of bradyzoites. This has earlier led to the development of an artificially immuno-compromised mouse model for the production of *N. caninum* bradyzoites, with obvious unpleasant side effects for these animals (McGuire et al., 1997; Gondim et al., 2001). However, this laboratory animal model-based

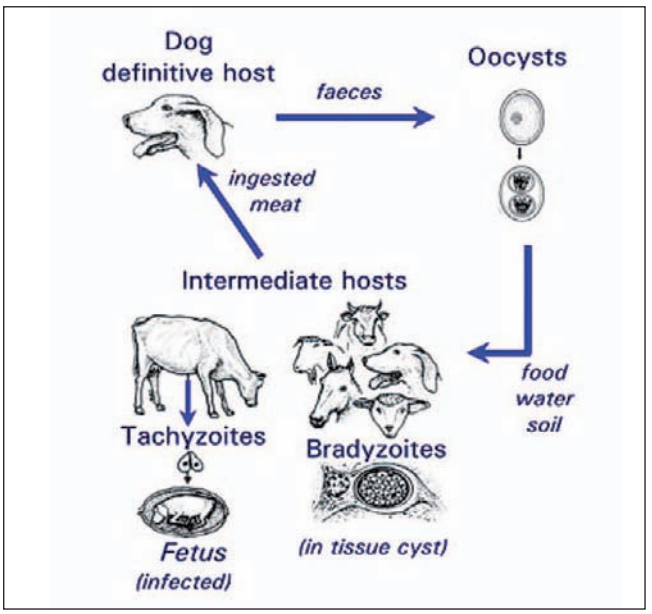

Fig. 1: Life cycle of the parasite *Neospora caninum*

method has proven rather unreliable, and requires large numbers of animals.

Nitric oxide-induced *in vitro* tachyzoite-to-bradyzoite stage conversion in cell culture

Preliminary experiments to achieve *N. caninum* stage conversion in cell culture were based on the hypothesis that the parasite would undergo tachyzoite-to-bradyzoite stage conversion under stress conditions that lead to a severe inhibition of proliferation, synthesis of tissue cyst wall components, and expression of bradyzoite-specific antigens. We systematically investigated the effects of variations in cell culture conditions for these parameters, including the use of different host cell types. Following a long phase of testing and optimizing conditions, *N. caninum* tissue cyst formation *in vitro* was first achieved by treatment of infected murine epidermal keratinocyte (MEK) monolayers with a high dose (70 µM) of the nitric oxide donor sodium nitroprusside (SNP)) for up to 8 days (Vonlaufen et al., 2002).

Subsequently, a modified and far more economical procedure was developed which enabled us to produce *N. caninum* tissue cysts containing bradyzoite stage parasites employing Vero cell cultures (Vonlaufen et al., 2004). Immunofluorescence using antibodies directed against a number of cyst wall antigens showed distinct staining of the cyst periphery, indicating that indeed a cyst wall-like structure was synthesized (Fig. 2). This was confirmed by electron microscopy, which clearly demonstrated the formation of a distinct cyst wall in bradyzoite, but not tachyzoite *in vitro* cultures (Fig. 3). Additionally, a protocol was elaborated to purify these bradyzoites from cell cultures, and this has now opened avenues to dissect this important life cycle stage of the parasite at the biochemical and molecular level without the extensive use of animal models.

Reduced infectivity of *in vitro* generated bradyzoites?

A major frustration has been the outcome of 2 separate experimental trials in which dogs were fed *in vitro* generated *N. caninum* tissue cysts obtained through mass production in Vero cells. While some of the dogs sero-converted, thus infection was initiated, we consistently failed to detect *N. caninum* oocysts in the faeces of these dogs. Either these oocysts were not produced at all, or only in low amounts and thus were not detectable using conventional coprology. These experiments actually raise concerns about the biological infectivity of these *in vitro* generated bradyzoites. However, experimental oral infection of dogs with contaminated meat does not always lead to detectable oocyst shedding (Gondim et al., 2005), thus this question remains to be clarified.

The *in vitro* approach facilitates investigations of mechanism

To our satisfaction, the application of 3R principles has come together with improved scientific impact. Other groups have taken up the methodology to generate *N. caninum* bradyzoite

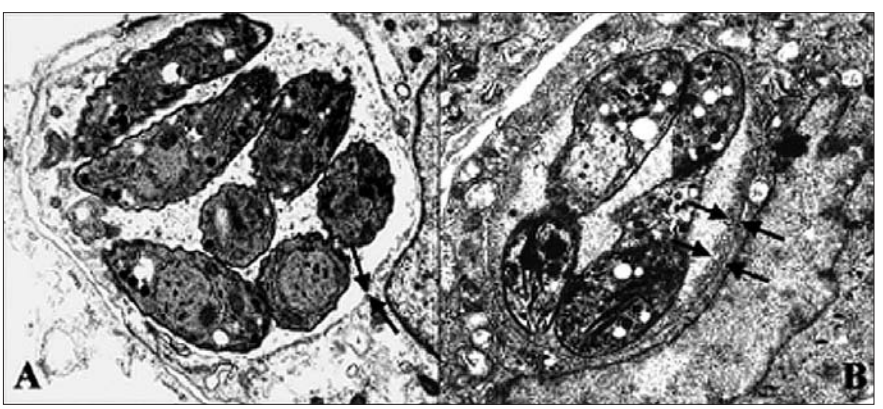

Fig. 2: *In vitro* stage conversion of *N. caninum* investigated by double-immunofluorescence.
Note the downregulation of expression of the tachyzoite antigen NcSAG1 in those parasites which exhibit expression of the bradyzoite antigen NcBAG1. Bars = 20 µm

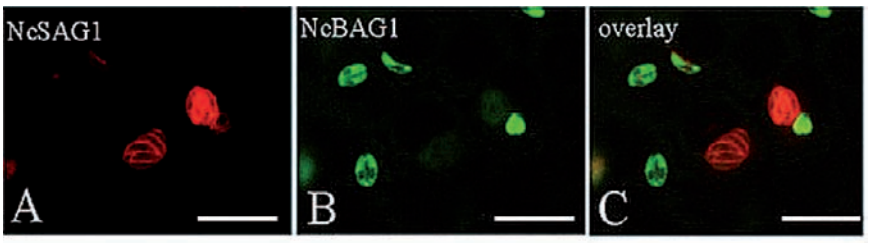

Fig. 3: Electronmicroscopical comparison of *N. caninum tachyzoites* (a) and bradyzoites (b) cultured *in vitro*.
Note that both stages are located within an intracellular vacuole, but bradyzoite vacuoles (b) exhibit a defined cyst wall (thick dark structure between the arrows), which is lacking in tachyzoites (a) (cf. arrows). Bars = 1,3µm in (a) and 0.8 µm in (b).

tissue culture models by essentially the same method, in some cases, however, employing other cell types (for review see Hemphill et al., 2006). In addition, we have been able to apply this *in vitro* model to investigate differential expression of *N. caninum* antigens in several studies (Hemphill et al., 2004; 2006) and also investigated the differences in bradyzoite- and tachyzoite-host cell receptor-ligand interactions (Vonlaufen et al., 2004; 2007).

The *N. caninum* bradyzoite tissue cyst model, in conjunction with conventional tachyzoite culture, is currently successfully used:

- as a first-round *in vitro* screening tool to assess the effects and optimize the efficacy of anti-protozoal drugs such as thiazolides (Esposito et al., 2007)
- and pentamidines (unpublished)
- for the identification of potential drug targets in *N. caninum*-infected host cells
-as a model to identify stage-specifically expressed bradyzoite antigens (for review see Hemphill et al., 2006)
-for improvement of immunodiagnosis and/or vaccine development
- to investigate the stage-specific gene expression and/or localization of *N. caninum* antigens to increase our understanding of the tachyzoite-to-bradyzoite conversion process and the events leading to reactivation (Vonlaufen et al., 2004).

Outlook

As a further development of this work, we have recently established and characterized a protocol for the tissue culture of canine intestinal epithelial cells, with the aim to generate an immortalized canine intestinal cell line (3R project 85/03; Golaz et al., 2007). The overall goal of this project has been to develop a tissue culture model to produce *N. caninum* oocysts, which would enable us to generate all stages of the life cycle of this parasite *in vitro*. This would render animal models largely redundant. Work is currently in progress to achieve this goal.

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Aggregating Brain Cell Cultures: Investigation of Stroke Related Brain Damage

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Summary
Aggregating brain cell cultures are three-dimensional primary cell cultures derived from embryonal rat brain cells. Within 3-4 weeks in culture under continuous agitation, they exhibit organotypic structures and functions. The transient arrest of agitation induced adverse effects comparable with those occurring in the ischemic brain in vivo. This culture system therefore offers a suitable in vitro model for the elucidation of ischemia-related pathogenic processes in the brain.

Keywords: brain, aggregate culture, ischemia

Background Information

Stroke related brain damage

The risk of a cerebrovascular accident such as a stroke doubles every 10 years from the age of 45 onwards. At the age of 75, the incidence reaches about 2%. Ischemia-related brain damage is thus one of the most important health problems in industrialised countries. This situation is likely to become further aggravated as the average age of the population continues to rise.

The extent of brain tissue damage depends on the brain region affected as well as on the duration and degree of decreased tissue blood supply. In regions of incomplete ischemia, selective neuronal damage and delayed cell death are observed.
A promising therapeutic strategy would be to develop medications which can interrupt early stages in the neuro-degenerative cascade. To find such medications, the cellular and molecular reactions involved in pathogenicity need to be fully elucidated. Accordingly, research in this area has been given high priority, in both areas of basic research and drug development.

The brain is highly vulnerable to ischemia

The interruption of blood supply to the brain (ischemia) deprives brain cells of glucose and oxygen, causing irreversible brain damage within minutes. The brain is particularly vulnerable to ischemia because of 1) the very high rate of oxidative metabolism in this organ, requiring a continuous supply of oxygen and glucose, 2) the metabolic interdependence of the two types of brain cells, neurons and astrocytes, and 3) the sensitivity of neurons to changes in ion homeostasis brought on by ischemia.

Cells in ischemic brain tissue undergo a number of changes: they rapidly lose their energy stores, their membranes become depolarised, calcium loads increase, reactive oxygen species are produced and excitotoxic effects are found. These biochemical changes are followed by irreversible structural changes and cell death by apoptosis and/or necrosis. The neurons are particularly sensitive to injury, whereas the reactivity of glial cells (astrocytes and microglia) tend to amplify and propagate the adverse effects.

Animal models *versus* cell cultures

Today, research on ischemia relies mainly on animal models. Unfortunately, such studies involve a high degree of discomfort for the animal. *In vivo* approaches involve procedures such as the surgical occlusion of major arteries, or (less invasive) the induction of reproducible infarcts in a selected area of the brain by means of artificially induced thrombosis. The animal models appear to closely reproduce the characteristics of gray matter ischemic injury in humans; however, the patterns of pathology in the body resulting from ischemia in a particular region of the brain can be quite different than those seen in humans.

Diverse cell culture models have been developed to study mechanistic aspects of cerebral ischemia. These include monolayer cell cultures and three-dimensional cultures such as aggregated brain cells and brain tissue slices. Each model has specific advantages and disadvantages (Tab. 1). However, neither animal models nor culture systems are able to reproduce all the characteristics of ischemic pathologies encountered in humans.

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Ischemic pathways can be induced....

Aggregating brain cell cultures are prepared from embryonic rat or mouse brain tissue (Honegger and Pardo, 1999). The cells can be maintained for several weeks as floating (suspension) cultures under continuous gyratory agitation (swirling at 80 rpm) in an incubator (Fig. 1).

Ischemic conditions can be induced in the cultured aggregates simply by stopping the gyratory agitation for different lengths of time, and then restoring again normal culture conditions (Pardo and Honegger, 1999a). The effects of transiently stopping the circulation of medium and air to the cells are assayed one week after the insult. Using cell-type specific biochemical markers of normal brain cell function (for example, enzyme activities such as glutamic acid decarboxylase (GAD) and glutamine synthetase), it was found that the transient immobilisation caused selective neuronal cell death and delayed glial reactions similar to those found in incomplete ischemia *in vivo* (Pardo and Honegger, 1999b). Similar adverse effects were observed in cultures subjected to hypoxia or hypoglycemia (Pardo and Honegger, 2000). The latter condition also showed significant alterations in the metabolism of amino acids and the accumulation of neurotoxic ammonia (Honegger et al., 2002).

....and prevented

Glutamate stimulation and excessive calcium influx are thought to be critical events in ischemia-induced neurotoxicity. Accordingly, it might be possible to protect neurons by inhibiting these early steps along the ischemic cascade. Our results

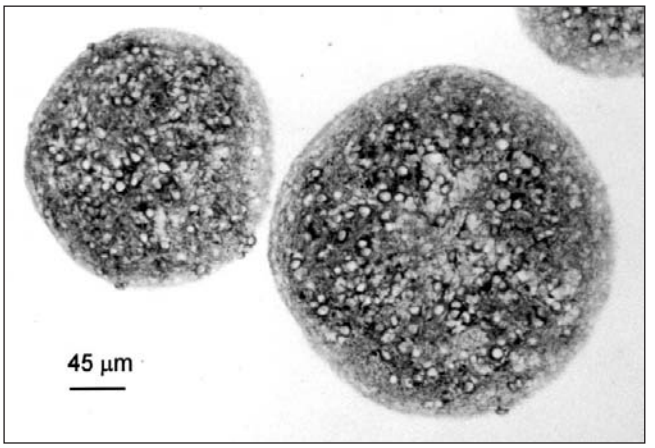

Fig. 1: Each flask contains over a thousand of spherical cell aggregates. Sections of differentiated aggregates showing stained neurons – cell bodies and neurites stained for microtubule associated protein (MAP-2), surrounded by unstained glial cells.

Tab. 1 Comparison of *in vitro* culture models

In Vitro Model	Specific advantages	Disadvantages
Monolayers		
tumor cells	- defined cell population - direct access for manipulations	- high variability - transformed cells
embryonic brain cells	- direct access for study of morphology immunocytochemistry - biochemical analyses	- partial loss of brain specific functions - ischaemia inducible only under harsh conditions - limited cell-cell interactions
Cell Aggregates		
3D-cultures with brain embryonic cells and cell-cell interactions	- high yield and reproducibility - undergo extensive maturation - cell-cell interactions involved in cascade of ischemia induced adverse effects	- limited access for microscopic observations and electrophysiology
Tissue Slices		
from postnatal brain	- preserve to some extent the organotypic structure of brain regions - direct access for manipulations	- low yield and low reproducibility - repetitive sampling not possible

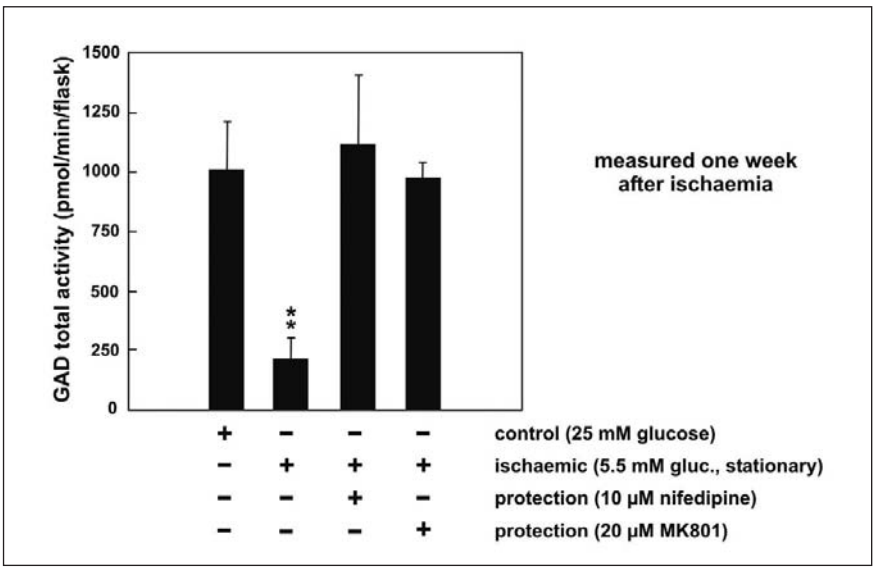

Fig. 2: Protection from ischemia induced neurotoxicity by a calcium channel blocker (nifedipine) or an NMDA receptor antagonist (MK801). GAD activity is used as a marker of normal brain cell function.

with aggregating brain cell cultures indicate that this is indeed the case: Nifedipine, a blocker of voltage-gated calcium channels, and MK801, an antagonist of the NMDA ionotropic glutamate receptor, were both able to inhibit the loss of GAD activity (Fig. 2).

Aggregating brain cell cultures, a promising *in vitro* model

In summary, aggregating brain cells exhibit unique features useful for the study of pathogenic mechanisms involved in cerebral ischemia. The culture system permits easy handling, repetitive sampling and post-injury follow-up studies. Data obtained so far demonstrate that many of the fundamental pathogenic processes involved in ischemia can be studied and recognised in aggregating cell cultures. A thorough knowledge of the mechanisms involved in ischemic cell damage will allow us to precisely identify the critical steps and cellular and subcellular targets in this process. This in turn will enable the development and testing of potential therapeutic agents designed to inhibit or counteract specific steps in the ischemic cascade of neurodegeneration.

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Formation of New Blood Vessels in the Heart Can be Studied in Cell Cultures

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Summary

Controlled induction of the formation of new microvessels, i.e., therapeutic angiogenesis, may be used one day to treat patients that for example had suffered a myocardial infarction. Experimental models of angiogenesis in the heart in vivo substantially stress the animal. We therefore developed a model of angiogenesis of the heart in vitro, where mouse and rat heart pieces are stimulated under controlled conditions in a three dimensional matrix. Capillary-like sprouts emerging in these cultures represent early to midterm steps of angiogenesis and can be quantified to study potential angiogenic compounds and underlying mechanisms.

Keywords: therapeutic angiogenesis, ischemia, neovascularisation, three-dimensional matrix, heart, capillaries

Background Information

Formation of new blood vessels, a new therapeutic concept

Angiogenesis is a process of creating new capillaries 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 insufficiently oxygenated. 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 during angina pectoris or after a heart attack. In patients with coronary heart disease hypoxia and myocardial ischemia often affect the function of the heart and results in a significant morbidity and mortality. Stimulation of angiogenesis by pharmacological or molecular methods (therapeutic angiogenesis) would generate the formation of new coronary collaterals in the myocardium, i.e., small branches of arteries and veins that newly develop to bypass narrowed or blocked segments. Improving coronary collateral circulation would limit myocardial ischemia and is therefore a promising new concept (Cao et al., 2005).

Accordingly, substantial research resources are currently being invested into studying the therapeutic stimulation of angiogenesis. Multiple animal studies and some preliminary human studies have confirmed the concept of stimulation of collateral circulation by pharmacological or molecular methods. 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.

Studying angiogenesis in three-dimensional ell cultures

Models of angiogenesis *in vitro* are based on the capacity of activated endothelial cells (cells forming the walls of blood vessels) to invade three-dimensional substrates. These substrates (Matrix) may consist of collagen gels, plasma clot, purified fibrin, Matrigel, or a mixture of these proteins with others. It is possible to embed and culture pieces of blood vessels (vascular explants such as aortic rings) and to observe cells sprouting and forming capillary-like structures (CLS) (Nicosia and Ottinetti, 1990). These models allow the preservation of the vessel architecture during the *in vitro* assay, and thus are close to an "*ex vivo*" model (Kiefer et al., 2004). We have further developed the original angiogenesis *in vitro* model for aortic explants by Roberto Nicosia (Nicosia and Ottinetti, 1990), for the use with heart explants. Briefly, pieces (ca. 1 mm^3) of left ventricular myocardium of rat or mouse hearts are embedded in a fibrin-gel, overlayed with growth medium and angiogenic stimulants (agonists) and/or inhibitors (antagonist). These cultures are then incubated for 7 days. Active molecules are added again every third day, and oxygen saturation can be lowered down to 1% O_2. A single mouse heart allows for assessing more than 20 different samples, each tested in octuplicates. After 7 days CLS quantification is carried out by visual scoring according to a scale that was defined by measuring the area of capillary sprouts by morphometric software (Fig. 1).

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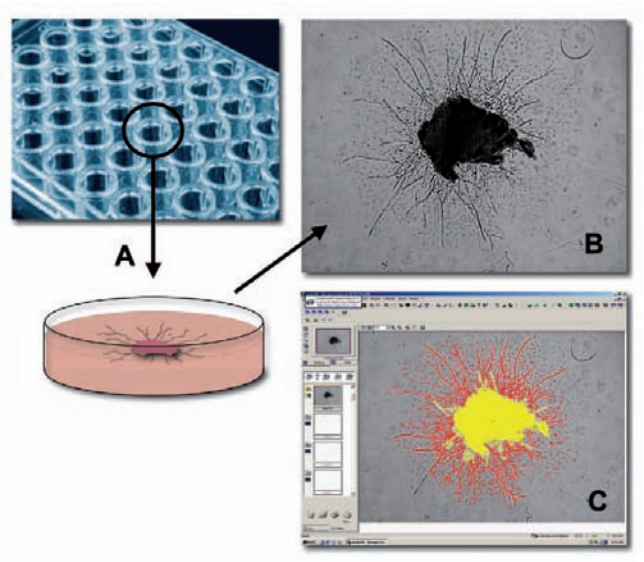

Fig. 1: Three-dimensional angiogenesis assay *in vitro*.
During *in vitro* culture of heart or aortic tissue in fibrin gels, endothelial cells within the tissue are activated (by growth factors, hypoxia), limited proteolysis of surrounding matrix occurs, endothelial cells elongate, migrate and proliferate into the matrix and attract pericytes to stabilise the nascent capillary tube. Capillary tubes bifurcate and give rise to a primitive capillary tube-network (A). After 7 days of incubation in 48 well plates, pieces of heart are photographed digitally at 4X magnification (B). The total surface of sprouts and the piece of heart is measured (C). The fraction of heart and sprouts are set in relation to the total surface. Example: surface of sprouts is 81356 µm2 (red colouring) and surface of the heart is 152580 µm2 (yellow colouring); total surface is 233936 µm2; thus sprouts are 35% of the total surface corresponding to an angiogenic index of 4.

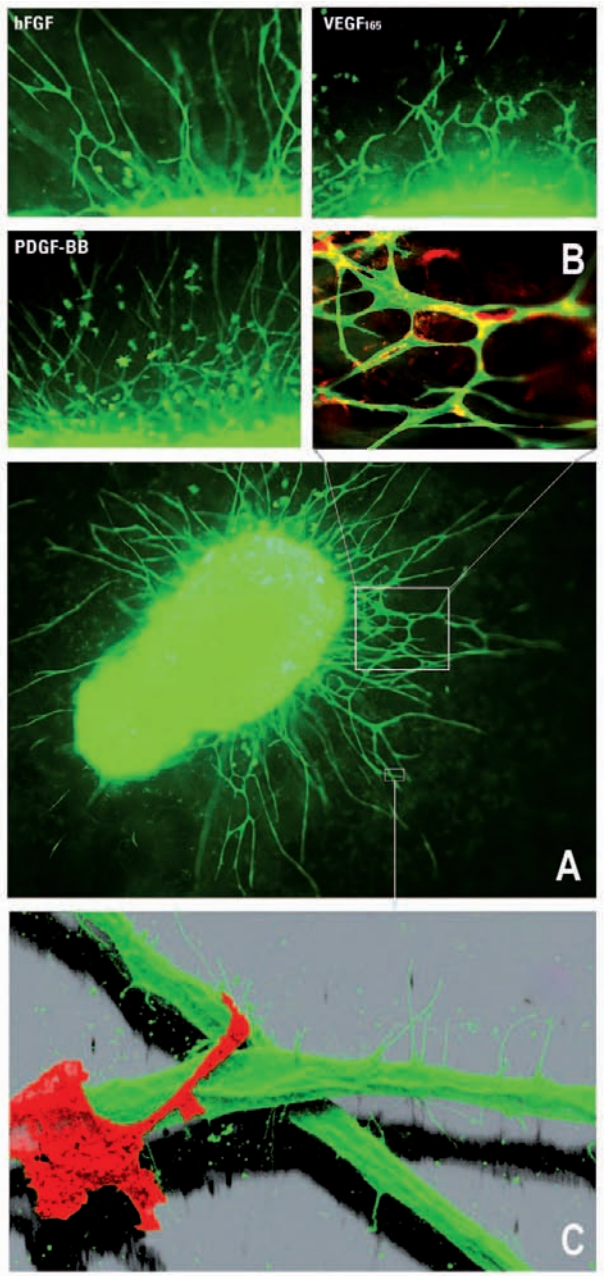

Fig. 2: Differential morphology of CLS can be observed after administration of different angiogenic molecules to mouse heart explants: basic Fibroblast Growth Factor – bFGF (10 ng/ml), Platelet-Derived Growth Factor B-dimer -PDGF-BB (10 ng/ml), Vascular Endothelial Growth Factor – VEGF (5 ng/ml).
A) In-gel characterization of CLS by FITC-coupled lectin G. simplicifolia (green fluorescent) for endothelial cells and B) magnification including Cy3-coupled anti α-smooth muscle actin-staining (red staining) for smooth muscle cell. C) Confocal micrograph of two crossing endothelial tubes. Tiny filpoodia reach out and sense the environment for neighboring endothelial tubes.

Reproducibility of the *in vitro* system

First we tested basic culture conditions to ensure reproducibility of the assay. In contrast to aortic explants forming CLS in serum-free conditions, CLS formation of heart explants requires fetal calf serum (5%) to ensure survival, whereas higher FCS concentrations mask the activity of angiogenically active molecules. In heart explants from mice younger than 8 weeks, CLS formed spontaneously – an unwanted response for testing the stimuli of angiogenic active factors. In 12-week-old adult mice however, virtually no CLS was formed under normoxia (standard culture condition, 20% O_2). Sex of the animals did not account for differences observed in CLS formation. Thus, for our future experiments, we chose adult mice, older than 12 weeks, to abolish the effect of active angiogenesis during adolescence of animals. Under hypoxia (1-3% O_2) however, CLS formation substantially increased, an established feature of angiogenesis, observed *in vivo* and *in vitro*.

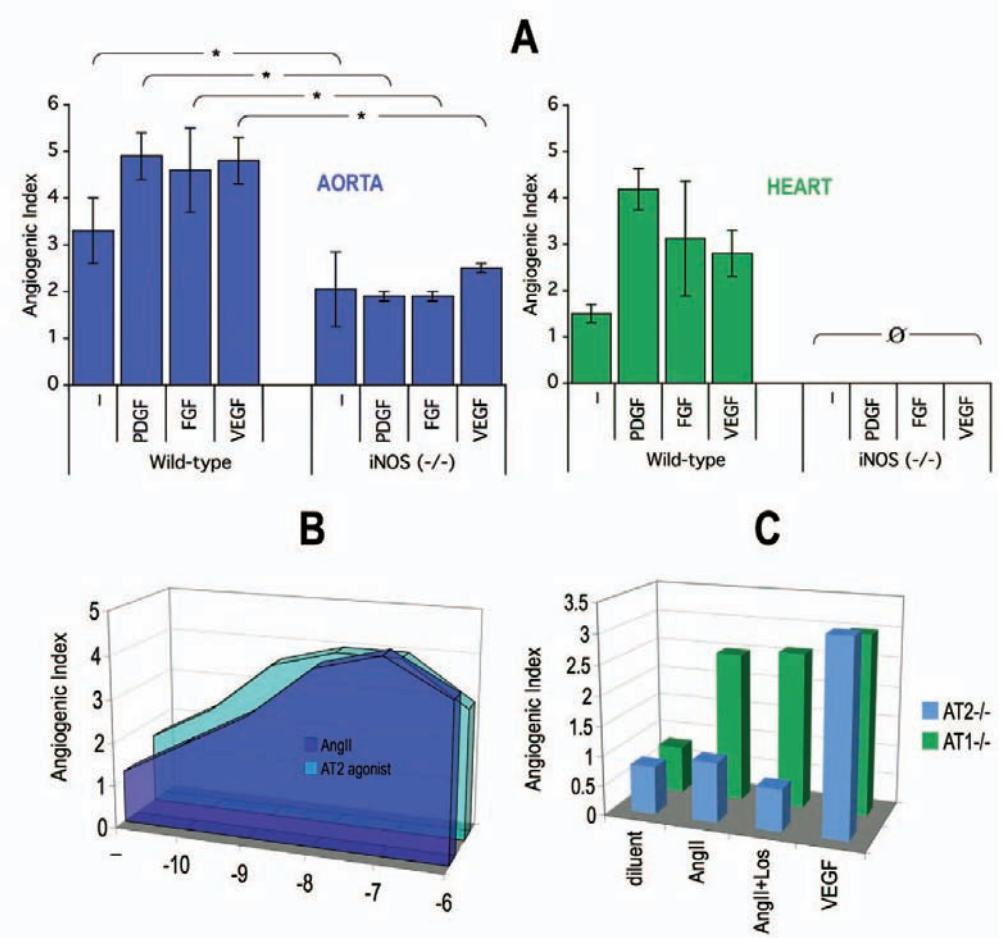

Fig. 3A: *In vitro* angiogenesis of the heart is abrogated under hypoxia in iNOS (–/–) mice.
In vitro angiogenesis of aortas (blue columns) and hearts (green columns) 7 days after addition of growth regulatory molecules: platelet-derived growth factor (PDGF)-BB (10 ng/ml), fibroblast growth factor (bFGF) (10 ng/ml) and vascular endothelial growth factor (VEGF)164 (5 ng/ml).
A standardized scale ranging from 0 to 6 indicates the degree of sprouting (angiogenic index). Data points given represent the mean of three experiments ± SEM. Ø – Sprouting undetectable; iNOS – inducible nitric oxide synthases.

Fig. 3B: Ang II induces dose-dependent sprouting *in vitro* in adult mouse hearts in hypoxia.
Pieces of mouse heart were stimulated with Ang II (blue graph) or AT2 agonist CGP-42112 (green graph) from 10-10 to 10-6 mol/L and incubated under hypoxia for 7 days. Data points represent the mean of 5 independent experiments. C. Ang II–induced angiogenesis is impaired in hearts from AT2-/- but not from AT1-/- mouse. Pieces of mouse heart from AT2-/- (blue columns) and AT1-/- mice (green columns) were stimulated with Ang II (10-7 mol/l) alone or in combination with AT1 blocker losartan (Los; 10-6 mol/l) and incubated under hypoxia for 7 days. VEGF164 (10 ng/ml) was used as a positive control. Data points represent the mean of 5 independent experiments.

Differential and *in vivo* like response toward angiogenic factors

Different classic angiogenic molecules induced diverse CLS patterns: Platelet Derived Growth Factor (PDGF-BB) induced outgrowth of a mixture of organized branched endothelial sprouts, unorganized single endothelial cells and pericytes/ smooth muscle cells. In contrast, basic Fibroblast Growth Factor (bFGF) induced mainly unbranched, elongated CLS. Vascular Endothelial Growth Factor (VEGF165) induced elongated CLS, and branching appeared complex (Fig. 2; bFGF, PDGF-BB, VEGF165). Independent of the morphology induced by different agonists, outgrowing sprouts were composed of endothelial cells by more than 90% when outgrowing cells were subcultured and assessed by endothelial cell marker CD31, Dil-Ac-LDL. Double in-gel-staining with FITC-coupled lectin G. simplicifolia (green fluorescent) and Cy3-coupled antibody against α-smooth muscle actin (red fluorescence) revealed endothelial sprouts with single attached smooth muscle cell-like cells (pericytes) (Fig. 2A-2C). Pericyte attachment in forming endothelial tubes has been observed *in vivo* and contributes to vessel remodeling, maturation and stabilisation. Also tiny filopodia, emerging from endothelial sprouts, can be observed by confocal microscopy (Fig. 2C). These filopodia sense neighbouring endothelial tubes and potentially guide network formation.

Nitric oxide as inducer of angiogenesis (Munk et al., 2006)

Nitric oxide (NO) promotes not only blood vessel relaxation and regulates vascular tone, but is critical for angiogenesis initiation and modulation (Sieber et al., 2001; Sumanovski et al., 1999). In this study we have investigated whether inducible NO synthase (iNOS) is required for angiogenesis *in vitro* of the heart versus angiogenesis originating from aorta.

We found that *in vitro* angiogenesis of the heart in mice lacking the iNOS gene (iNOS–/–) under hypoxia was totally abrogated and endothelial sprout formation could not be restored with classic angiogenic growth factors (Fig. 3A). In contrast, *in vitro* angiogenesis in aortas was still present, albeit reduced and without a response to angiogenic growth factors (Fig. 3A).

Thus, our results suggests that angiogenesis is regulated in an organ specific way and requires iNOS in the heart. iNOS-independent pathways may exist that can regulate angiogenesis in aorta and that are not present or less active in the hypoxic heart. This supports the need of organ specific models for angiogenesis and the concept that the angiogenic response in the adult mouse heart is far more restricted than in other organs.

Vasoactive peptides and their role in angiogenesis (Munk et al., 2007)

The vasoactive peptide angiotensin II (Ang II) is a key regulator of blood pressure. Most of the Ang II cardiovascular effects, for example, vasoconstriction, are attributed to angiotensin receptor 1 (AT1). AT1 is ubiquitously expressed, whereas the AT2 receptor is upregulated in response to ischemia and inflammation. Previous studies have shown that the AT2 receptor may interact with the bradykinin receptor, the B2 kinin receptor (BK2), during signaling. We have therefore investigated the mechanism of heart angiogenesis in response to Ang II under conditions of normoxia and severe hypoxia by dissecting the role of AT1 and BK receptor subtypes using the model of angiogenesis *in vitro*. We found that Ang II and AT2 receptor agonists dose-dependently induced angiogenesis *in vitro* of the heart and required hypoxic conditions (Fig. 3B). However, Ang II was not angiogenic in mice hearts lacking AT2 receptor or, interestingly, the BK2 receptor, whereas no impairment of angiogenesis was observed in hearts from AT1-/- mice (Fig. 3C). We could corroborate these findings when using specific pharmacological antagonists against AT1, AT2, BK1 and BK2 receptors. We concluded that angiogenesis induced by Ang II requires signaling through the AT2 receptor and is mediated by an increase in BK production and activation of the BK2 receptor. Currently, we are using the assay to assess mechanisms of BK2-dependent angiogenesis.

A versatile *in vitro* model

Thus, a wide range of questions connected with therapeutic angiogenesis can be answered without the use of *in vivo* experiments: (1) Induction and repression of early to midterm morphogenic steps in angiogenesis can be investigated in mouse and rat hearts. (2) Pharmacological compounds can rapidly be screened using only a small number of animals. (3) Assays can be performed under controlled experimental conditions, such as under a distinct oxygen saturation. (4) The role of specific genes in heart angiogenesis can be investigated by using tissue from knockout mice.

This assay has gained attention by other research laboratories, particularly because of the possibility to use capillary sprouts for *in vitro* immunolocalization of proteins (Fig. 2).

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The Tick Blood Meal: From a Living Animal or from a Silicone Membrane?

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Summary
An artificial feeding unit with a reinforced silicone membrane to replace host skin provides ticks with a perch over blood with a tick attachment rate of 75-100%. Some 5 mg of an acaricide like fipronil is sufficient to establish survival curves over different doses down to ppb levels in blood. This in vitro feeding assay for hard ticks is more advantageous than in vivo trials on animals.

Keywords: reduction, replacement, veterinary drugs, tick, diagnostic, parasite, ectoparasites

Background Information

The tick blood meal

The prevention of tick bites in animals reduces tick-transmitted diseases such as anaplasmosis, babesiosis, theileriosis and heartwater disease. The duration of the tick's blood meal takes 2 to 14 days depending on whether it is a larva, nymph or adult. During the first days of feeding, only small amounts of blood are imbibed. However, this a period during which the tick undergoes a variety of physiological changes to accommodate the blood meal in females. Numerous physiologically active agents are injected by the parasite into the feeding lesion inducing strong inflammatory, vasodilatory and immunological responses by the host. The tick's engorgement occurs during the last 24 hours of feeding during which the females imbibe 2 to 8 times as much blood as they finally gain in weight and multiply their body mass by as much as 100 fold with protein and lipid rich nutrient for the production of thousands of eggs. Males do not engorge themselves with blood. In some ticks (e.g. the European tick, Ixodes ricinus), every life stage feeds on a different host: larvae and nymphs mainly on small vertebrates and females on sheep, deer and other larger mammals. As man is occasionally parasitised, this contributes to this tick's role as a vector of spring-summer meningoencephalitis and bacterial (Lyme Borreliosis) diseases.

Animals as hosts for ticks

Animal husbandry could not be practised over large areas of the planet without acaricides. This persistent reliance on pesticides has led to the development of resistance in ticks against the major classes of acaricide treatments. There is a continual requirement for new types of molecules to target physiological processes that are crucial to tick survival. The development of animal health products against ticks requires hundreds of cattle, dogs, rabbits and gerbils for *in vivo* trials with acaricides, placing the annual worldwide use of animals in acaricide research in the tens of thousands. Small mammals used in such trials may suffer from skin inflammation and anaemia, and may be submitted to restrictions by the Elizabethan collar inhibiting grooming behaviour. For controlled studies, dogs have to be kept in small cages, cattle are kept in isolation, in climatic boxes, where their movement is confined. Apart from the ethical aspects of using experimental animals, the costs of maintaining suitable hosts for ticks are high.

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In vitro feeding: outwitting the ticks

Tick control on animals is achieved either through contact with a product applied either topically or orally. Ideally, an *in vitro* assay should permit both the assessment of products that either affect a tick's capacity to attach for a blood meal, or that restrict feeding once the tick has started to take blood. When ticks start to attach to the skin, they penetrate the uppermost keratin rich *stratum corneum* with outward lacerating movements of their cutting mouthparts. Strong retrograde food canal denticles anchor the tick in the skin (Fig. 1) allowing the cutting mouthparts to move deeper until the corium containing blood vessels is reached. The artificial feeding unit, that we have developed, has a silicone membrane that replaces host skin and provides the tick with a similar perch over blood (Fig. 2).

The feeding unit consists of a silicone membrane reinforced with cellulose rayon glued across one end of a piece of acrylic glass tubing (44 mm high and 26 mm i.d.). The membrane is a modified version of one developed in a previous 3R project (Kuhnert et al., 1995; Kuhnert 1996). We rendered the membrane softer to facilitate feeding by the European tick, *Ixodes ricinus* (Kröber and Guerin, 2007a). The new membrane

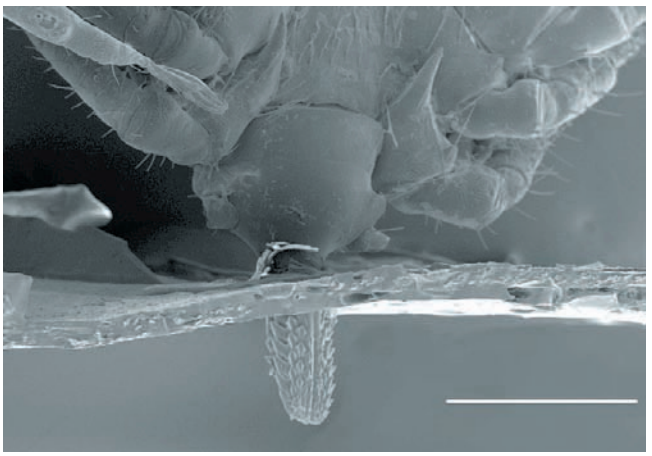

Fig. 1: *Ixodes ricinus* female attached to the silicone membrane.
Half of the 500 µm long food canal has pierced the membrane.

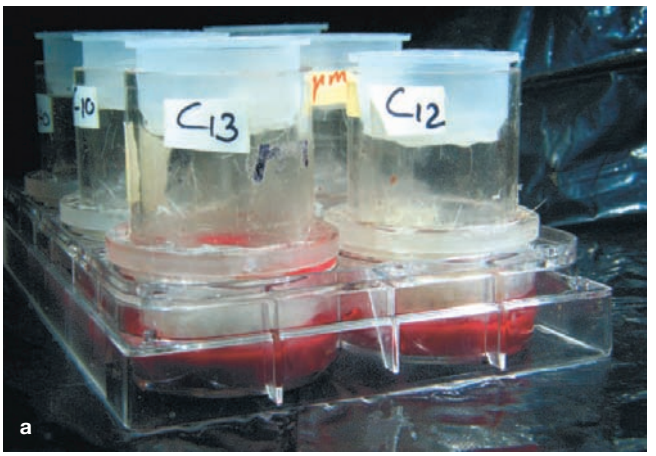

Fig. 2: Six-well plate with feeding units over blood (a) and schema of a section through one feeding unit depicting the ticks feeding on blood through the membrane (b).

allows ticks with shorter mouthparts (500 µm in the case of female *I. ricinus;* Fig. 1) to be accommodated, and the mouthparts of ticks to be withdrawn and reattach elsewhere. The previous penetration site closes by elastic retraction forces in the membrane preventing leaking of blood into the unit (Kröber and Guerin, 2007b). The feeding units are set up in six well plates (Fig. 2) where each is equipped with an outer ring that limits the depth to which it sinks into the 3.1 ml of blood applied per well. Tick feeding proceeds at body temperature on a support in a water bath. Bovine blood, collected weekly from an abattoir, is manually defibrinated and supplemented with glucose, an antibiotic and ATP (as a feeding stimulus). Blood is exchanged twice daily.

Attachment by ticks at predilection sites on hosts is preceded by a behavioural sequence that depends on the presence of an appropriate array of mechanical, olfactory and contact chemostimuli (Guerin et al., 2000). We achieved a 75-95% attachment rate by *I. ricinus* in these feeding units by applying a combination of chemical and mechanical stimuli. A cow hair extract was applied to the membrane in addition to mosquito netting and a layer of cow hair (Fig. 2, 3).

Testing an acaricide *in vitro*

Fipronil, an odourless phenyl pyrazole, is toxic to a broad range of arthropods *via* contact or ingestion. This acaricide disrupts ion flow by interacting with GABA-gated chloride channels of the CNS, causing hyperexcitation. The effect of different doses of fipronil dissolved in the bovine blood on I. ricinus mortality was assessed over 9 days in our feeding units. Fipronil killed all females within two days at 10 µg/ml of blood, at 1 µg/ml no females survived longer than four days, and at 0.1 µg/ml all females were killed by day 7 (Fig. 4). These are similar to the doses of fipronil required to protect companion animals against ticks. At the lowest doses of 0.01 and 0.001 µg fipronil/ml female ticks survive but their feeding activity is reduced by 35% and reproduction is inhibited. Females feeding on 0.001 µg fipronil/ml laid eggs but none of these hatched.

Since fipronil, like other acaricides, is also applied to animals as a spot-on in the fur, we tested the contact effects of this

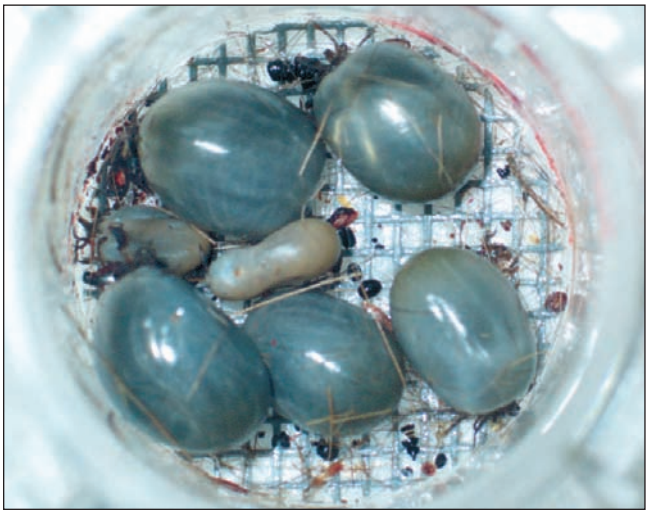

Fig. 3: Feeding unit with partially engorged female *Ixodes ricinus* sucking bovine blood for over a week.
Less engorged females can readily reattach to complete the blood meal.

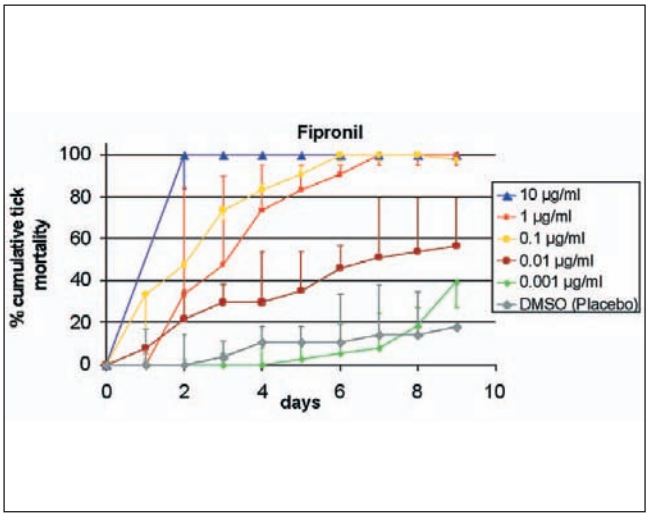

Fig. 4: Cumulative mortality of *Ixodes ricinus* females feeding on bovine blood through a silicone membrane with dimethylsulfoxide (DMSO) added (placebo) and with increasing doses of fipronil in DMSO.
The error bars indicate the range between repeated trials.

acaricide in our feeding units by applying it to the surface of the silicone membrane before the ticks were introduced to the feeding units. Fipronil applied in this manner inhibited feeding and strongly affected tick survival: mortality reached 69% at 10 ng fipronil/cm^2 and 100% at 1 µg fipronil/cm^2 within 30 h (controls 19%, $P \leq 0.001$ and ≤ 0.0001, respectively, Fisher's exact test).

Advantages of the *in vitro* feeding assay

In vivo trials of acaricides with animals as hosts require repetitions with 10 to 20 animals due to the inherent variation between them. Furthermore, large amounts of products and ticks are needed for such tests. By contrast, our *in vitro* assay requires approximately one hundred fold less of the test product and only about 40 ticks are required per dose. Survival curves calculated over the different doses of fipronil in different feeding experiments showed that the observed effects were significant, were obtained within 5-6 days and highly reproducible (Kröber and Guerin, 2007a). In addition, this *in vitro* assay permits setting up more standardised conditions since the placebo, a reference acaricide and test products can be tested in blood from the same donor animal (Kröber and Guerin, 2007b). Together these reasons suggest that the *in vitro* feeding assay for hard ticks is preferable to *in vivo* screening trials on animals.

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Generation and Use of a Mouse Kupffer Cell Line

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Summary
Kupffer cells are the most abundant population of tissue macrophages, involved in the pathogenesis of various liver diseases. We generated a murine Kupffer cell line from H2Kb-tsA58 transgenic mice, which has proved useful for the study of drug delivery, of phagocytosis of pathogens and apoptotic cells and of the role of KC in transplantation.

Keywords: liver macrophages, Kupffer cell line, mouse

Background Information

What are Kupffer cells?

Kupffer cells account for 80 to 90% of resident macrophages in the body, they constitute about 15% of the liver cells. Kupffer cells are located along the sinusoid and exhibit different phenotypes and functional capacity in the periportal, intermediate and central area of the liver lobule (Fig. 1A). They have a life span of approximately 14 months and only a small fraction, amounting to 3%, is responsible for cell population renewal in vivo.

The principal role of Kupffer cells is phagocytosis and mediation of the innate immune response in the liver. Kupffer cells are able to remove from the blood any particulate or soluble gut-derived stimulus (Fig. 1B); upon uptake they release reactive oxygen species, nitric oxide, cytokines and lipid mediators. The best known stimulus of Kupffer cells is the gram-negative cell membrane constituent, Lipopolysaccharide (LPS) or endotoxin, which is a normal component of portal venous blood. Depending on the timing, frequency and intensity of endotoxin stimulation, sensitisation or tolerance to endotoxin results. Excessive stimulation of Kupffer cells leads to liver damage via release of cytokines and toxic oxygen products.

Impact of Kupffer cells in disease

Kupffer cells play an important role in a number of diseases:
Sepsis: During early sepsis, Kupffer cells – together with granulocytes, which accumulate in liver sinusoids – participate in bactericidal activity and release reactive oxygen and proteolytic products, which are a cause of liver injury.
Alcohol induced injury: Due to alcohol induced, impaired gastrointestinal epithelial barrier function, Kupffer cells become exposed to increased levels of endotoxin, which cause a sustained production of inflammatory mediators. Consequently, Kupffer cell sensitivity to LPS is enhanced and contributes to chronic alcohol hepatitis.
Hepatectomy: After partial hepatectomy for liver tumor resection, liver regenerates and all cells including Kupffer cells proliferate, these have a controversial effect on the growth of other liver cells.
Liver transplantation: Kupffer cells interact with circulating T cells in the transplanted liver. Recent experiments in allograft models have shown, that Kupffer cells induce tolerance by suppressing T cell proliferation and inducing T cell apoptosis. Also, Kupffer cells isolated from an accepted allograft, prolong liver survival in a rejection model.
Ischemia/reperfusion injury during liver surgery or transplantation: During liver surgery, perfusion may be interrupted by periods of ischemia; this causes irreparable damage to which Kupffer cells participate. Pre-exposure of the liver to transient ischemia increases the tolerance to reperfusion. This process is mainly mediated by Kupffer cells, which produce less proinflammatory cytokines and attract less granulocytes after ischemic preconditioning.

Together this demonstrates that it is highly relevant to know more concerning the function and role of this particular liver cell population.

Why immortalised Kupffer cells?

Investigations concerning Kupffer cells are hampered, because in humans, Kupffer cells are only accessible for immunohisto-

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chemical analysis, from biopsies or autopsies. From rats and mice they are difficult to isolate and after purification only approx. 5 million cells can be obtained from one mouse. Furthermore, these Kupffer cells are not homogeneous, form syncytia, rarely proliferate and survive for not more than 10 days. These reasons have made it mandatory to search for a way to immortalise Kupffer cells while preserving their original function.

How to generate a Kupffer cell line

Kupffer cells were isolated from H-2K^b-tsA58 transgenic mice, which stably express a thermolabile mutant of the Simian virus 40 (SV40) large tumor antigen under the control of a histocompatibility gene promoter (H-2K^b). Cells isolated from this mouse grow continuously at the permissive temperature of 33°C, at which the mutant tsA58 is active, but don't grow, or grow less under the normal culture temperature of 37°C. Growth is initiated by the incubation of cells with interferon-γ, which activates histocompatibility genes.

In the present project, we harvested Kupffer cells by collagenase perfusion of the liver, gradient centrifugation and subsequent counterflow centrifugation. Four lines were generated by culture at 33°C, in a medium containing interferon-γ and conditioned media from a hepatocyte and an endothelial cell line. In the absence of these paracrine growth factors, we observed a gradual loss of phenotype and secretory function.

One out of 4 clones (KC13-2) obtained by limiting dilution from the line, grew stably at 33ºC without interferon-γ and also – although slower – at 37°C, which indicates an interferon-γ and temperature-independent regulation of SV40T Ag. Specific Kupffer cell characteristics of the KC13-2 clone were confirmed by comparing phenotypes and functions between peritoneal macrophages, primary isolated Kupffer cells and our clone. We had to use 44 mice to establish and characterise the clonal line. In comparison: When working with primary Kupffer cells, a single experiment requires the livers of 10 mice and the variability between experiments is large.

What are these cells?

The generated cell line has been growing in culture in a stable manner for more than 6 years. The cells of the clone, in contrast to the line and primary cell, were uniform, survived detachment and could therefore be analysed by flow cytometry. The KC13-2 clone, like the primary Kupffer cells, constitutively expressed a number of specific functions and structures. These include: a) the classical macrophage enzymes non-specific esterase (Fig. 2A) and peroxidase; b) two macrophage-specific antigens of unknown function, MOMA-2 (Fig. 2B) and BM8 (identical to the antigen known as F4/80); c) the pattern recognition receptors, which are activated by pathogen-associated conserved molecules, including scavenger receptor A, CD14 and Toll-Like-Receptor 4/MD-2 (TLR4/MD-2) (Fig.3); d) the antigen presenting molecules MHC class I and II, CD40, CD80 and CD1d; e) Kupffer cells endocytosed Dextran-FITC, which is another characteristic of immature antigen presenting, dendritic cells; f) the lack

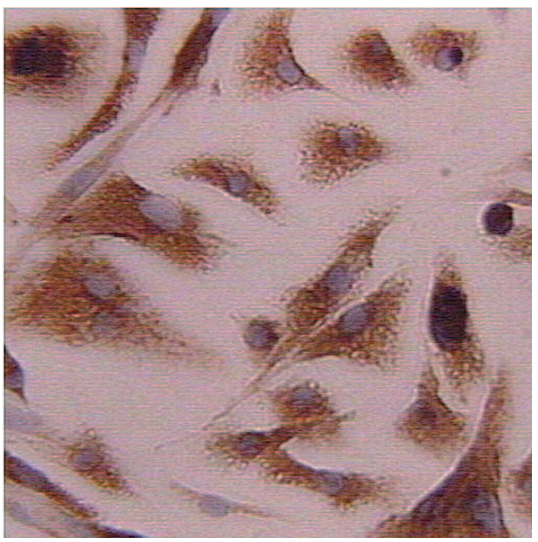

Fig. 1A: Primary Kupffer cell cultured *in vitro*: Stain with macrophage-specific antibody.
Cell size is not homogenous, because Kupffer cells from the periportal area are larger than those from intermediate or central-lobular area.

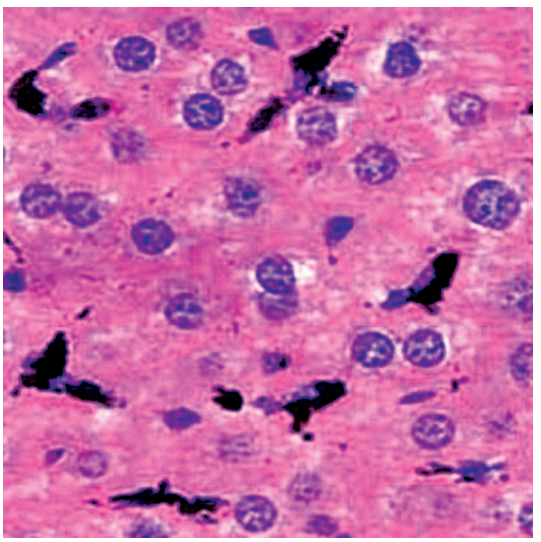

Fig. 1B: Histology of Liver Sinusoid:
Kupffer cells are stained in black after ink uptake, they are interposed between hepatocytes.

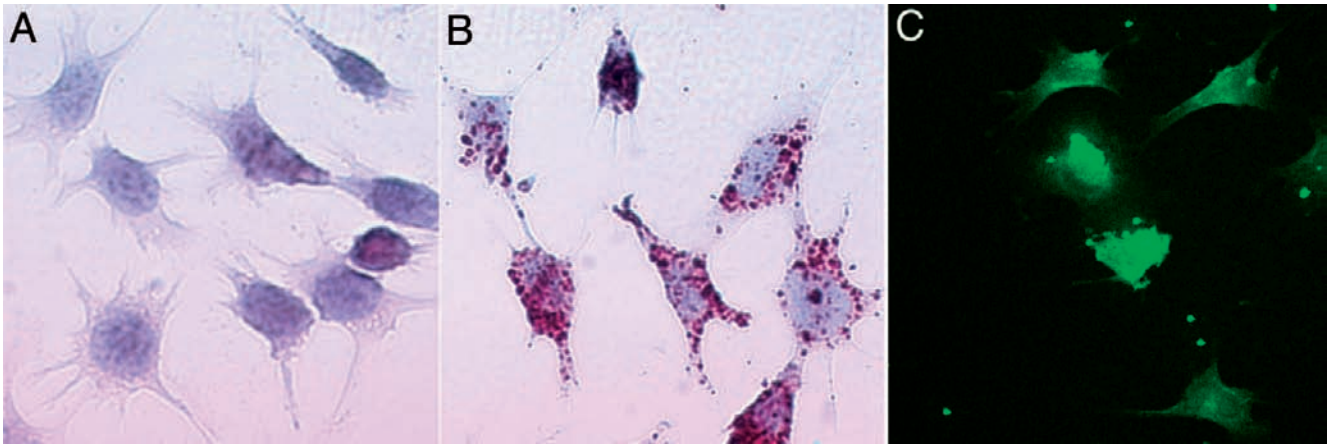

Fig. 2A: Enzymatic activity (esterase) of Kupffer cell line (KC 13-2).

Fig. 2B: Staining of Kupffer cell line KC 13-2 with macrophage specific antibody MOMA-2.

Fig. 2C: Phagocytosis of fluorescent *E.coli* by the Kupffer cell line KC 13-2 1h at 37°C.

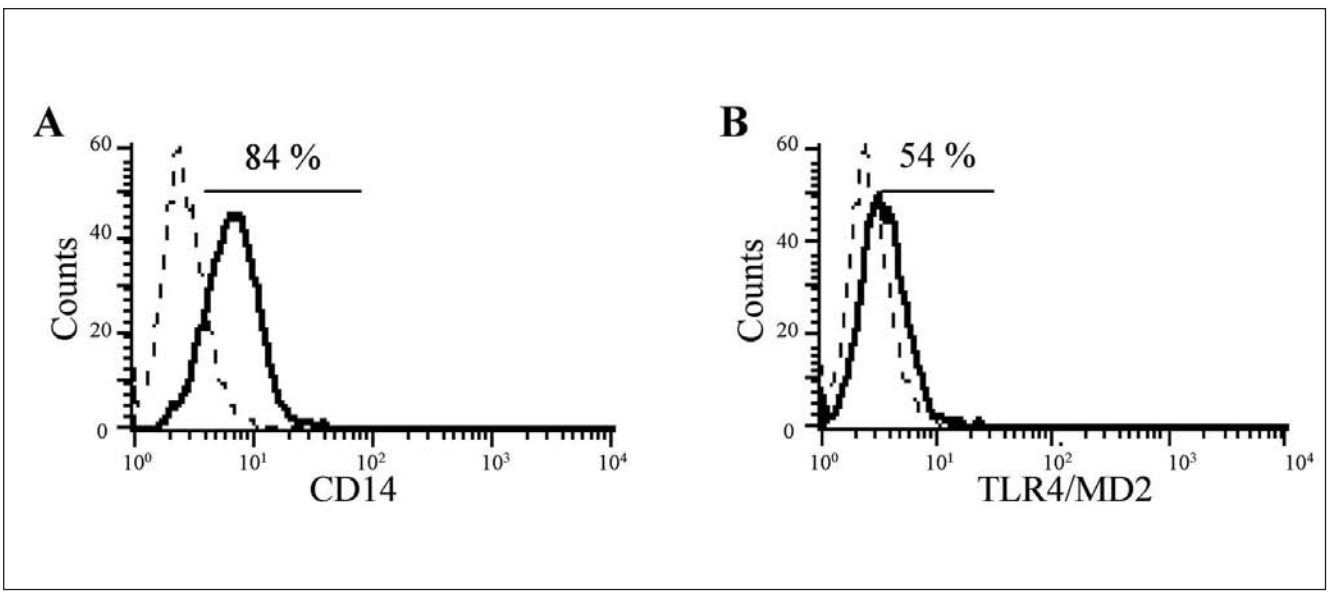

Fig. 3: Pattern receptor expression of CD14 (left) and TLR4 (right) in Kupffer cell line KC 13-2, dotted line: isotype control antibody staining.

of the phagosomal coat protein TACO, which is in all macrophages, except Kupffer cells; g) exhibition of CD14- and TLR4/MD2-independent, plasma-dependent lipopolysaccharide (LPS) binding. h) *E. coli* (Fig. 2C) and *S. pneumoniae* phagocytosis and LPS- and IFN-γ-induced NO production, but no TNF-α, IL-6 or IL-10 release (Dory et al., 2003).

In summary, the large size, surface marker expression and capacity to clear gram-negative and -positive bacteria, but absence of cytokine release, indicates that the clone was derived from the periportal large Kupffer cell subpopulation.

A valuable tool for mechanistic investigations

For the first time it has been possible to generate a stable, clonal Kupffer cell line representing a subpopulation within the Kupffer cells of rodent livers.

The applications for the use of the Kupffer cell line in disease models are multiple and scientists from all over the word asked for the line to study e.g.:

Liver disease after parasite infection: signalling in Kupffer cells after stimulation with bacteria or bacterial products is analysed

(Peng et al., 2006). Furthermore, parasite entry into Kupffer cells and the immunological response after uptake of apoptotic cells is followed.

Liver transplantation: The set of antigen presenting molecules in the clone allows the study of the consequences of antigen presentation to MHC- and CD1-restricted T cells. Furthermore it allows the investigation *in vitro* of xenogeneic interactions e.g. between murine Kupffer cells and human erythrocytes and consequences of antigen presentation, such as T cell apoptosis e.g. in liver transplantation.

Liver tumors: The role of Kupffer cell line in liver tumor formation is studied.

Drug delivery: Firms interested into drug delivery have started using the line for uptake studies.

The clone allows molecular studies of the antiinfective and immune functions of Kupffer cells. It will reduce and replace studies with primary Kupffer cells obtained from mice.

However, control of *in vitro* derived knowledge has to be proven in selected cases, in an intact animal or directly in specific clinical applications.

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Welfare Assessment and Phenotype Characterisation of Transgenic Mice

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Summary

Induced mutations can cause new and unpredictable phenotypes and may impact the health and welfare of animals. Impairments may arise within normal husbandry and breeding regimes i.e. before starting to do experiments. In order to apply the 3R principles and to use transgenic animals under high scientific and welfare standards, two structured forms for individual health monitoring and strain characterisation have been developed. They are available at: www.vu-wien.ac.at/labortierkunde or www.altex.ch

Keywords: refinement, phenotype and health monitoring, score sheet, transgenic animals, mice, ethology

Background Information

Transgenic animals in animal experimentation

Variations from the mean or norm have always been interesting to biomedical research in providing clues as to the normal and pathological functions of living beings. Genetic mutations are particularly useful because they can be reproduced by breeding; thus they are accessible to a thorough investigation. With the advent of transgenic technology and its routine application in many laboratories around the world, the generation and use of genetically modified animals has increased dramatically in biomedical and pharmaceutical research and safety testing. This development has been additionally accelerated by the decoding of the genome of man, mouse and rat; currently about 10% of the mouse's genes have been modified experimentally and incorporated into genetically engineered organisms (Austin et al., 2004). In 2005, 94.000 such animals - mainly mice - were used in Switzerland for experimentation, an increase of 16% over the previous year; 22% of all mice used were genetically engineered. In the period 1997 to 2004, 5.000 transgenic mouse strains were kept in Swiss laboratories (Bundesamt für Veterinärwesen, 2006) and new strains are being added continuously. However, transgenic animals have a potential for genetically derived health impairments and other welfare problems (e.g. developmental difficulties, behavioural abnormalities, problems of reproduction); in fact, 7% of all transgenic strains kept in Switzerland show a mild degree of innate suffering (severity score 1), 5% have a middle or high degree of suffering (severity scores 2 and 3; Bundesamt für Veterinärwesen, 2006). This needs to be addressed systematically in order to adopt 3R measures of refinement and to take ethical decisions about the continuation of heavily loaded strains.

Is there a particular health and welfare problem with transgenic animals?

Transgenic mice are valued models in biomedical research. Several strains are used to investigate human congenital diseases and disorders; these strains carry a genetic defect that may or may not be clinically apparent in the animals. In practice, altering the genotype has not necessarily an impact on the health of transgenic strains. However, the resulting phenotypic consequences can not be predicted in detail and a reduced viability or impaired health at the phenotypic level may be expected in several cases (Bundesamt für Veterinärwesen, 1998; 2006). The impairment is also present even if the animals are not used for experimentation, and is transmitted from one generation to the next by normal breeding. The degree of impairment and the symptoms of suffering will vary from minor to severe and may show up at various stages of ontogenesis in different strains. Within a single strain, the degree of suffering will depend primarily upon the genetic alteration, on the genotype (the alleles at a specific locus) of each individual and the genetic background, but can also be strongly influenced by environmental conditions.

It may or may not be possible to alleviate suffering by specific measures. In any case, each newly created transgenic strain has the potential to cause poor health and suffering in the animals. In this regard, transgenic technology poses a challenge for the 3R goal of "refinement": if a mouse strain is to carry certain dysfunctions at the genetic level for scientific reasons, everything has to be undertaken to minimise animal suffering.

Can transgenic animals offer a particular contribution to the goals of 3R?

In spite of the problems listed above, transgenic animals may represent a refinement in comparison to some other traditional

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experimental models of disease in which the animals bear a heavy load of suffering. It seems possible to create "elegant" models where – in the best case – a genotype is an excellent model of disease for selected body functions at the molecular or cellular level while the corresponding phenotype is completely healthy. However, in order to ensure that the well-being of a newly created transgenic strain is unaffected, a careful and comprehensive examination is indispensable.

Given these two problems and tasks at the level of the animal strain, i.e. (i) proof of absence of health problems due to a modified genotype and (ii) recognition, prevention, minimisation and therapy of reduced welfare, the first few generations of newly created mutant strains (founders, F1 and F2) need to be characterised with respect to their phenotype using health and welfare assessment guidelines.

What do we have to know about transgenic animals?

Animal care staff and scientists working with and responsible for transgenic rodents are often confronted with impairments to fitness of mutant animals. Published experimental results derived from new transgenic animals do not usually include detailed information about the requirements for breeding and maintenance of the strain. By using existing knowledge regarding an efficient breeding and husbandry program of a novel line, it will be possible to reduce the overall number of animals used and to minimise the potential for affected animals to experience pain, suffering or distress.

Forms for a standardised documentation of relevant information

The literature was first reviewed to collect clinical signs described as being useful for recognising health problems and suffering in laboratory animals. This body of literature was then analysed and a relatively large set of parameters selected for the characterisation of transgenic mice. The parameters had to fulfill two criteria: (i) feasible for conducting as a screening test on a routine basis, and (ii) indicative of health or animal welfare problems at the strain level.

Next, two standardised forms were developed to characterise transgenic mice comprehensively. The form "Data Record Form" (Mertens and Rülicke, 1999; 2000b) includes 10 different score sheets for litter-wise and individual health monitoring from birth until spontaneous death or euthanasia. This information about individual animals was then structured and evaluated for the health and welfare status of the strain at two levels of detail in the form "Strain Characterisation" (Mertens and Rülicke, 2000a; 2000b). The "Basic Information" will answer

Table 1: Form for strain characterisation: general structure

I. Basic information (5 pages)	II. Detail information (13 pages)
A. Main page	F. Gen expression at molecular and cellular level; constitutive or conditional expression
B. Genotype (overview)	G. Phenotype: manifest differences in comparison with the wild type of the same genetic background
C. Phenotype (overview)	H. Additional strain specific characteristics
D. Ethical and animal welfare assessment	
E. Recommendations for housing, breeding and transport	

Table 2: Form for strain characterisation, part C: Phenotype (overview)

	no	yes	?	for details p.
1. Are there useful breeding records?	☐	☐		9
2. Are there differences between hemi-/hetero and homozygous animals?	☐	☐	☐	10
3. Are there sex specific differences?	☐	☐		11
4. Is there an increased lethality (prenatal, perinatal, postnatal)?	☐	☐		11
5. Are there abnormalities in individual development?	☐	☐		12
6. Are there apparent malfunctions and deformations?	☐	☐		13
7. Are there malformations of inner organs?	☐	☐	☐	13
8. Are there disorders in individual behavior?	☐	☐		14
9. Are there disorders in social behavior (without reproduction)	☐	☐		14
10. Are there disorders in breeding behavior?	☐	☐		15
11. Are there abnormalities in reproductive success?	☐	☐		15
12. Are there strain specific diseases?	☐	☐		16
If yes: which?				
13. Is the immune status affected?	☐	☐		17
14. Cross breeding to a different background planed or in progress?	☐	☐		17
15. Generation of double or multiple mutants planed or in progress?	☐	☐		17
16. Is there any strain specific detail information not given under pt. 1 - 15?	☐	☐		18

Each question answered with ‚yes' has to be supplemented by the corresponding form for specific information.

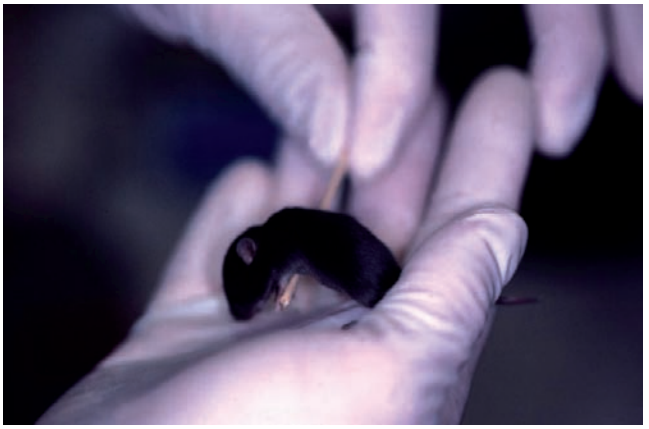

Fig. 1A, 1B: Neurobehavioral tests are easy to perform with each pup (approximately day 10-14) and indicate gross sensory-motor deficits. The pictures show the pole grasping test for motor (dis-)abilities and muscle strength.

the question if there is a welfare problem with a transgenic line (Tab. 1, Tab. 2). If so, a detailed description of the symptoms can be developed or is provided with the "Detail Information" part (Tab. 1).

Finally, the score sheets were tested for their "user-friendliness" and clarity in a pilot study that monitored and documented 106 mice from two established genetically modified strains in the first three months of life (Mertens and Rülicke, 1999). The results demonstrated the fundamental practicality of the protocols, provided that personnel were adequately instructed and able to invest additional time in the monitoring program. Meanwhile, similar recommendations for data record forms have been published by other groups (van der Meer et al., 2001; Wells et al., 2006).

Characterisation of transgenic animals improves animal welfare and science as well

A comprehensive characterisation of transgenic mice is indispensable for animal welfare, for the application of the 3R principles, for the correct interpretation of research results, and for official purposes (annual statistics). The characterisation sheet presented here is feasible for routine use as a standardised procedure. It will contribute to increased scientific accuracy and efficiency in the laboratory. Transgenic animals, whether commercially distributed, transferred to new facilities or used in animal experimentation, should always be accompanied by their score sheet and strain characterisation form. Up to now, a lack of awareness of the problem and a lack of supporting regulations has prevented such a procedure from becoming standardised. The complete forms can be downloaded as pdf.files at www.vu-wien.ac.at/labortierkunde or www.altex.ch

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Predicting Drug Hypersensitivity by *In Vitro* Tests

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Summary
Recently it was found that drugs causing drug hypersensitivities do not only rely on the formation of hapten-carrier conjugates but can stimulate T cells directly via their T cell receptors for antigen. This new mechanism was termed pharmacological interaction of drugs with immune receptors (p-i concept). It is frequent in systemic drug hypersensitivity reactions and has major implications for predicting them. First experiments to identify such drugs able to interact with T cells are presented.

Keywords: T cell, allergy, hypersensitivity, TCR

Background Information

T cell immune response

Certain chemicals (including biological agents and drugs) are immunotoxic, which means they can stimulate or suppress the human immune system. The adverse side-effects of a stimulated immune system can be sensitisation or autoimmunity with an inflammatory reaction. In rather rare cases, sensitised individuals can experience an allergic reaction to a second exposure of the stimulus (antigen, allergen) and become hypersensitive. Well known are compounds which, when given on the skin, cause inflammation of skin areas (allergic contact dermatitis). The side effects can be even more severe when an immune stimulant enters the body and reaches the blood system (e.g. after oral ingestion) inducing a T cell-mediated immune response.

T cells are a subpopulation of lymphocytes and are involved in most allergic reactions (hypersensitivity). T cells are able to recognise peptides, lipids, metals and complexes consisting of chemicals and peptides as antigens. The immunogenicity of chemicals (but not of biological agents = proteins) is generated by the previous covalent binding of the hapten to a protein, which is then processed to a peptide and presented on MHC-structures (= HLA-molecules). Such hapten-peptide complexes are recognised by some T cells with the fitting T cell receptors (TCR) for the particular antigen.

However, W.J Pichler argues that this hapten model is not a sufficient explanation for many drug-induced side effects. He and his group showed that a direct non-covalent binding of drugs to the TCR is possible and under certain circumstances stimulatory for T cells (p-i concept). Binding of the drug to the TCR activates T cells, whereby MHC interaction (regardless of the enclosed peptide!) with the TCR supports this drug mediated signal. The T cells start to divide and organise an inflammatory response in the body by secreting cytokines and killing other cells. These immune-mediated inflammatory responses may cause mild symptoms such as maculopapular exanthema, but a substantial fraction of these reactions are severe, causing Stevens-Johnson-Syndrome, toxic epidermal necrolysis, hepatitis, pancreatitis, fever, vasculitis, eosinophilia, and even death.

Predicition of systemically applied compounds

There is an urgent need to improve the prediction of immune-mediated side effects of drugs, biological agents and chemicals. Available animal and *in vitro* tests (e.g. skin sensitisations and lymph node assays) are mainly positive with haptens or pro-haptens, which rapidly become haptens. Nevertheless, these tests lack a reliable prediction of generalised forms of drug hypersensitivity (Bala et al., 2005). Furthermore, immune-mediated side effects appear only in a minority of patients which might have a special genetic predisposition (Chung et al.,2004) which is not present in animal models. Finally, new concepts of drug hypersensitivity, such as the presented p-i concept, are hardly covered by animal experiments or *in vitro* studies using animal cells (Pichler, 2001).

Consequently, one might favor *in vitro* testing with human material. For the safety assessment of chemicals or compounds which are applied topically and sensitise *via* skin or lung, *in vitro* tests seem to be promising (www.sens-it-iv.eu). However, they focus exclusively on haptens or prohaptens and the pathway *via* dendritic cells. For testing the safety of systematically (orally or parentally) applied drugs inducing allergic reactions *via* the p-i concept, *in vitro* tests require human material, since these reactions are exquisitely specific. Already small alterations of the T-cell receptor (TCR), of the structure of the drug, of the MHC molecule, which interact with the TCR, alter the reactivity of T cells dramatically. Moreover, animals may have a greater resistance to the immune stimulation *via* the p-i concept.

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The p-i concept

This concept postulates that drugs can bind to the TCR like to other receptors – and that this interaction might stimulate T cells directly. It does not require a previous hapten-carrier formation (Fig. 1). The consequence is a reaction mimicking an immune reaction, however not triggered by a classical antigen, but by a drug. It does not (!) require the generation of an own immune response to the hapten (as the drug is no hapten) or the involvement of the innate immune system. This additional (alternative) pathway is called pharmacological interaction with immune receptors (p-i concept) (Pichler, 2003). Considering this pathway might be highly relevant for detecting the potential hypersensitive (allergic) activity of a given compound and provides a good explanation for a number of open questions in drug hypersensitivity reactions (Pichler, 2003).

Initiating an immune response *in vitro*

The p-i concept was originally established based on comparing the immune response of patients allergic to sulfamethoxazole (SMX) to the parent, non-hapten like compound and to its main metabolite, the hapten Sulfamethoxazole-Nitroso (SMX-NO). SMX-NO binds covalently to proteins or peptides.

Hapten-like drugs and chemicals can easily induce an *in vitro* immune response by culturing peripheral blood mononuclear cells (PBMC) of non-sensitised individuals with the hapten for a prolonged time period. We could confirm this finding (Engler et al., 2004) and tested ten healthy non-sensitised individuals and found nine responders to SMX-NO.

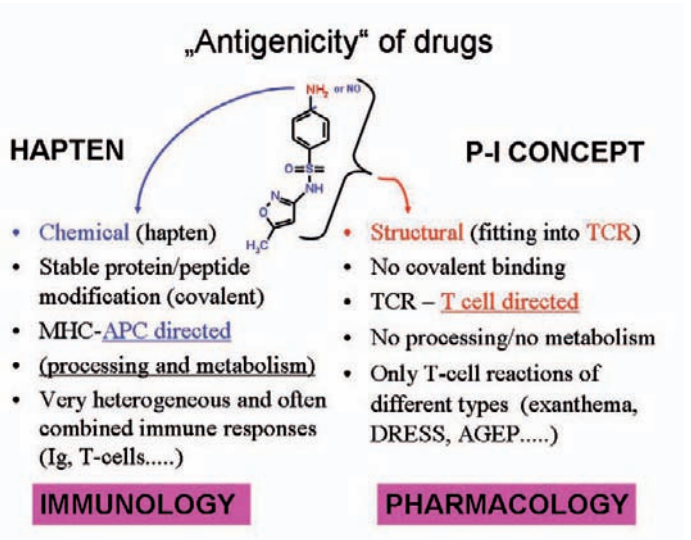

Fig. 1: Antigenicity of drugs: Drugs gain antigenicity by two pathways.
A) they can bind covalently as haptens to peptides and proteins, modify them and make them immunogenic. This pathway is APC directed and determined by the chemical reactivity of a drug. B) the p-i concept relies on the fitting of the drug into a certain T cell receptor for an antigen which happens to have a stimulating effect and leads to T cell expansion (for details see Pichler, 2003).

Initiating an immune response *via* the p-i pathway

But what happens if not the hapten SMX-NO, but the parent compound SMX is added to the cell culture? According to the p-i-concept, T cells are the main target cells for the reaction (instead of dendritic cells). We were able to show that the metabolism of SMX to SMX-NO does not occur *in vitro* using peripheral blood mononuclear cells (PBMC). The addition of SMX to PBMC of non sensitised individuals (previously never exposed to SMX) did not induce a proliferative response in PBMCs. However, by extending the cell culture period to 4-6 weeks and the repetitive addition of PBMCs as antigen presenting cells (APC) and IL-2, we could detect some reactivity in three of ten healthy non-sensitised volunteers (Fig. 2). The immune response was clearly directed to the non-reactive parent compound (SMX) itself and not to SMX-NO (Engler et al., 2004). One of these volunteers was tested several times, always giving the same positive response. We even cloned SMX-reactive T cells from this non-sensitised individual, which were specific for SMX (but not for SMX-NO) and had the same characteristics as SMX-specific T cells obtained from SMX-sensitised individuals. Thus, under certain culture conditions, SMX stimulates T cells *via* their TCR. However, only certain individuals seem to react in this assay, and a massive co-stimulation of the T cells seems to be required (Engler et al., 2004).

Long lasting immunological memory

It is well known that a previous drug hypersensitivity reaction poses a risk for a new one. This implies the existence of an immunological memory. But how many cells are actually involved? By analysing the precursor frequency to five different drugs in five different patients with different forms of drug hypersensitivity reactions (sulfamethoxazole, carbamazepine, phenytoin, vancomycin and amoxicillin) (Beeler et al., 2006) we were able to detect specific T cells in patients which had reacted 12 years to 4 months before the analysis was performed.

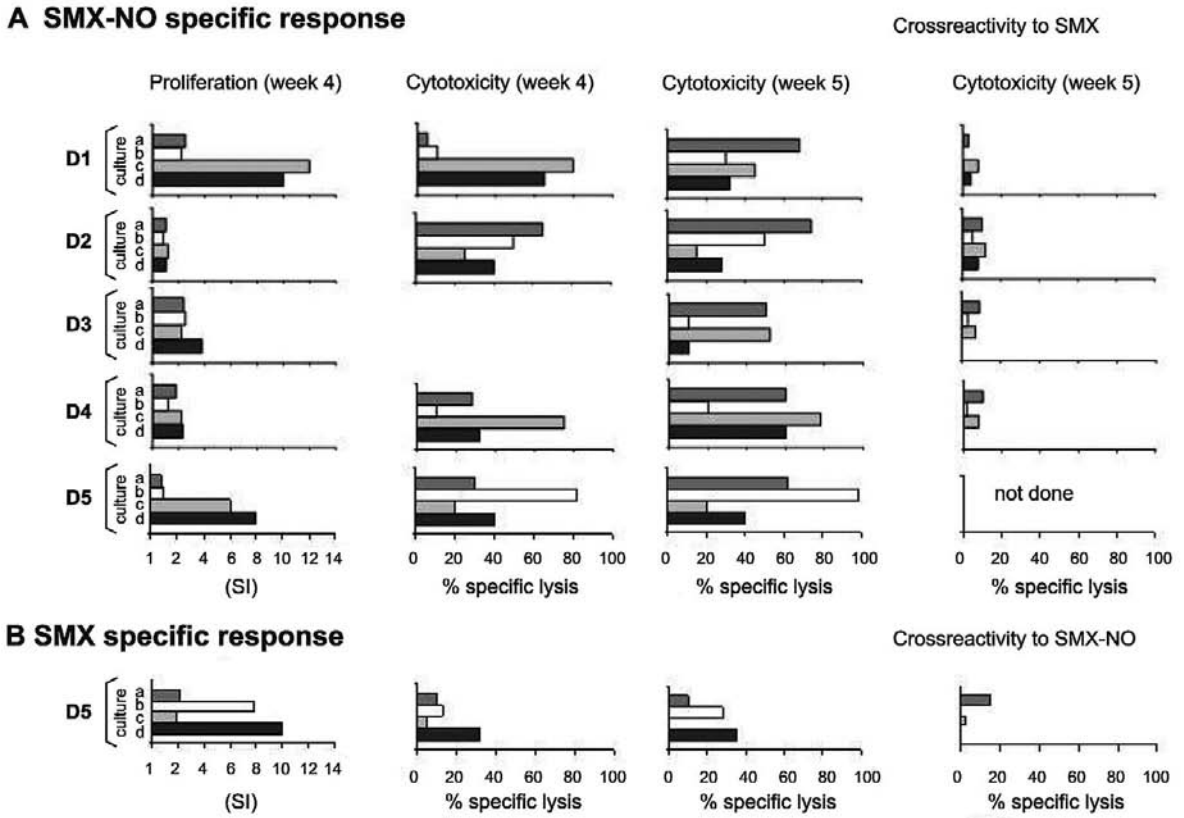

Fig. 2: Induction of a primary immune response or expansion of SMX-reactive T cells from non-sensitised individuals. The induction method consisted of the weekly stimulation of naive PBMCs with irradiated autologous PBMCs, IL-2 and the drug. The proliferative and cytotoxic response of 5 donors (D1-D5) to the reactive drug metabolite SMX-NO and its inert parent compound SMX is shown. During week 4 and 5, 4 individual induction cultures from each donor (a-d) were tested for specific proliferation, cytotoxicity and during week 5, also for cross-reactive cytotoxic responses. The stimulation index (SI) represents the factor of specific proliferation – measured by thymidine incorporation after 2 days of stimulation – in presence and absence of the drug. Cytotoxicity was analysed by standard chromium release assay using EBV lines as targets. Mean values of duplicates are indicated for an effector-to-target ratio of 40:1 (for details see Engler et al., 2004**)**

High precursor frequency of drug-specific T cells

The frequency of drug-reactive T cells was measured with two assays and compared to the frequency of T cells reactive with tetanus toxoid, which is a common recall antigen in Switzerland since the whole population is regularly vaccinated. CSFE labeling of peripheral blood lymphocytes allowed to measure the proliferation, as this CSFE fluorescein cell stain is halved at each cell division, allowing a precise calculation of how many cells have divided in a certain time period. In the ELISPOT analysis the cytokine production of drug-reactive T cells was determined after 36 hours of cell culture with the drug or tetanus. Both analysis gave quite similar values: a high frequency of drug-specific T cells were found in individuals with an allergy to the corresponding drug whereby the analysis was always negative to other drugs to which the patients had been exposed but not sensitised. The frequency was actually higher than the simultaneously measured tetanus response, as 1:250 to 1:10.000 of $CD4^+$ T cells reacted with the different drugs. This detailed analysis of drug precursor frequencies is a good basis to establish tests to detect such cells for the *in vitro* diagnosis of delayed drug hypersensitivity reactions (Beeler et al., 2006).

From an individual (clinical) to a predictive test

The present data shows the ability to stimulate T cells "pharmacologically" *via* the T cell antigen receptor (p-i concept). This stimulation of T cells of non-sensitised individuals required repetitive stimulations and was strong enough to cause the T cells to divide and to expand. This drug-mediated stimulation via T cell receptors is per se a quite astonishing finding as it underlines the high potency of certain drugs to stimulate the immune system.

Clinical data indicate that the p-i pathway is probably more relevant than the hapten concept in eliciting generalised drug

hypersensitivity reactions, while contact dermatitis is more due to the hapten mechanism.

In order to develop a predictive test based on the p-i concept and suitable for preclinical testing, several technical problems remain to be solved: The test must become far simpler, more robust and needs to be standardised. The human cells used for the *in vitro* tests must be carefully characterised. But the most important aspect is a better understanding of systemic drug hypersensitivity reactions in general. These questions are: What is the relationship of T cell stimulation to the clinical picture and why do only some individuals react - both *in vivo* as well as *in vitro*: What is the role of co-stimulation of T cells to enhance reactivity to the drug? Is the ability to react to drugs via the p-i mechanism due to the T cell receptor repertoire, immune regulation, genetic background, or is it, as some data would indicate, a combination of all? With this knowledge, one could create sophisticated *in vitro* models with human cells which might even replace a substantial number of animal tests for the detection of hypersensitivity inducing drugs.

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From Blood to Brain and Vice Versa: Transport Processes in Choroid Plexus Can be Studied *In Vitro*

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Summary

The choroid plexus represents the second barrier between blood and brain besides the blood brain barrier. The barrier function is set up by plexus epithelial cells, which are equipped with a variety of active transport systems. However, in contrast to the epithelial organs location and extent of expression of these transporters may be different. Studying the choroid plexus (CP) epithelium in living animals is difficult due to its complex morphology, anatomical location and small size. Therefore, an in vitro monolayer model of choroid plexus was established from porcine brain in order to study the functional contribution of transport proteins to drug transport across CP tissue with special focus on ABC-proteins.

Keywords: choroid plexus, cerebrospinal fluid, ABC-transporter, p-Glycoprotein, MRP1

Background Information

Choroid plexus (CP) epithelium

The brain is most sensitive to changes in its surrounding environment. Separated from blood flow it maintains stability, protects itself from injury and actively removes endogenous metabolites and xenobiotics from the central nervous system (CNS). The treatment of CNS diseases is difficult for several reasons. Besides poor delivery to the target, metabolism, rapid efflux and drug-drug and drug-metabolite interactions reduce the therapeutic potential of drugs in the brain. The blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier formed by the CP regulate type and concentration of molecules transported to and from brain extracellular fluid, cerebrospinal fluid (CSF) and intracellular fluid.

CP are highly vascularised tissue patches located within the ventricles. CP makes up 0.2% of total brain in weight or 2-3 g in humans. In contrast to the BBB, the CP endothelium (specialised epithelium) is fenestrated. The surrounding monolayer of epithelial cells is connected by tight junctions, separating apical membranes facing the ventricular space, and basolateral membranes facing the blood. Thus, the CP epithelium (cells lining outside and inside cavities and lumens), but not endothelium forms the barrier structure separating blood and CSF.

- Choroid plexus function: The CP performs vital functions, including the production of CSF (liquor) the synthesis and secretion of proteins and neurogenic and endocrine regulation. Its supplies the brain with nutrients and other solutes and removes proteins and catabolites. Compounds reach the CSF by crossing the CP from the bloodstream. The CP can either take up compounds from CSF or wash them out into the bloodstream. This makes the CP an effective detoxification system within the brain.

- Transport across CP-epithelium: The transport of xenobiotics and endogenous metabolites is highly regulated. Morphometry measurements reveal a relatively comparable surface area for BBB and CP. In the rat, the apical CP membrane surface area is three times as large as the basolateral membrane surface area and only just half the brain's endothelial surface. Factors influencing transport across the CP include barrier characteristics, physiological, physicochemical and pharmacokinetic parameters.

Transport across choroid plexus tissue

The choroid plexus is atypical in its distribution of membrane proteins acting as transporters. Many transporters do not follow the functional pattern observed in kidney or brain endothelium; rather protein localisation is reversed. Excretory transporters present at the basolateral membranes in kidney, liver or brain are distributed on the CSF-facing side in CP epithelia (apical) and efflux transporters are found at basolateral membranes. Thus, not only presence and density, but also the polarised distribution of relevant transport proteins has to be considered when examining transport at the CP. To date, a total of eleven transporter families with almost 30 individual proteins are known to be expressed at the CP (Choudhuri et al., 2003).

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Establishment of the monolayer system

Epithelial cells from porcine CP were prepared from fresh CP tissue desintegrated into single cells as described (Baehr et al., 2006). Cells were seeded either on laminin-coated Transwell® filter membranes or in flasks. In general, CP cell growth was slow and proliferation rates were low. Addition of cytosine arabinoside suppressed growth of contaminating cells and epithelial cell cultures developed into an integer monolayer within 9 days in culture (9 DIC), displaying the typical cobblestone appearance. Then, serum was removed from the medium and monolayers were allowed to fully differentiate for another 4-5 days (14 DIC) as shown with specific markers such as for TTR mRNA and protein expression, alkaline phosphatase and γ-glutamyl transferase activities (Choudhuri et al., 2003).

Cerebrospinal-fluid secretion

The high rate of CSF secretion is fueled by rapid blood flow through the CP (5 ml/g/min), which is about tenfold faster than average cerebral blood flow. *In vitro*, the CSF secretion was analysed with cells grown on 6-well Transwell“ filter systems. 67 kDa FITC-dextran was added and secreted CSF volumes (in µl/cm2) calculated as a function of FITC-dextran concentration change.

Figure 1 shows CSF volumes secreted by cultured CPEC for up to eight hours. Volumes of CSF secreted by the cell monolayer increased and reached a plateau after five hours, with approximately 150 µl/cm^2 produced.

Choroid plexus monolayer permeability

Values of CP monolayer transepithelial electrical resistance (TEER) were measured using the Millicell®-ERS and STX-2 electrode system before starting permeability analyses. In our porcine CP cell model, monolayers displayed TEER values in the range of 100 to 150 Ωcm^2. For paracellular marker analyses, CP monolayers were incubated with 5-carboxyfluoresceine and FITC-dextrans of different size from either the apical or basolateral side and accumulation was measured in the corresponding compartment. Permeability coefficient values ranged from (4.56 ± 0.26) x 10-5 cm/s for smallest down to (1.42 ± 0.13) x 10-5 cm/s for largest molecules.

ABC-transport protein expression and function

Active excretion by ABC efflux transporters (transporter proteins with ATP-binding domains encoded by the ABC-gene superfamily) not only removes endogenous metabolites and waste products from the brain, but also limits uptake and penetration of many therapeutic compounds. Two proteins contributing to multidrug resistance are of particular importance due to the diversity of compounds transported: the multi-drug resistance (MDR) phenotype Pgp, part of the ABCB subfamily, and Mrp1, part of the ABCC subfamily. Pgp and Mrp1 gene expression was analysed on a qualitative and quantitative basis and the distribution and localisation of both proteins on polarised CP cell membranes was determined. Comparative RT-PCR experiments from isolated CP tissue and cultured CP monolayers indi-

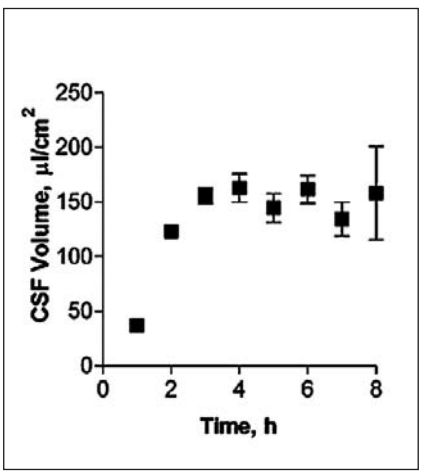

Fig. 1: CSF secretion by CP cell monolayers at 14 DIC.

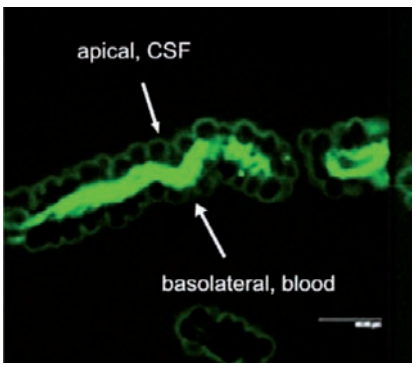

Fig. 2a: Excretion of Fl-MTX (Mrp-substrate) from brain to blood (=apical to basolateral) in intact CP tissue.

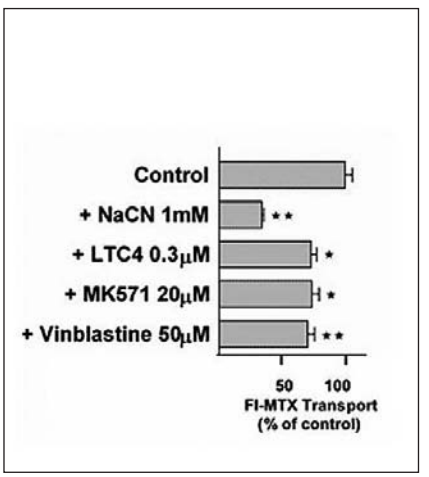

Fig. 2b: Transport of the Mrp-substrate Fl-MTX (Fluorescein-Methotrexate) across CP monolayers (apical to basolateral) in absence and presence of Mrp-modulators (NaCN = sodium cyanide, LTC4 = Leukotriene C4, MK571 = antagonist of Leukotriene 4 (LTD 4) receptor and Mrp1-Inhibitor; Vinblastine = alkaloid).

cated the presence of Pgp, but fresh and cultured CP cells expressed Pgp mRNA at low levels. The signal for Pgp was weaker in cultured epithelial cells than in tissue. Mrp1 mRNA was also expressed in freshly isolated and cultured cells. In contrast to Pgp mRNA, there was no significant difference between Mrp1 gene expression in cultured cells 14 DIC and freshly isolated cells. Immunostaining indicated that the localisation of Pgp in CP may be subapical and different from that in other epithelial cells and that Mrp1 was clearly present in CP epithelium. *In vitro* we demonstrated that Pgp is not functionally active in the membranes of the cultured cells, similar to findings in intact tissue whereas Mrp1 was active (Fig. 2a, 2b).

3R benefits

In order to interpret transport processes across the CP, appropriate models of investigation are required. *In vivo* techniques are complicated since they require surgical skill and it is difficult to monitor data. Common methods include the serial sampling of CSF after drug administration and deconvolution of data to determine transport profiles. Therefore, the development of adequate *in vitro* methods which allows the examination of drug transport from blood to CSF and *vice versa* is essential in order to reduce investigation in intact animals. *In vivo* studies allow only very limited insight into mechanistic aspects. Present data demonstrates that CP epithelium can be isolated and cultured, with cells growing into intact monolayers, fully differentiating and with properties resembling the tissue *in vivo*.

The present investigations *in vitro* provide the first data on Pgp and Mrp1 expression and activities without the use of animal experiments. An apical localisation of Pgp in the CP trafficking certain substrates from blood into the CSF seems to oppose its action at the blood-brain barrier. The lack of Pgp function would be in contrast to other organs such as liver, kidney, small intestine or blood brain barrier, where Pgp is documented to play a major role in efflux of compounds and/or transport into the bloodstream. Results obtained *in vitro* with Mrp1 function mimicked the distribution observed *in vivo* indicating that in CP epithelium Mrp1 may act as a barrier for certain drugs coming from the blood.

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Establishing a 3D *Ex vivo* Culture System for Investigations of Bone Metabolism and Biomaterial Interactions

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Summary
A 3D load-providing culture system is presented, which enables human cancellous bone explants to be studied ex vivo. Bone integrity and activity was demonstrated during 2 to 3 weeks of culture. Viable osteocytes and bone specific markers could be identified. Culture conditions were improved using a serum free medium containing TGF-β3. Therefore this system gives the opportunity to study bone biology, hormonal effects as well as biomaterial interactions ex vivo prior to animal studies.

Keywords: human, bone, osteocytes, osteoblasts, loading culture chamber, reduction, replacement, toxicity testing, biomaterials, implants, validation

Background Information

Importance of 3D *ex vivo* bone studies using a mechanical load providing bioreactor

It is well known that strain is one of the main factors that can influence bone architecture and mass. The correct dosage of an applied mechanical load can therefore act as an anabolic stimulus on bone. On the other hand disuse of bone (astronauts at zero gravity; bed rest patients; immobilisation) can cause a severe loss of mass and strength in normal load bearing bones. It is hypothesised that the cells which can sense and transduce the information produced due to mechanical loading are the osteocytes. These cells are connected to others via their fluid-filled lacuna-canalicular system and are proposed to communicate with each other as well as with adjacent osteoblasts, bone lining cells and osteoclasts. Commonly, 2D cell cultures are used to investigate bone related questions. These systems lack mechanical load and the complex interplay between the different cell types. Therefore, there is a need for a 3D bone culture system as the naturally occurring bone cell interaction, in combination with the presence of the different cell types, can not be achieved in 2D. With the use of a Zetos bioreactor (created by DB Jones, Marburg, Germany and EL Smith, Madison, Wisconsin, USA) load, that mimics the naturally occurring pattern, can be applied to ex vivo cultured cancellous bone explants, which can be supplied with their individual culture medium. This opens new possibilities in bone research. Beside the use in pretests of biomaterial integration, this system will also be a potential means to study basic bone biology, bone diseases and effects of different drugs on e.g. osteoporosis. It has been demonstrated that this model can be used to maintain viable human osteoporotic bone ex vivo. Current methods of studying osteoporosis which use sheep models aim to simulate osteoporotic bone using steroids, special diets and sometimes removal of the ovaries which are usually very distressing for the animal. This culture system enables more rigorous in vitro testing, reducing the number of animals needed within such a study.

The system – model systems, material processing and usage

Cancellous bone due to its porosity is the bone of choice for a 3D culture system. Diffusion of fluids and nutrient supply can be achieved easier than using 3D cortical bone. Cancellous bone is present in sufficient mass at different origins. Three model systems are used within our working group – distal end of ovine femurs, bovine distal metacarpals and human femoral heads (approved by Ethic Commission Graubünden 18/02). No animals were sacrificed specifically for this work, bovine material was collected from the local slaughterhouse and ovine material was used from animals after sacrifice for other experiments. The processing of each bone includes cutting 7 mm thick slices with the use of an Exakt 300 band saw, then cores of 9.5 mm diameter are bored from the sections with a Synthes drill bit. Finally these are cut parallel to 5 mm height with a Leica annular saw (Davies et al., 2006). After removing debris with repeating washing steps, each core is placed in its individual culture chamber and perfused with its individual culture medium with a flow rate of 0.1 ml/min. Mechanical loading can be applied with the Zetos bioreactor (Jones et al., 2003) (Fig. 1) by inserting a chamber into the load-

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ing device. The loading procedure used is performed daily for a duration of 5 min using a complete jump wave form (300 cycles, 1 Hz, 4000 µstrain). After long term culture cores can be harvested and analysed in order to answer the specific question.

Bone characterisation and protein synthesis on cultured bone explants

After culture, cores can be either fixed in 70% ethanol prior to dehydration and Technovit 9100 embedding (Yang et al., 2003) for histochemical analysis; or fixed and decalcified prior to cryosectioning, to perform immunohistochemical evaluation on 6-12 µm sections. Histological sections (6 µm thick) of bovine, ovine and human material after culture are comparable to fresh bone concerning matrix and cell integrity, as demonstrated by a variety of stains. The presence of noncollagenous proteins, such as osteopontin, osteonectin and osteocalcin could be localised immunohistochemically.

Protein synthesis could be detected using [3H]-glycine incorporation combined with SDS-page and Western blot analysis as well as autoradiography of embedded sections, demonstrating cell viability and activity during 14 days of culture within the system. In addition, the presence of the unstable propeptide of collagen-I could be detected on 12 µm cryosections of 14 days cultured human bone cores (Fig. 2).

Influence of loading and long term culture on bone viability

Investigating bone viability of 3D bone explants after culture can be difficult. The bone matrix autofluorescence interferes with many fluorescence labelling techniques. Radioactive labelling of protein synthesis has inherent risks and can take many weeks to develop images. Both of these methods require embedding or decalcification procedures prior to cutting. We have focused on using a Lactate dehydrogenase assay (LDH) to detect an essential cytoplasmatic enzyme. The high stability of LDH allows cutting of fresh cores prior to staining. After fixation, visualisation of purple stained viable cells can be performed. We could quantify osteocyte viability per area of bone matrix with an optimised method using the natural autofluorescence of the bone matrix to enhance the staining contrast (Stoddart et al., 2006) (Fig. 3).

Using the LDH viability method on human cancellous bone cores, it could be demonstrated, that a daily applied load could maintain higher osteocyte viability after 7 and 14 days of culture

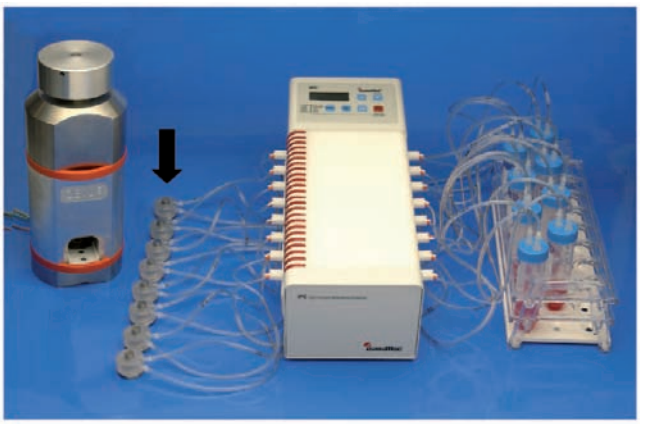

Fig. 1: Zetos culture system which comprises of a loading device (left), and a set of diffusion culture chambers (arrow), each with their own media supply fed from a reservoir (right) through Tygon tubing with the aid of a peristaltic pump (white box).

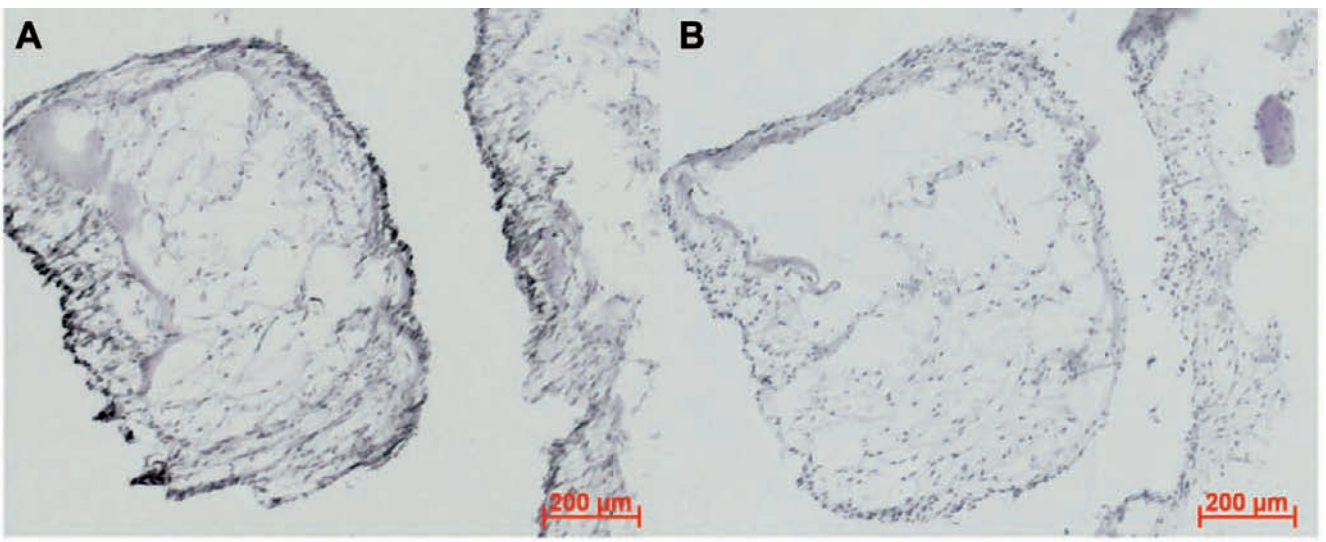

Fig. 2: Immunohistochemistry of procollagen-I on cryosections from 14 days cultured human bone explant (64 years, male) within the Zetos system.
A) Procollagen-I staining, B) Negative control without primary antibody

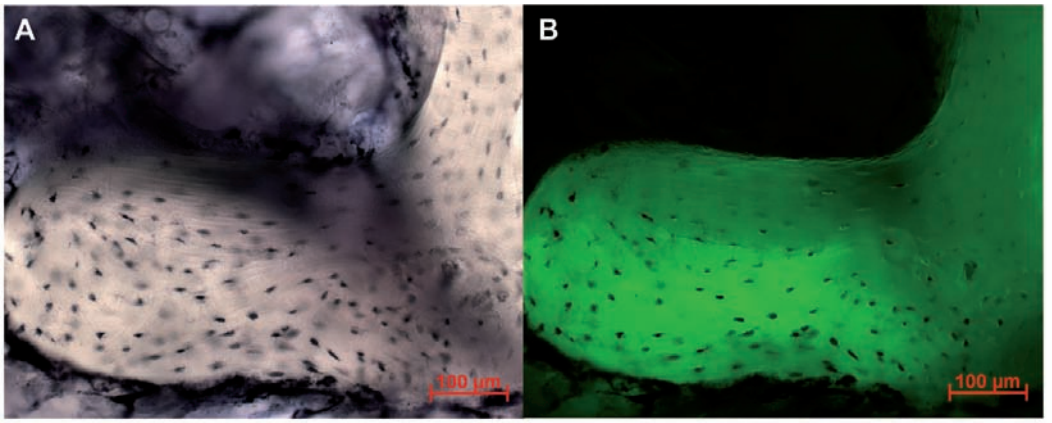

Fig. 3: Two representative pictures from LDH stained middle sections of one human (64 years, male) loading experiment visualised with Axioplan microscope.
A) Brightfield image, B) Fluorescence image (515 – 565 nm emission filter)

in comparison to unloaded control cores. Therefore the bioreactor itself improves 3D culture conditions.

Effect of TGF-β3 on osteocyte viability and the use of a new defined culture medium

The TGF-β superfamily consists of cytokines that can affect different cellular events, such as cell growth, differentiation, apoptosis etc. In bone TGF-β was shown to increase collagen-I synthesis as well as matrix apposition and therefore enhance the total bone mass. We chose to look at the effects of TGF-β3, as it was demonstrated by ten Dijke et al. to be more potent, when comparing general DNA and collagen synthesis to the more commonly studied TGF-β1 (ten Dijke et al., 1990). During the culture of human bone cores with a DMEM based culture medium containing 10% fetal calf serum (FCS) and 15 ng/ml TGF-β3, higher osteocyte viability maintenance could be achieved which exceeded the effect caused by daily loading alone. This led us to investigate the effect of this factor within a medium without serum, which is normally thought to cause a higher cell death rate due to reduced factor concentrations found within serum containing medium. Serum free medium including 15 ng/ml TGF-β3 was supplemented with insulin, transferrin, selenium and a defined lipid source to provide a defined environment of factors essential for cell survival and activity. Analysis of the centre of cores cultured for 14 days either within serum containing or serum free medium, revealed a similar level of osteocyte viability (Fig. 4). A common phenomenon during culture of 3D bone explants is the formation of an inconsistent fibrous tissue on some / all surfaces of the cores. The fibroblast-like cells covering the core will decrease nutrient supply of the underlying cells as well as secrete factors that could influence the culture conditions. Using a serum free medium the chance of surface fibrous tissue formation was reduced in comparison to serum containing medium. Therefore the culture within a serum free medium achieves a more defined culture environment and maintains the level of osteocyte viability achieved with the use of FCS within culture medium. Additionally, this medium was also proven to show procollagen-I synthesis during 14 days of culture as seen with the serum containing control, demonstrating cell activity.

Improved media distribution with the use of a new chamber design

Further improvements of culture conditions were focused more technically on modifying the existing culture chambers. As sticking of bone cores to baseplate or piston surfaces of the chambers can occur, this may cause declined media distribution on the core surfaces. Therefore baseplate and piston surfaces where modified by applying 0.2 mm deep channels in a negative honeycomb pattern. Perfusion experiments using a disulfide blue containing medium revealed a more even media distribution using these “honeycomb surfaced” chambers. Viability investigations using these chambers with the use of the defined serum free medium are currently under process.

Conclusion and future possibilities

The *ex vivo* loading Zetos culture system was validated to culture cancellous bone with osteocytes, osteoblasts, osteoclasts and bone marrow cells in their natural 3D relationship to each other. This system also has advantages in reducing the variability, cost and ethics behind *in vivo* studies. Most experiments evaluating biomaterial integration with bone and soft tissue are conducted within animals. Therefore implants for human use can now be tested *in vitro* with applied load (and this is underway). Only the most successful biomaterial/implant would continue for animal trials, reducing the number of animals required for such studies. The development of a realistic 3D loaded system, whereby the candidates could be eliminated before *in vivo*

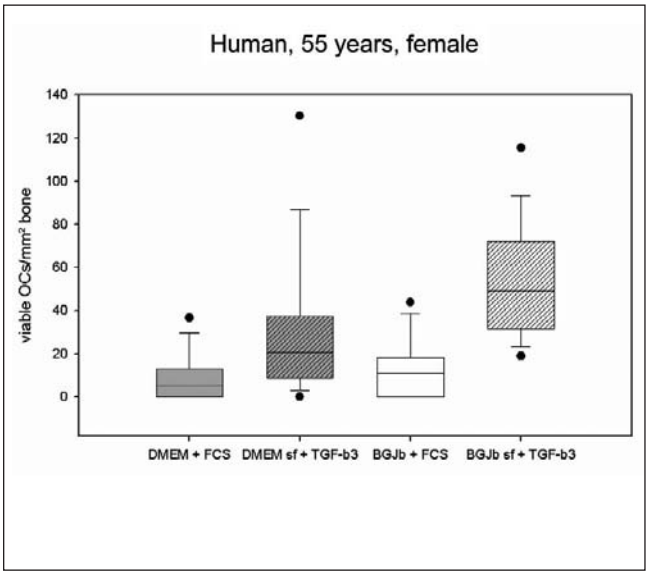

Fig. 4: Graph of one human loading experiment (55 years, female) investigating the effect of serum free medium on osteocyte viability.
The number of viable osteocytes per mm2 bone matrix area at the centre of the cultured bone explants was compared between DMEM + 10 % FCS (grey bar) *versus* DMEM serum free + 15 ng/ml TGF-β3 (striped grey bar); and between BGJb + 10 % FCS (white bar) *versus* BGJb serum free + 15 ng/ml TGF-β3 (striped white bar).

studies would greatly reduce the number of animals. Therefore the system has potential study bone-biomaterial interactions, as well as bone biology in normal and osteoporotic human or animal bone, bone biomechanics, and the effects of drugs, hormones or growth factors on cancellous tissue, making it an essential laboratory aid for the future.

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Fever in the Test Tube – Towards a Human(e) Pyrogen Test

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Summary

The human whole blood IL-1 test exploits the reaction of monocytes/macrophages for the detection of pyrogens: human whole blood taken from healthy volunteers is incubated in the presence of the test sample in any form, be it a solution, a powder or even solid material. Pyrogenic contaminations initiate the release of the "endogenous pyrogen" Interleukin-1ß determined by ELISA after incubation. In order to understand any differences between the pyrogenic activity in this test and the existing live rabbit test (species differences versus aberrant response of the particular blood sample), the rabbit whole blood test was developed. This approach could also help to avoid the use of putatively infectious human blood for pyrogen testing in vitro.

Keywords: whole human blood test, pyrogen in vitro test, rabbit whole blood test, IL-1ß

Background Information

Pyrogen testing – *in vivo* and *in vitro* test methods

Pyrogens are a chemically heterogeneous group of fever-inducing substances derived primarily from gram-negative and gram-positive bacteria but also from viruses and fungi. They provoke an immune response by producing endogeneous pyrogens such as prostaglandins and the proinflammatory cytokines interleukin-1, interleukin-6 and tumour necrosis factor-α. Pyrogen testing of any pharmaceutical product for parenteral application is therefore imperative. Different methods using different protocols are in use.

In vivo Rabbit Test: The animals are injected i.v. with the drug and monitored for any reaction in the form of fever. This test is currently legally required by health authorities. The test, however, is subject to inherent problems, since the sensitivity of different species towards endotoxins varies by a factor of up to 10,000. Pyrogen testing currently requires about 200,000 rabbits each year in Europe. After a recuperation period of 2-3 weeks, the animals can be used again to test a new drug, providing that the test substances cause no permanent changes in the immune system of the rabbits.

Limulus Amoebocyte Lysate Test (LAL): This test measures the coagulation of the amoebocytes of the horseshoe crab, initiated by cell wall components (LPS) of gram-negative bacteria with a molecular weight of > 8000 daltons. The test cannot detect smaller LPS nor the LPS equivalents of gram-positive bacteria or fungi. Furthermore, the test cannot distinguish between the different types of endotoxins from gram-negative bacteria, which can vary in their fever-inducing potential in the mammal by a factor of 10,000 (Fennrich et al., 1999).

Human whole blood test (WBT): This test, using an ELISA, was developed in 1996 by Hartung and Wendel (Hartung and Wendel, 1996) and internationally validated by ECVAM (Hoffmann et al., 2005). It measures cytokine production, in this case IL-1ß, by human monocytes following a challenge with pyrogens. It is less expensive and more sensitive than the rabbit test and has the additional advantage of being able to examine the reaction strength directly in human material. Unlike the LAL, this test can detect not only endotoxins, but also lipoteichoic acids, fungi, and superantigens such as SEB (enterotoxin of Staphylococcus aureus).

Most recently, additional tests using interleukin-6 release as endpoint and peripheral blood mononuclear cells (PBMC) or the monocytoid cell line MONO MAC 6 (MM6) as a source for human monocytes were established. All these tests were scientifically validated (Hoffmann et al., 2005) and the validity approved by the ECVAM Scientific Advisory Committee.

Pyrogens induce the release of pyrogenic cytokines

The guaranteed absence of pyrogens is a critical safety precaution for all drugs administered parenterally, since these contaminants can pose a life-threatening risk of shock to the patient.

Contact with minute concentrations of endotoxia, the best known pyrogen – as low as 5 IU/ kg, i.e. 500 pg/kg bodyweight – cause multiple reactions in the patient: human monocytes release several cytokines, the most important being IL-1β, IL-6 and TNFα. The release of these cytokines can cause chills, rigors and hypotension. Furthermore, platelets can aggregate and the coagulation system become activated, resulting in dissemi-

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nated intravascular coagulation and organ hypoxaemia, multiple organ failure and death by shock.

Human whole blood test (WBT) can replace animal tests

Several tests are currently available to detect pyrogenic agents. Of these, a commercially available test using human whole blood (IPT) can detect a wide variety of pyrogens and is suitable for a broad range of applications (Fennrich et al., 1999; Hartung and Wendel, 1996; Jahnke et al., 2000).

Rabbit whole blood test: Bridging the gap between animal tests and WBT

Unfortunately, the response to pyrogens in the WTB can be affected by the donor of the blood sample. In order to establish the validity of the WBT for the pharmacopoeia, it is important to be able to understand any differences between the pyrogenic activity in the human whole blood test and the existing live rabbit test and to be able to attribute these differences to species differences or to an aberrant response of the particular blood sample (Fig. 1). As a link between the existing live test in rabbits and the WBT, the rabbit whole blood test was thus developed. This test uses the same species as the established *in vivo* test, but the same material and endpoint as the new *in vitro* test with human blood.

Several steps were necessary to establish the rabbit whole blood test: i) Production of recombinant rabbit IL-1β in *E. coli* (positive control for the ELISA, substance to immunise the animals in step two), ii) Immunisation of a sheep with the IL-1β antigen, iii) Immunisation of mice with IL-1β and production of monoclonal antibodies, iv) Establishment of a sandwich ELISA (Enzyme Linked Immunosorbent Assay) with the antibodies and the antigen.

These steps were successfully completed: recombinant IL-1β was produced in a reliable quality and sufficient quantity. Monoclonal (mice) and polyclonal (sheep) antibodies against rabbit IL-1β were isolated. The ELISA assay allows the quantitative determination of the endogenous rabbit fever signal IL-1β (Fig. 1).

Response to a pyrogen

The *in vitro* blood test allows the pyrogenic activity of various drugs and agents to be tested (Fig. 2), e.g. pentaglobin. Pentaglobin is a clinical human immunoglobulin preparation which is painful to the animal when administered intravenously in the live rabbit test. The rabbit whole blood test takes only four hours to perform and requires only 100 µl of blood per sample. Currently (in contrast to the human WBT), fresh blood has to be used; however, 7 ml of blood (i.e. enough for 70 samples) can be collected from one rabbit without any harm.

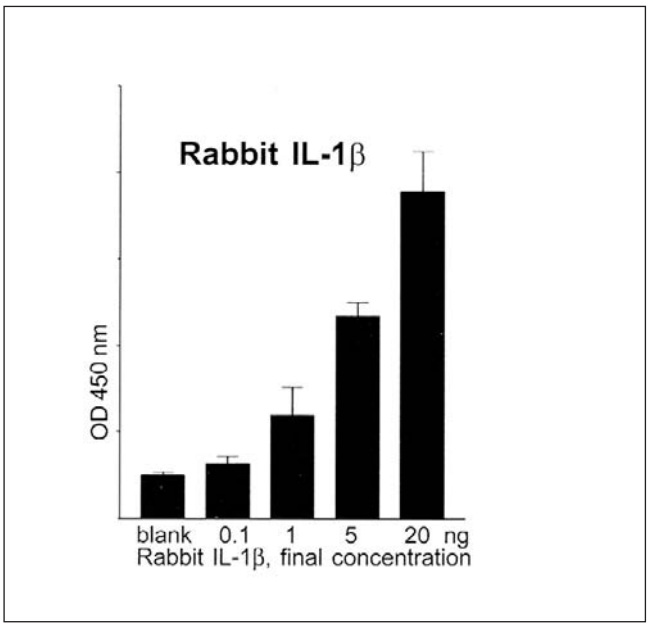

Fig. 1: Quantitative measurements of rabbit IL-ß with an ELISA

Tab. 1: Comparison of three pyrogen tests

		Test:	Rabbit	LAL	WBT
Pyrogens	Bacteria gram-negative		+	+	+
	Bacteria gram-positive		+	-	+
	Fungi		+	-	+
Applications	**Biologicals**	+		-	+
	Pharmaceuticals	+		+	+
	Medical Devices	-		+	+
	Air quality	-		(+)	+
	Blood components	-		-	+

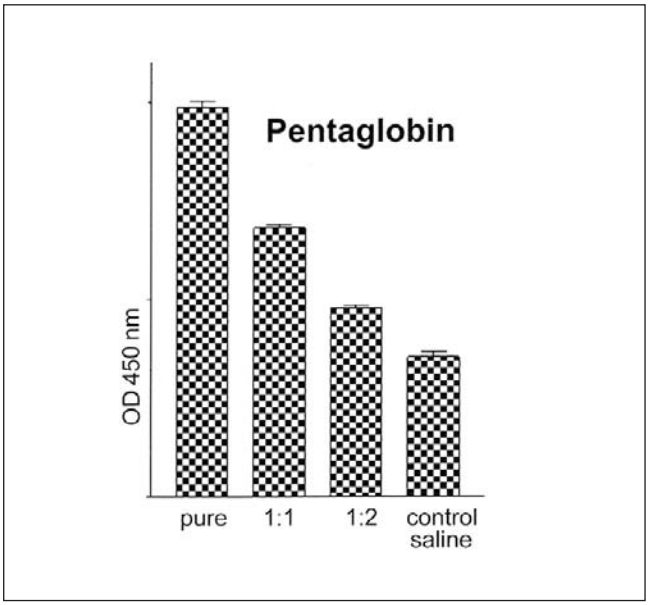

Fig. 2: TNFα release after incubation with a batch of pentaglobin (pure = final concentration: 10%) and serial dilutions

A promising approach

The whole blood test in rabbits can help to explain false-positive and false-negative results when comparing the WBT with pyrogen test results in live rabbits. Furthermore, pyrogen testing in animal blood makes it possible to examine species differences and test veterinary drugs in the target species (Schindler et al., 2002). In the future, this approach could also help to avoid the use of putatively infectious human blood for pyrogen testing *in vitro*.

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Predicting the Toxic Potential of Drugs and Chemicals *In Silico*

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Summary
Based on the 3D structure of the target protein (ERαβ, AR, PPARγ, TRαβ, GR; CYP3A4) or a surrogate thereof (AhR), the Biographics Laboratory 3R has generated a series of virtual test kits and validated them against 693 compounds. In a pilot project (ToxDataBase), both existing and new drugs or environmental chemicals can be screened for their endocrine-disrupting potential or the probability to trigger drug-drug interactions in silico. After peer testing (2007–8), it is planned to make the database available on the Internet.

Keywords: in silico, QSAR, toxicity, receptor, REACH, TocDataBase, Quasar, Raptor

Background Information

Quantifying the binding of a drug or chemical to a target protein *in silico*

In the past decade, computer-based (in silico) concepts matured into powerful tools for simulating and quantifying biochemical processes at the molecular level. This became possible due to over 40,000 protein structures known at atomic resolution, a more detailed understanding of the forces governing molecular interactions and the nowadays available computing power. The philosophy of structure-based design is based on the lock-and-key analogon — recognized as early as 1894 by Emil Fischer (Nobel Laureate, 1902) — the three-dimensional complementarity of drug and its target receptor or enzyme. In absence of structural information on the target protein, receptor-mapping technologies were developed allowing to construct 3D surrogates of the binding pocket. In a QSAR context, those can act as substitutes for the structure of the true biological receptor.

Poor pharmacokinetics, side effects and compound toxicity are not only frequent causes of late-stage failures in drug development but also a source for unnecessary animal tests. In silico methods are nowadays routinely used in the early stages of drug development. In the context of the REACH (Registration, Evaluation and Authorization of Chemicals) initiative of the European Union, computer-based experiments have received additional attention as they can predict the toxic potential of existing and hypothetical compounds. In silico techniques are fast, reproducible, and are typically based on human bioregulators, making the question of data transferability between species obsolete.

Reception of chemicals at biological relevant structures

Nuclear receptors are an important protein class in living organisms. They comprise a family of ligand-dependent transcription factors that transform extra- and intracellular signals into cellular responses by triggering the transcription of target genes. In particular, they mediate the effects of hormones (ligand) and hormonally active compounds (endocrine disruptors). Nuclear receptors are specific for the various steroid hormones, e.g. the estrogens (ER), androgens (AR), progesterones, and glucocorticoids. A number of receptor-mediated adverse effects by xenobiotics have been identified in the past. This includes toxicity mediated by the thyroid hormone receptor, the epidermal growth factor and aryl hydrocarbon receptor (AhR). The concern about chemicals which bind to these receptors and induce adverse, uncontrolled effects has created a need to both screen and monitor compounds before they are further developed as potential drugs or manufactured or released into our environment. At the Biographics Laboratory 3R, we have developed and validated a series of virtual test kits for the AhR, ERαβ, AR, PPARγ, TRαβ, and the GR. Models for the pregnan-X (PXR) and mineralocorticoid receptor are in preparation (Fig. 1).

Metabolic transformation

Competition of drugs for metabolization at Cytochrome P450 3A4 (CYP3A4) may result in undesired drug-drug interactions in patients. In addition CYP3A4 might transform chemicals into reactive metabolites. The development of a computational model to accurately predict the docking potential of a diverse set of ligand molecules was based on the X-ray crystal structure of the human CYP3A4 enzyme and a total of 48 structurally diverse

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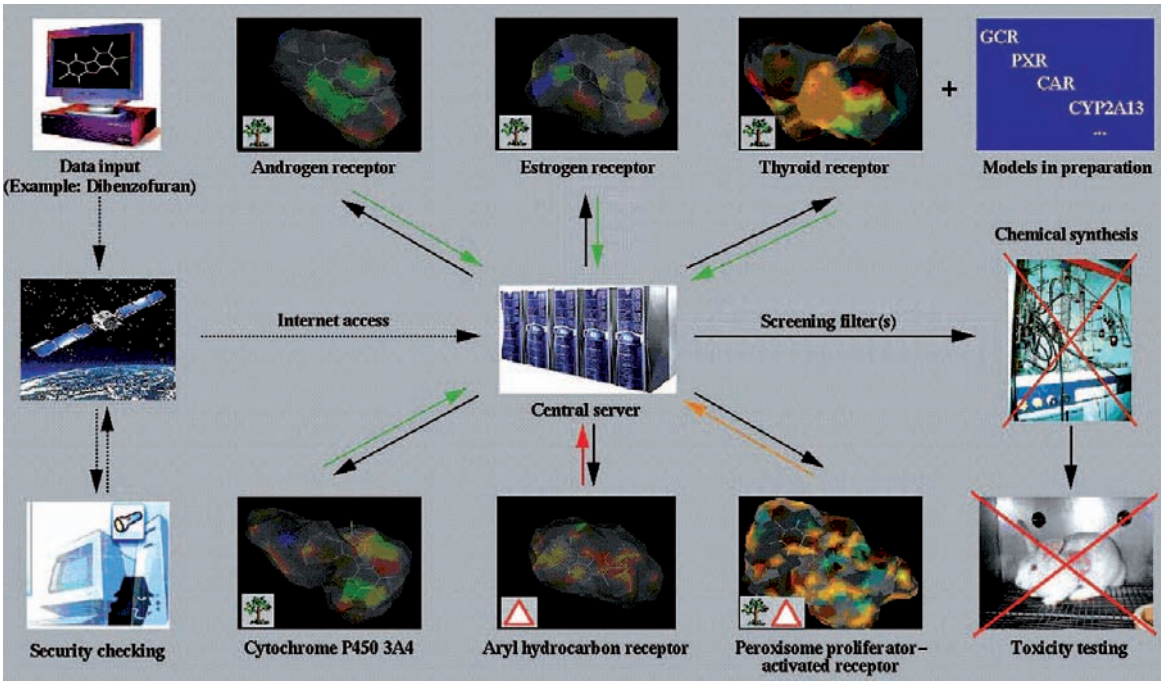

Fig. 1: Flowchart of the virtual laboratory with five receptor surrogates (estrogen, androgen, thyroid, and aryl hydrocarbon receptor and cytochrome P450 3A4) shown.
The hypothetical test compound (a dibenzofurane) is submitted *via* the Internet to a central server, which schedules the processors with the various receptor surrogates.
No significant binding affinities are calculated for the estrogen and androgen receptor and CYP3A4 (IC_{50} > 0.1 mM); against PPARγ, an IC_{50} of 58 µM is calculated. The interaction with the aryl hydrocarbon receptor (IC_{50} = 8 nM) indicates a high toxic potential of the compound; in this test example, the compound should consequently be removed from the evaluation pipeline at this point.

Tab. 1: Validation of virtual test kits.

System	# of compounds training+test=total	q^2	rms training	max. training	p^2	rms test	max. test
Aryl hydrocarbon	105 + 35 = 140	0.782	2.2	15.8	0.766	2.2	12.5
Estrogen α	80 + 26 = 103	0.895	2.0	8.6	0.892	2.9	9.5
Estrogen α*	80 + 23 = 106	0.787	0.9	2.5	0.505	1.7	7.3
Estrogen β*		0.703	1.4	6.7	0.561	1.9	5.7
Androgen	88 + 26 = 114	0.858	1.7	7.8	0.792	1.6	13.9
Thyroid α	64 + 18 = 82	0.919	1.8	4.3	0.814	2.5	10.0
Thyroid β		0.909	2.0	7.7	0.796	2.7	8.8
PPARg	75 + 20 = 95	0.832	1.4	6.2	0.723	1.4	3.9
Glucocorticoid**	80 + 30 = 110	0.743	1.2	6.1	0.623	2.3	6.1
CYP3A4	38 + 10 = 48	0.825	2.7	7.0	0.659	3.8	7.1

q^2 = cross-validated r^2, p^2 = predictive r^2; the rms and maximal deviation from the experimental binding affinity is given as factor in Ki or IC_{50}.
* model under development (ongoing PhD thesis); different compounds than for the 80/26 model above
** model under development (ongoing PhD thesis)

molecules (39 training and 9 test compounds). The results are given in Table 1 and were used to validate the predictability. A model for CYP2A13 is in preparation.

Construction of receptors

Model construction and optimization was achieved by combining protein and receptor modeling. First, the binding mode of each investigated compound was identified using the 3D structure of the target protein (for the Ah receptor, where no experimental structure is available, we used 4D pharmacophore generation instead) by automated, flexible docking combined with dynamic solvation of the binding pocket. Typically, the four lowest-energy orientations were composed in a 4D data set. In contrast to a 3D representation, each compound can be represented as an ensemble of conformations, orientations, protonation states, tautomers and stereoisomers (Tab. 2). This ligand superposition (the binding hypothesis) is then used for out multi-dimensional QSAR technologies named Quasar and Raptor. It is based on a quasi-atomistic model representation and explicitly allows for induced fit – the ligand-induced adaptation of the topology of the macromolecule (see, for example, www.biograf.ch and www.modeling.unibas.ch). Next, the models are validated using test compounds (ligands different from those in the training set), scramble testing and consensus scoring.

Validation of the virtual test kits

Vedani et al. (2006) gives details of model construction and validation. For generating a model a series of compounds (training set) is needed for which experimental binding affinities (Ki or IC_{50} values are available). The quality of the reproduction of these values is reflected by the q^2 value – the cross-validated r^2. Next, a series of ligands different from those of the training set (test set) is used to validate the model. The p^2 (the predictive r^2) value indicates the predictive power of the model. The predictivity can be given as $100 \times \sqrt{p^2}$ – e.g. 87.5% for the AhR.

Testing via Internet

The Biographics Laboratory 3R is presently implementing an Internet database for the screening of adverse effects triggered by drugs and chemicals *in silico*. The bioregulators described so far in this account (AhR, ERαβ, AR, PPARγ, TRαβ, GR and CYP3A4) represent the backbone of this Internet Database; PXR, MCR and 2A13 are in preparation. Within this framework, hypothetical or existing compounds can now be tested for their activity towards the various virtual test kits (Fig. 1) and their toxic potential may be estimated therefrom. Adverse effects mediated by receptors other than those compiled in the database can, of course, not be identified. Accordingly, the present approach based on receptor modeling will result in the production of false-negative results for classes of toxic chemicals which do not interact via receptor or which interact via so far unknown receptor-based pathways. Therefore, QSAR technologies may be used to identify the harmful potential of a drug or chemical and no false positives are produced. However, they are not (yet) suited to prove its harmlessness.

Outlook

Up to date, our concept has not produced any false-positive results. At the current level, however, false-negative predictions are still obtained, as a compound of interest cannot be tested against all potential receptors it may bind to *in vivo*. Some macromolecular targets will remain unknown, for others no experimental structure exists or too few affinity data are available (prerequisites for a QSAR study). We are therefore extending the current concept by implementing a set of virtual filters, which can recognize benign compounds. These filters are based on criteria such as the molecular weight, drug-like properties, and the presence or absence of characteristic structural motifs. After successful completion of a peer testing, it is planned to make the database – along with all supporting software – freely available to universities, hospitals, governmental agencies and regulatory bodies worldwide.

3R relevance

The envisioned Internet laboratory and the already functional virtual test kits can contribute to a significant reduction in animal testing. In drug development, it allows for an early – even before compound synthesis – recognition of potentially harmful substances. By removing those candidate substances from the evaluation pipeline, they will not be forwarded to any *in vivo* toxicity tests. These expectations are supported by the fact that

Table 2. Dimensionality of QSAR approaches.

Dimension	Method	Protein
1D-QSAR	Affinity correlates with pKa, logP, electronic properties, etc.	no
2D-QSAR	Affinity correlates with structural patterns (connectivity, 2D pharmacophore)	no
3D-QSAR	Affinity correlates with the three-dimensional structure of the ligands	possible
4D-QSAR	Ligands are represented as an ensemble of conformers, orientations	typical
5D-QSAR	as 4D-QSAR + representation of different induced-fit models	yes
6D-QSAR	as 5D-QSAR + representation of different solvation scenarios	yes

our virtual experiments have so far not produced any false-positive results. In testing of industrial chemicals for toxicity – for example the 30,000 compounds that have to be retested within the REACH framework – and causing an estimated toll of 10 Million laboratory animals, our approach can be used to safely identify the most harmful compounds *in silico* and prevent their further testing *in vivo*.

Of course, with only a limited number of enzyme/receptor systems known to mediate adverse effects and even fewer accessible in a QSAR context (due to lacking experimental affinity data), false-negative results will always be present. It will selectively recognize potentially hazardous compounds associated with major mechanisms (e.g. metabolic degradation, endocrine disruption) and allow for discarding them early on. Second, a widely used database of this kind might reduce the number of otherwise doubly-conducted toxicity tests at research laboratories focusing on closely related biomedical targets. The main advantage of the proposed virtual laboratory is that it can be applied to hypothetical substances, produces reliable results and is fast and cheap.

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Housing and Husbandry Conditions Affect Stereotypic Behaviour in Laboratory Gerbils

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Summary
An artificial burrow was developed which fits into standard laboratory cages and significantly reduces stereotypic digging in gerbils. Also, the causes of bar-chewing were assessed experimentally. Neither the lack of gnawing material, nor the spatial proximity of cage-lid bars and food in the hopper, nor the routine husbandry procedure of transferring juvenile gerbils to a fresh cage, but premature separation of juvenile gerbils from their parents before the birth of younger siblings significantly increased bar-chewing.

Keywords: gerbil, stereotypies, housing, enrichment, refinement

Background Information

Refinement of rodent husbandry conditions, a relevant issue?

- Animal welfare laws require that the environment of captive animals should meet their physiological and behavioural needs. Housing conditions must facilitate the performance of natural behaviour patterns and allow for adequate social contacts. Unfortunately little is done about behavioural and often the social needs of laboratory rodents, usually only the physiological needs are covered.
- Refinement of experimental procedures relies on animals free of chronic stress and abnormal behaviour. If housing conditions already represent a stressful situation, the validity of experimental results gained from such animals is questionable (Würbel, 2001; Garner, 2005).
- Standardisation of experimental procedures requires a reduction of intra- and interindividual variation. Notwithstanding, huge individual variation of stereotypic behaviour patterns (for example: bar-gnawing, jumping, somersaulting in laboratory mice) can be observed in animals under barren housing conditions, reflecting their different strategies to cope with the situation (Mason and Rushen, 2007). In this case the validity of experiments conducted on such animals is also affected.
- Current debate suggests that animals living in a more complex environment, as for example an enriched laboratory cage, show more natural behaviour patterns, exhibit less intra-individual variation and can cope better with novel situations, resulting in less stress reactions and robust results in standard behavioural paradigms, without masking phenotypic differences between strains (Wolfer et al., 2004).

Artificial housing conditions and behavioral abnormalities

It has long been known that inadequate housing and breeding conditions promote the development of abnormal behaviour such as stereotypies and replacement activities in farm, zoo and laboratory animals. The mechanism leading to behavioural abnormalities is hypothesised as follows: under natural conditions in the wild, animals interact with their biotic and abiotic environment, whereby internal and external stimuli serve to regulate the frequency of certain behaviour patterns. This enables organisms to adapt flexibly to differing environmental conditions. In domesticated animals, many behavioural mechanisms are still regulated in the same manner and by the same stimuli as in their wild ancestors. Under artificial housing conditions, the animals may not be able to adapt to the barrenness of their environment or cope with the lack of appropriate sets of regulating stimuli. As a result, they often show stereotypies, defined as the repeated performance of the same behaviour without apparent goal or function. In farm and zoo animals, stereotypies have successfully been treated by supplying the animals with an appropriate set of artificial environmental stimuli, also called environmental enrichment. In the laboratory, environmental enrichment has long been neglected and should be implemented more frequently.

Investigations in laboratory gerbils

Mongolian gerbils (*Meriones unguiculatus*), which are often used for parasitological and neurological research, represent a useful model for stereotypies. Gerbils growing up under standard laboratory housing conditions typically develop two distinctive

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behavioural abnormalities: stereotypic digging and chewing on the bars of their cage. We analysed the causes of this behaviours by adding specific enrichment factors to the standard housing conditions or by modifying the handling of the gerbils during breeding. Afterwards we observed these animals weekly from their birth to adulthood to quantify the frequency and duration of bouts of stereotypic behaviour. In addition, we measured cortisol levels in fresh faeces collected once a week (ELISA) as an indicator of chronic stress, but found no increased cortisol levels depending on the housing conditions.

1. Simulation of burrows

In the wild, gerbils live in vast subterranean burrow systems which provide shelter against the climate and predators. Standard laboratory cages lack a burrow-like structure into which the animals can retreat. In such a situation, the gerbils are highly motivated to attempt to create a burrow by digging. Their efforts remain unsuccessful, which then leads to stereotypical repetition of digging motions (Wiedenmayer, 1997). Based on Wiedenmayer's results, we developed a prototype artificial burrow system integrated into a standard laboratory cage and tested its efficiency in reducing stereotypic digging. In gerbils grown up with such a burrow system stereotypic digging was significantly reduced.

2. Separation of the juveniles from their family

Initially we hypothesised that bar-chewing in gerbils was either a replacement activity caused by the lack of appropriate chewable nesting material or a reinforced bar-manipulation caused by the close proximity of their food pellets to the bars of the cage-lid in the food hopper. Our first experiments showed, however, that neither of these factors had an influence on the development of bar-chewing (Waiblinger and König, 1999; Fig. 2). Upon closer observation, we found that bar-chewing significantly increased after juvenile gerbils were routinely separated from their families and housed in a separate cage with fresh bedding material at the age of 35 days ("Separation" in Fig. 2; Waiblinger and König, 2004). In a second experiment, we therefore tested the influence of both the process of separation from their family and the transfer to a fresh cage on the development of stereotypic bar-chewing in a two-way factorial design. Juvenile gerbils significantly increased bar-chewing only if separated from their family before the next litter ("younger siblings" in Fig. 3) was born, but not after a transfer to a fresh cage together with their family or after a separation after the birth of younger siblings (Tab. 1). Bar-chewing might therefore reflect the juvenile animals' motivation to return to their families, as long as there are no younger siblings present. By separating family and juveniles only after the birth of a younger litter in the family, the development of stereotypic bar-chewing can thus be reduced ("Separation" in Fig. 2 and 3). The time interval between litters averages 35 days in gerbils, therefore we suggest not separating juveniles from their parents before the age of 5 weeks.

Simple refinement is effective

Our experiments have shown that rather simple refinement in animal housing conditions and flexible timing of separation of juveniles can successfully reduce or even prevent specific stereotypic

Tab.1: Results of a repeated measures ANOVA showing the effects of transfer (to a clean cage) and/or separation (from parents and siblings), weight at the age of 35 days, and presence of younger siblings (within subjects factors) on the development of bar-chewing before (days 33 and 34) and after transfer and/or separation (days 36 and 37); age was used as within-subjects factor.

Factor	F	df	P
age (days 36/37 compared to days 33/34)	**34.661**	**1**	**0.0001**
Transfer to a clean cage	*0.758*	*1*	*0.396*
Separation from parents and siblings	***7.180***	***1***	***0.016***
presence of pups, i.e. younger siblings	*0.018*	*1*	*0.895*
weight at 35 days	*1.959*	*18*	*0.086*
Age x transfer	0.017	1	0.898
Age x separation	**12.213**	**1**	**0.003**
Age x presence of pups	**4.594**	**1**	**0.047**
Age x weight	1.911	18	0.094
Age x transfer x separation	0.133		0.720
Age x transfer x presence of pups	0.003		0.957
Age x transfer x weight	0.887		0.493
Age x separation x presence of pups	2.160		0.160
Age x separation x weight	**3.355**		**0.023**
Age x presence of pups x weight	**3.169**		**0.041**

Between subjects factors are indicated in italics, significant effects are indicated in bold type.

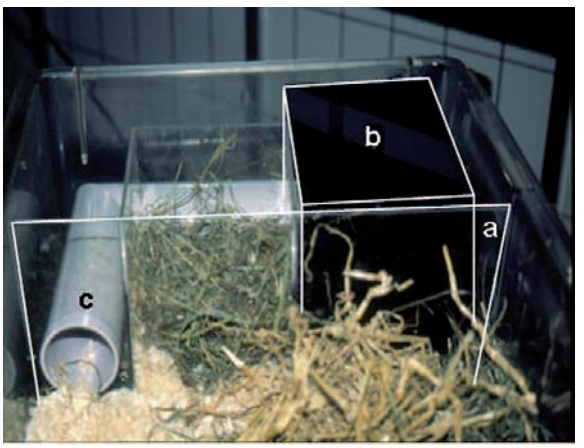

Fig. 1: Prototype of an artificial burrow that consists of three modules (a: separation wall, b: dark nestbox with removable lid and c: access tunnel) and can be easily integrated into a standard Makrolon cage type IV.

behaviour. Delayed separation of the juveniles will not affect other aspects of animal management since juvenile gerbils do not usually reproduce as long as they remain with their parents.

Stereotyping animals are poor models for biomedical research

Stereotypies are not only aberrant behaviours or bad habits, but indicate more in-depth abnormalities in the central nervous system of animals performing stereotypies. Garner (2005) showed that stereotyping animals exhibit cognitive deficits such as behavioural disinhibition similar to schizophrenic human patients if tested in appropriate behavioural paradigms such as the spatial extinction task (i.e. the animal has to learn that a previously learnt path is not rewarded any more). If such animals are used in behavioural tests that require any form of learning and extinction of learning, activity, response latencies or behavioural variability, then stereotyping animals will perform much poorer than non-stereotyping animals. Also, this suggests that there are underlying neuronal bases in stereotyping and behaviourally disinhibited animals, i.e. changes in neuronal pathways and brain metabolism, which make these animals poor models in biomedical research.

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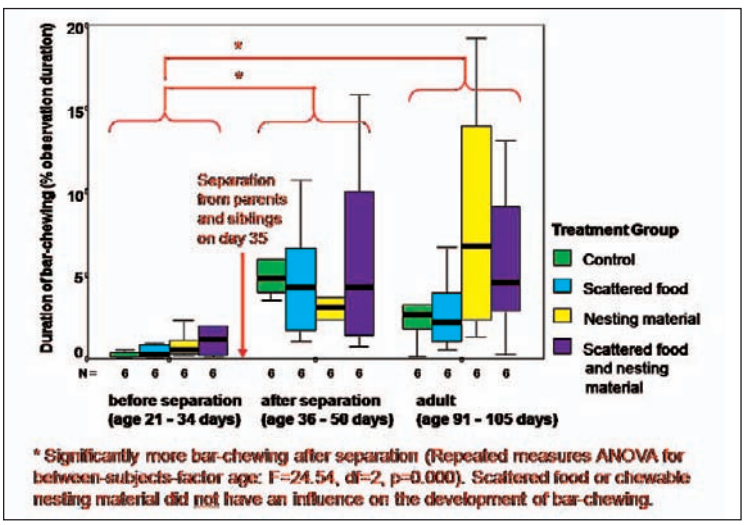

Fig. 2: First experiment.
Influence of age, chewable nesting material and scattered food in bedding on the development of bar-chewing in juvenile laboratory gerbils. The latter factors do not have an influence on the development of bar-chewing (measured over a 42 min observation period/animal/2 days once a week from day 21 to day 105 after birth). However, bar-chewing increased significantly after the gerbils had been separated from their parents and siblings and been placed in isosexual groups of two in clean cages – a normal husbandry procedure.

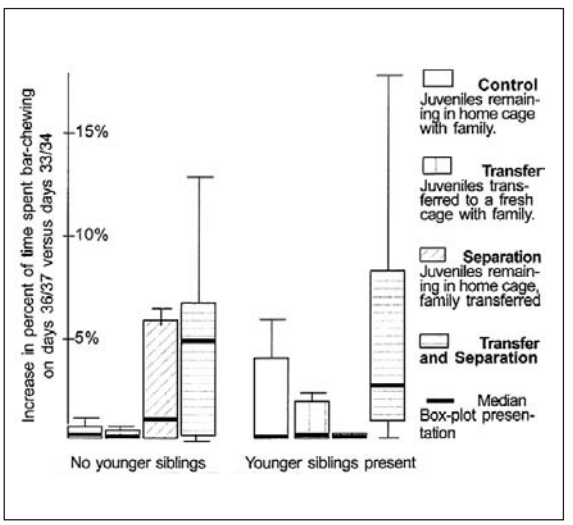

Fig. 3: Second experiment.
Influence of separation from parents and siblings, transfer to a clean cage and the presence of younger siblings on the development of stereotypic bar-chewing measured over a 21 min observation period/animal/day on day 33/34 and 36/37. Positive values mean an increase; negative values a decrease in bar-chewing.

Environmental Enrichment does not Disrupt Standardisation of Animal Experiments

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Summary
Environmental enrichment can prevent abnormal behaviours and improve the well-being of laboratory mice, but concerns have been raised that it might disrupt the standardisation of experiments. Based on a multi-laboratory study, I show that animal welfare can be improved by environmental enrichment without disrupting standardisation.

Keywords: ethology, laboratory animals, transgenic animals, enrichment, mice, standardisation, refinement, reduction

Background Information

Standard rodent housing: impaired brain development – abnormal behaviour – poor well-being

Environmental enrichment refers to the addition of structure and resources to the barren standard cages for laboratory rodents with the aim to stimulate active interaction with and exploration of the environment and to facilitate the expression of species-typical behaviour. Accumulating evidence indicates that mice and other rodents housed in barren laboratory cages (which are still "standard" in many laboratories) show impaired brain development due to sensory and motor deprivation (van Praag et al., 2000); develop abnormal repetitive behaviours (i.e. stereotypies), originating from chronically thwarted attempts to gain access to highly valued resources (e.g. shelter) or to perform highly motivated behaviours (Würbel, 2001); and exhibit an anxiogenic behavioural profile compared to mice from more enriched environments (Chapillon et al., 1999). Thus, converging evidence indicates that the well-being and normal brain development of mice housed in barren cages may be seriously impaired, which also questions the validity of research done with these animals (Würbel, 2001). All of these effects could be attenuated, if not eliminated, by adequate environmental enrichment, especially during early ontogeny.

Testing effects of enrichment on standardisation

For many years, concerns have been raised that environmental enrichment might disrupt standardisation by increasing variation in data obtained in animal experiments. Previous studies found variable effects of enrichment on variation in the data depending on the variable studied (Tsai et al. 2003), indicating that enrichment has no consistent effect on variation in data.

However, none of these studies provided conclusive evidence since they were all based on single experiments instead of several independent replicates. In contrast, we used a multi-laboratory approach involving nine independent replicates (three in each of three laboratories) to study the effects of enriched *versus* barren housing on (i) variation in behavioural endpoints and (ii) reproducibility of behavioural differences between three strains of mice across these independent replicates. Each replicate involved eight mice per strain and housing condition, amounting to 48 mice per replicate and 432 mice in total. Enrichment was a combination of more space, additional resources, increased environmental complexity, and novelty (novel items and environmental change). We used mice of two common inbred strains (C57Bl/6J, DBA/2) and their F1-hybrids (B6D2F1). They were housed in either small barren or large enriched cages from weaning to 9 weeks of age (Fig. 1a, 1b).

Behavioural tests

At 10 weeks of age, all mice were subjected to four behavioural tests commonly used in drug screening studies and behavioural phenotyping of mutant mice (Fig. 2). We used identical test systems in all three labs and standardised test conditions as good as possible. Test performance in all tests was video-tracked using EthoVision 3.00 (Noldus Information Technology, Wageningen NL) and the data was analysed using a 4-way factorial ANOVA model with housing (barren versus enriched housing), strain (DBA/2, C57Bl/6, B6D2F1), laboratory (Lipp, Nitsch, Würbel), and replicate (1, 2, 3) as between subject factors.

Variation and reproducibility are unaffected

To test the effects of enriched housing on the detection and reproducibility of strain differences in behaviour, we split the data by housing conditions and calculated for each replicate the propor-

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tion of variance in behavioural measures contributed by within-group variability and by laboratory x strain interactions. Figure 3 presents a synoptic summary of the results. Within-group variability contributed between 40 and 84% (average 60%) to total variance. With an average of 7.6%, the contribution of strain x laboratory interactions was considerably smaller and also less variable. However, within-group variability was unaffected by enriched housing (except for fecal bolus counts on the O-maze). This indicates that enrichment did not decrease the sensitivity of the tests to detect genetic differences. It also shows that barren housing conditions fail to reduce individual variability in behavioural endpoints. Furthermore, enrichment had no significant effect on the proportion of variance contributed by strain x laboratory interactions, and the direction of differences varied across measures, indicating that enrichment did not increase the risk of obtaining conflicting results between laboratories.

Similar to an earlier multi-lab study (Crabbe et al., 1999), we found significant strain x laboratory interactions in many variables. However, closer inspection of the data revealed that these were mainly of quantitative rather than qualitative nature; reflecting differences in effect magnitude rather than direction of the effects (data not shown; see Wolfer et al., 2004).

Is environmental standardisation ineffective?

Between-laboratory effects (contributing on average 5.2% to total variance) and replicate effects (3.1%) made similar contri-

Fig. 1: Housing conditions
a) Barren housing: Makrolon type II cages with sawdust as bedding and food and water ad libitum. b) Enriched housing: Makrolon type IV cages with sawdust, food, water and shelter ("Mouse House"). Twice a week one enrichment item was added, some of which were removed after one week (e.g. paper tissue, straw, shredded paper), while others remained in the cage until the end of the housing period (e.g. tunnel, wooden branches, cardboard house).

Fig. 2: Example of a behavioural test
Water Maze Test: A circular pool (diameter: 150 cm) filled with opaque water, containing a goal platform (14x14 cm) hidden 0.5 cm below the water surface at a constant location. The mice performed 16 training trials (4 per day) from varying start positions. On day 5, they performed a 60 s probe test without the goal platform.

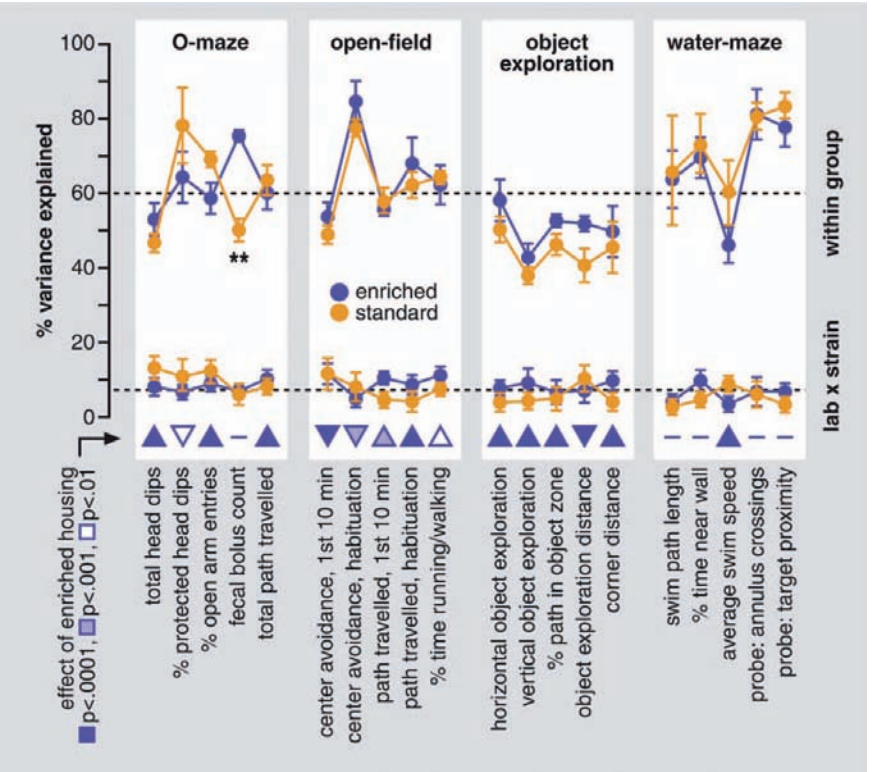

Fig. 3: Effects of enrichment on variation and reproducibility of behavioural endpoints.
Mean (± 1 s.e.) proportion of variance (%) in representative measures of the four behavioural tests contributed by within-group variability and laboratory x strain interactions. Data was pooled for the 3 strains (total N=432). (**: p<0.01). Triangles illustrate direction and significance of enrichment effects on each variable.

butions to total variance, indicating that standardisation between laboratories was nearly as good as standardisation within laboratories. This was surprising since nothing but cage-type, enrichment protocol, light phase, test equipment, and test protocols were equated across labs. This casts doubt on the effectiveness of excessive environmental standardisation to improve between-laboratory replicability of results from animal experiments (Würbel, 2002). On the other hand, it may simply reflect thtat an enriched environment may be as standardised as a barren environment. It says, however, nothing about the external validity of the results. The many significant strain x housing effects (see also below) indicate that barren as well as enriched housing conditions may produce idiosyncratic results that are valid with respect to the specific housing conditions only, and that therefore housing conditions need to be systematically randomised to produce externally valid results (Würbel and Garner, 2007).

More exploratory, less anxious

Enrichment also had significant effects on many measures of exploration and anxiety (Fig. 3). Importantly, enriched mice showed higher exploratory activity and less anxiety-related behaviour in all three tests of exploration. Figure 4 gives an example from the Elevated O-Maze Test, indicating that enrichment effects were consistent across strains and laboratories (see Wolfer et al., 2004 for more details).

Beneficial for animals and research

Our findings reject concerns that environmental enrichment might disrupt standardisation. These are important findings in the light that such concerns have hindered the implementation of enriched housing, despite its known advantages to the animals (Würbel, 2001). Our findings should be generally applicable, for example to drug screening, lesion studies, and the phenotyping of mutant mice. They should also apply to morphological or physiological measures, which are likely to be less sensitive than behavioural measures to environmental perturbations. It remains to be seen whether our findings also apply to male mice who may sometimes respond with enhanced aggression to certain forms of enrichment (Würbel and Garner, 2007). At least for females, however, our results

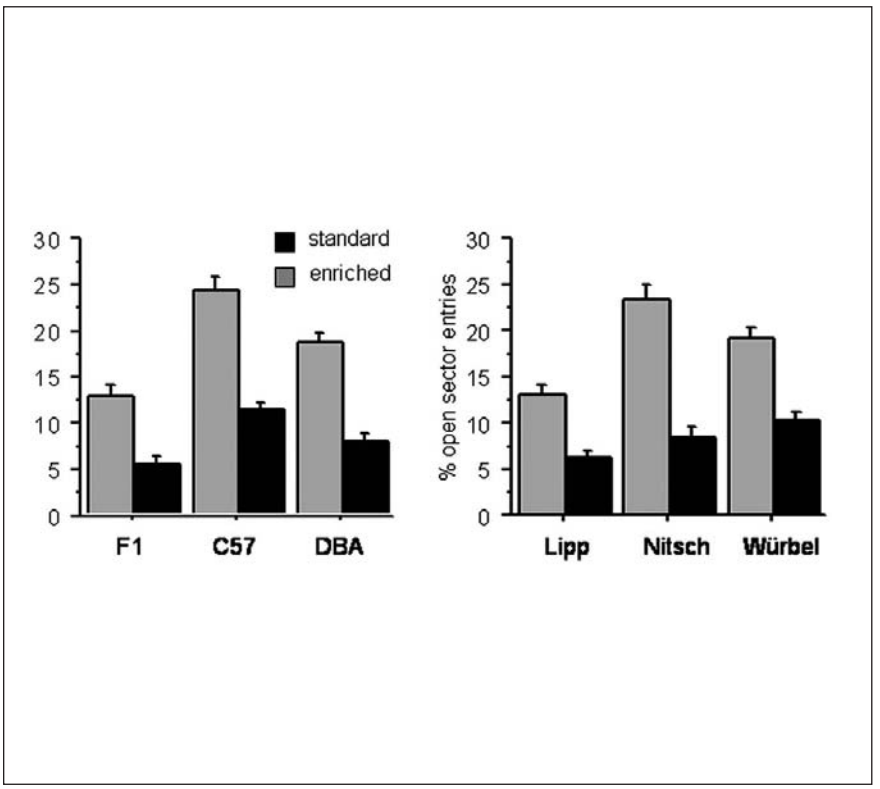

Fig. 4: Enrichment reduces anxiety in all strains and laboratories
Effect of enrichment on mean (± 1 s.e.) proportion (%) of entries to the unprotected sectors on the Elevated O-Maze displayed by strain (left panel: data of the three labs pooled) and lab (right panel; data of the three strains pooled).

demonstrate that environmental enrichment may be used to improve animal welfare without reducing precision and reproducibility of the data, while at the same time attenuating abnormal brain function and anxiety – two potential confounds in animal experiments.

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Isolated, Autologous Blood-Perfused Heart: Replacement of Heterotopic Heart Transplantation

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Keywords: rat, heart, transplantation, ischemia, reduction, replacement

Background and aim

The aim of this project is to develop a novel *ex vivo* model of rat heart perfused with autologous blood (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 20 % O_2, 5% CO_2 and 75% N_2 (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 ECG electrodes.

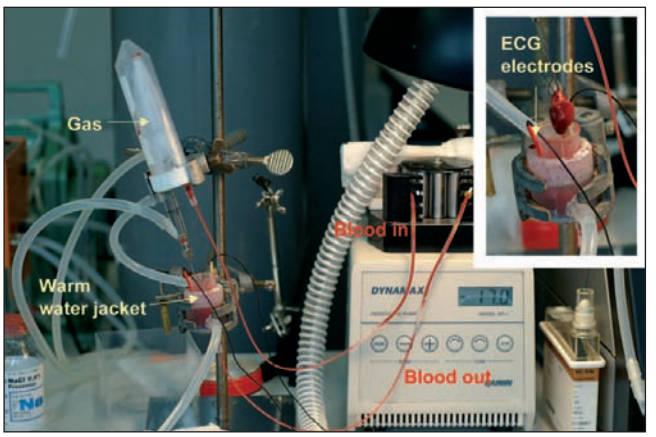

Fig. 1: Experimental set-up.

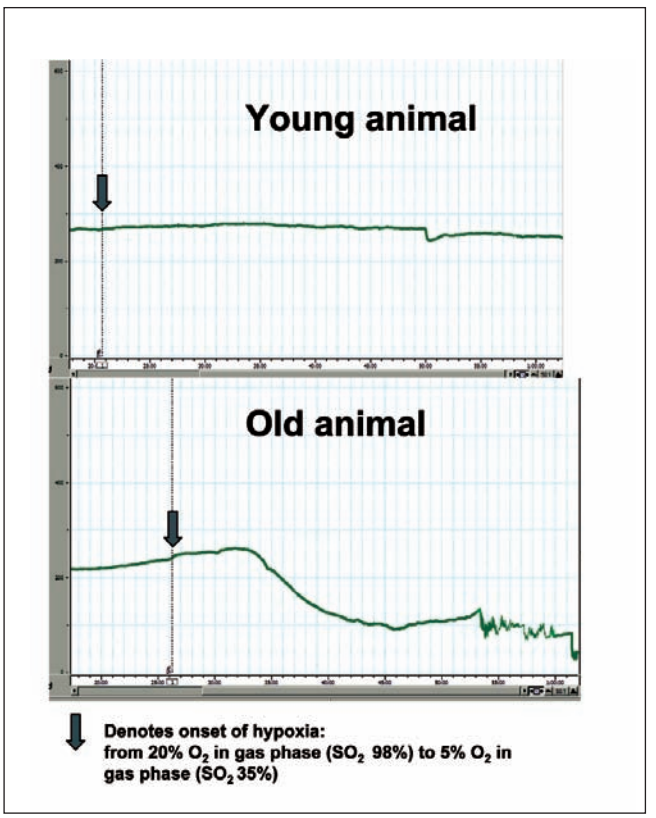

Fig. 2A: Hypoxia-induced changes in heart rate in *ex vivo* perfused heart of old and young rats.

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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-}), brain natriuretic (BNP) and cardiac troponin (TnT) thus assessing mechanical stress and tissue damage. In a separate set of experiments, the utilisation 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 heart 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, heart rate of the young animal's heart remains unaltered (Fig. 2A, 2B). Hypoxia-induced decrease in heart 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.

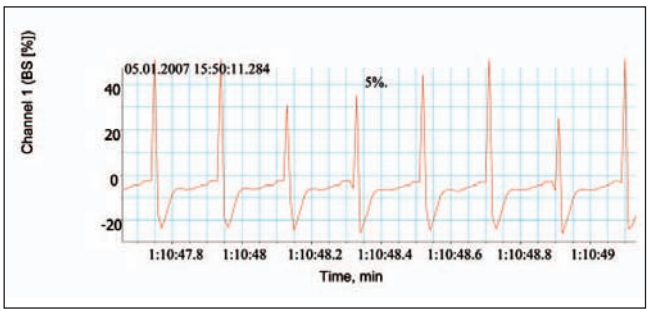

Fig. 2B: Original recording of an electrocardiogram of isolated blood-perfused heart

Conclusions and relevance for 3R

At the moment we are in the process of characterising a novel experimental model showing promising potential. Based on the results, we are planning to be able to not only replace a common Tyrode-perfused isolated heart model (Langendorff model) but also to reduce the use of in vivo heterotopic heart transplantation model in the near future. The latter required at least twice as many animals and was significantly more stressful (severity grade 2-3 for the recipient rats instead of 0-1 for the *ex vivo* model).

Furthermore, the animals' age plays a key role in their ability to resist a decrease in oxygen supply. The comparison of hypoxic responses of aged and young animals' hearts will be important to correlate the obtained data with clinical observations in young and old patients.

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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

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 www.forschung3r.ch/en/projects/pr_71_00.html). 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.

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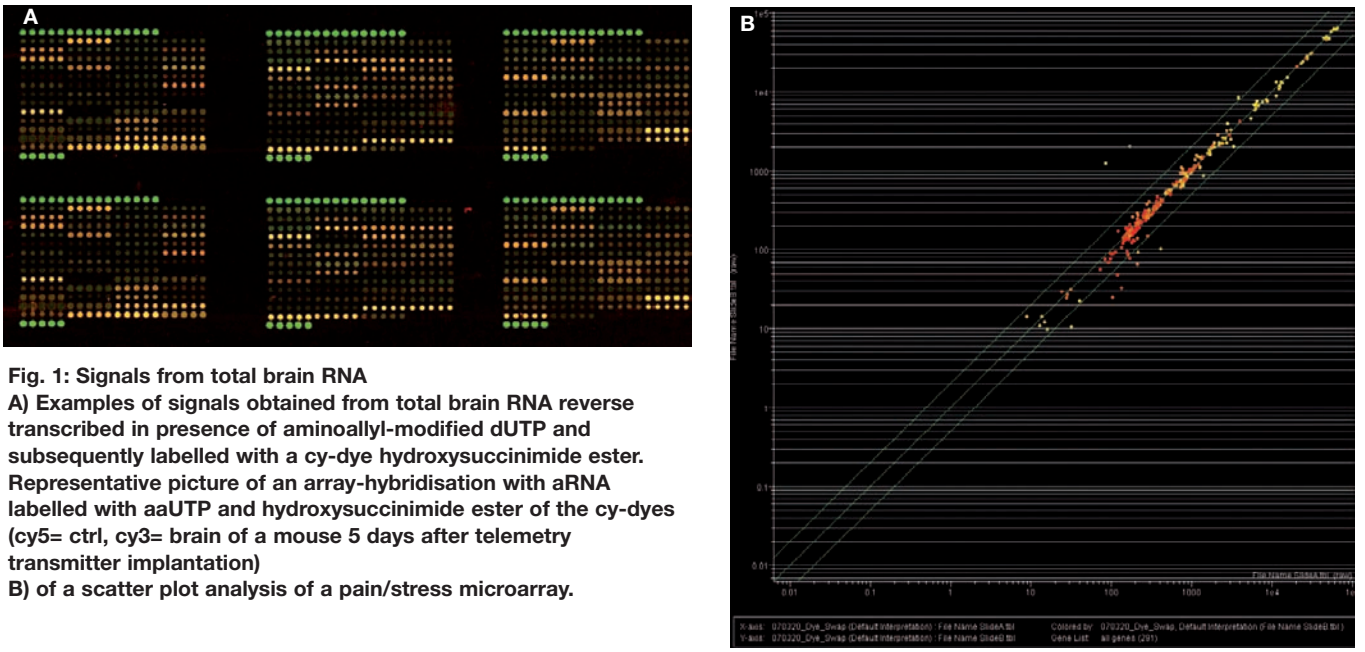

Fig. 1: Signals from total brain RNA
A) Examples of signals obtained from total brain RNA reverse transcribed in presence of aminoallyl-modified dUTP and subsequently labelled with a cy-dye hydroxysuccinimide ester. Representative picture of an array-hybridisation with aRNA labelled with aaUTP and hydroxysuccinimide ester of the cy-dyes (cy5= ctrl, cy3= brain of a mouse 5 days after telemetry transmitter implantation)
B) of a scatter plot analysis of a pain/stress microarray.

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 minimisation of the secondary structure, high Tm's, and therefore a normalised hybridisation temperature (Fig. 1).

We defined time points in postoperative mouse models for microarray analysis using a telemetric system. We are currently analysing 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 analysing expression pattern changes in total RNA pools from different tissues (e.g. spinal cord, brain stem, hippocampus, cortex).

Preliminary hybridisations 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 minimisation of pain and stress. The same holds true for breeding of mutant animals. The microarray analysis will be used in parallel to behavioural 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 artefacts due to the presence of pain/stress in the analysed 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 minimise the number of affected mutant animals in breeding colonies.

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A Non-Mammalian System to Study Bacterial Infections

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Keywords: bacteria, protozoa, amoeba, infectious diseases, reduction, replacement, infectiosity

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 analyse the complex relationship between bacteria and phagocytic cells. Our initial work was focused on the mechanisms controlling phagocytosis of bacteria by amoebae (Cornillon et al., 2000). We have more recently focused on the interaction of *Dictyostelium amoebae* with pathogenic bacteria (Fig. 1). We have shown that *Pseudomonas aeruginosa* makes use of a number of virulence factors to inhibit the growth of *Dictyostelium amoebae* (Cosson et al., 2002). 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 characterised 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* (Benghezal et al., 2006). Based on our current results it is clear that, firstly this system can be adapted to study many different bacterial pathogens (Charette et al., 2005), 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-specialised laboratories.

Conclusions and relevance for 3R

As is summarised 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.

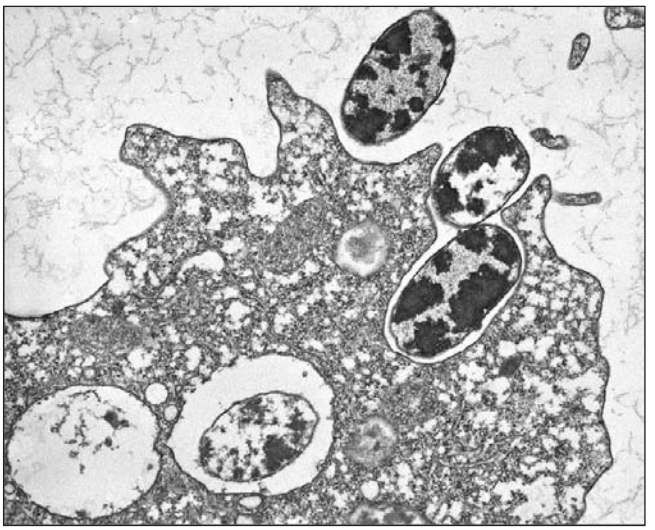

Fig. 1: A *Dictyostelium amoeba* eating bacteria.
Pathogenic bacteria often use the same mechanisms to defend themselves against unicellular amoebae and to infect mammalian animals. Consequently unicellular amoebae offer an alternative system to study pathogenic bacteria. This system could reduce significantly the need to use mammals to study pathogenic bacteria.

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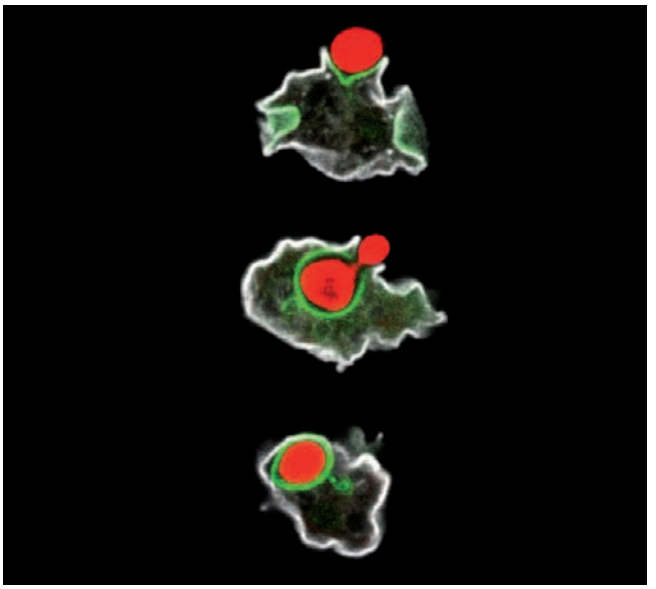

Fig. 2: To study infectious diseases, it is not always necessary to infect animals.
Researchers in the NEMO Network use more simple hosts such as amoebae, or drosophila flies. In this picture an amoeba (white) eating up a yeast cell (red).

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

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 prioritise 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.,

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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 prioritised 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 (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 generalise this descriptor will be developed. Then a first subset of Kinspec measurements of non phenolic compounds will be used to test if this generalisation is successful. The focus of the QSAR-model is to achieve a high robustness, i.e. an ability to generalise 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-modelling 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 legitimise 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.

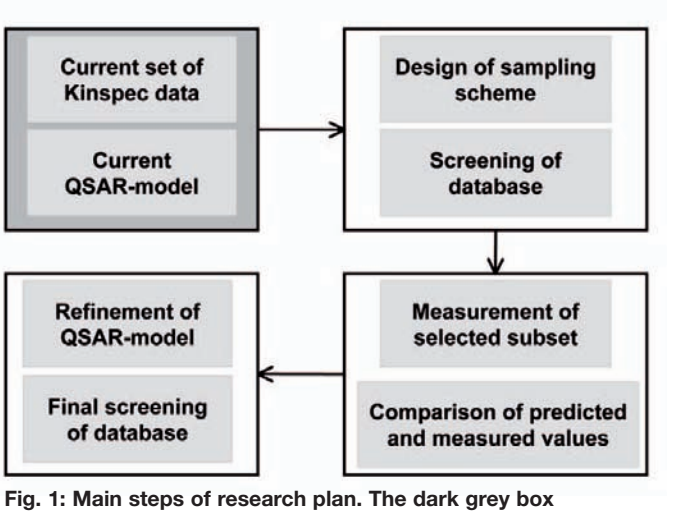

Fig. 1: Main steps of research plan. The dark grey box indicates the status at the start of the project.

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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, biochemical / analytical, in silico, reduction, replacement, drug screening

Background and aim

The pending implementation of the new European chemicals legislation (REACH) will produce the requirement of a lot of additional testing, including bioaccumulation assessment in fish and other aquatic species. 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 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) 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.

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. Very recently, the co-applicant of this project has used PAMPA for the first time to evaluate its potential for assessing passive absorption and elimination in small fish across fish gills. This work is very promising but the method still needs refinement, adjustment of conditions and a wider database before it can be applied as an alternative test method.

The PAMPA system is composed of two well-stirred aqueous compartments divided by a microporous filter that is impregnated with a solvent – phospholipids mixture (Fig. 1). The greatest advantage of PAMPA is that the microporous filter-supported bilayers or the substitute that we are presently using (PDMS) have similar properties to actual biological membranes. Measured permeability correlates well with percent absorption of orally administered drugs that passively permeate intestinal aqueous and lipid barriers. Also the system has the potential for high-throughput testing by using a microtiter plate format, a system that is commercially available in pharmaceutical sciences but needs refinement for use as fish model.

Passive uptake across the gill epithelium is not the only determinant factor for overall bioconcentration. Metabolic transformation reactions will also strongly influence the bioconcentration. In a second phase of the proposed project, we plan to include metabolic processes by combining passive membrane permeation and a metabolic degradation assay *in vitro* using an enzyme cocktail. This metabolic degradation assay is based on the S9 fraction of fish liver, which is another *in vitro* method that is presently undergoing method development for the validation phase.

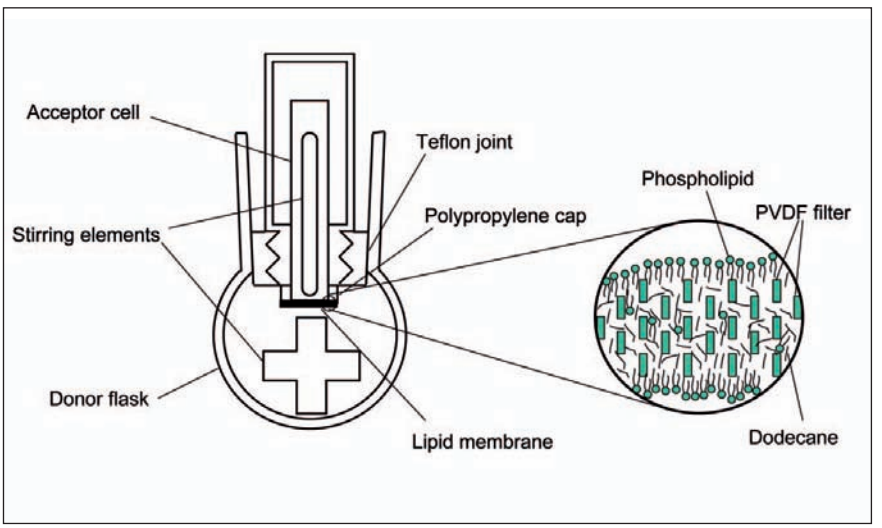

Fig. 1: Experimental set-up of the PAMPA system.

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Conclusions and relevance for 3R

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 will 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.

In addition, the presently availably Quantitative Structure-Activity Relationship (QSAR) models or partitioning-based bioaccumulation models only cover passive diffusion of hydrophobic chemicals. There is a clear need for new in vitro tests and prediction models that also tackle difficult substances such as ionisable material or readily biodegradable substances, thus lowering overall uptake.

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Assessing Animal Health and Welfare and Recognising Pain and Distress

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Keywords: rodents, animal welfare, animal health, pain, distress, 3R education, refinement

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 requires 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 and elsewhere.

Methods 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 (Fig. 1). 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 incorpora-

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tion 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 of 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 transgenic 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.

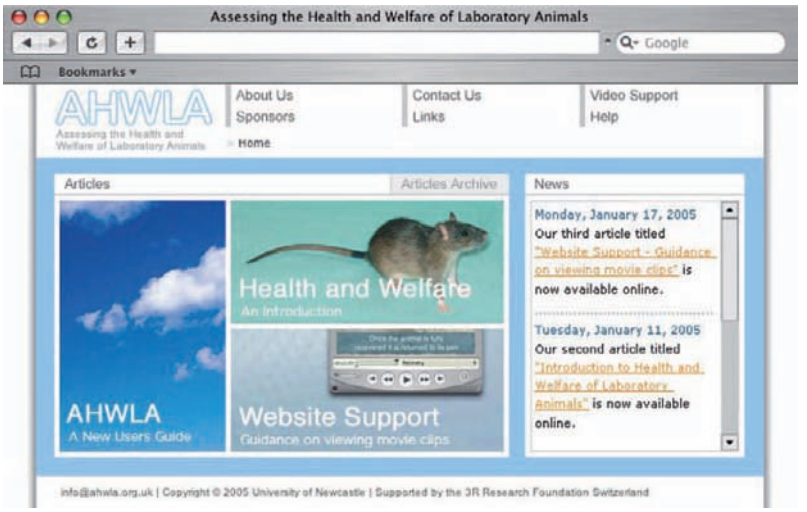

Fig. 1: www.ahwla.org.uk
Web-tutorial 2005 about: "Assessing animal health and welfare" and "Recognising post-operative pain in animals – an introduction"

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.

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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, lung, macrophages, toxicology, cell cultures, 3D cultures, reconstituted tissue and co-cultures, reduction, replacement, toxicity testing, aerosols, nanoparticles, ultrafine particles

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

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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. It contains a differentiated respiratory epithelium with ciliated and secretory cells and macrophages, grown at the air-liquid interface.

1) Epithelial cells are derived from porcine trachea, human bronchi, or from an immortalised human bronchial cell line and 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. Further, respiratory epithelia obtained by micro-dissection from porcine trachea are cultured on porous filter inserts and maintained at the air-liquid interface for direct use for aerosol experiments (Fig. 1).

2) Macrophages obtained by broncho-alveolar lavage (BAL) from donor lungs or pig lungs are added on top of the epithelium. Porcine BAL macrophages cultured on filter inserts are shown in Figure 2.

3) Respiratory epithelial cell cultures may not produce their own surfactant at the air-liquid interface. In that case, an artificial surfactant film has to be added.

The integrity of the 3D primary culture system and the state of differentiation are regularly monitored by morphological, physiological, and biochemical analyses.

The 3D primary cell culture system described is used in particle exposure studies to investigate possible health effects by environmental and manufactured nanoparticles. Various particles (different materials, size, and surface) are applied to the cell cultures as aerosols in a specially designed particle deposition chamber. The cellular responses upon the exposure to the aerosols are studied in a time series using light and electron microscopy for morphologic analyses, by ELISA to check for (pro-)inflammatory cytokine release (e.g. IL-6, IL-8 and TNF-α) and for cytotoxicity (e.g. LDH release), and eventually by molecular biological techniques to monitor gene expression patterns. So far, the cell system has been used in exposure studies with polystyrene (test) particles and (secondary) organic aerosols. Data evaluation is in progress.

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

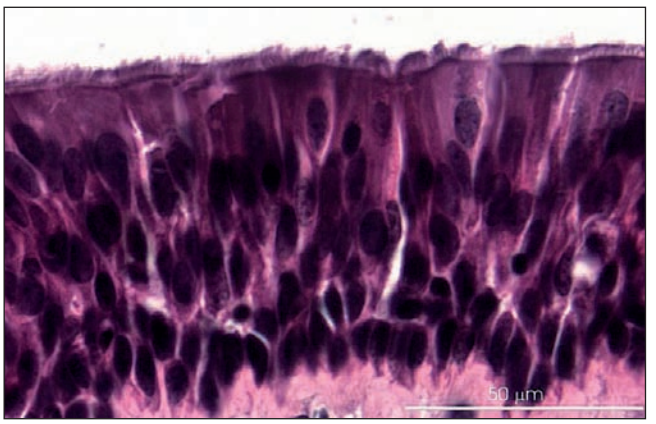

Fig. 1: Light micrograph of differentiated respiratory epithelium obtained by micro-dissection from a porcine trachea cultured for 3 days on filter inserts at the air-liquid interface. Paraffin section, hematoxilin-eosin staining.

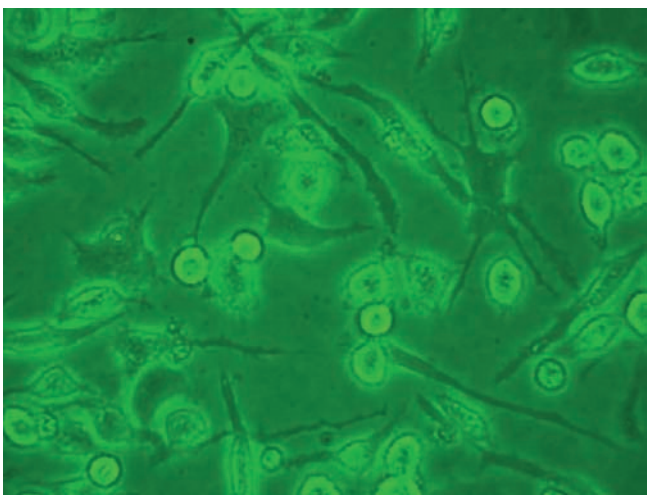

Fig. 2: Porcine macrophages obtained by bronchoalveolar lavage in cell culture. Micrograph of living cells.

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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Organotypic CNS Slice Cultures as an *In Vitro* Model for Immune Mediated Tissue Damage and Repair in Multiple Sclerosis

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Keywords: mice, brain, slices, reduction, replacement

Background and aim

Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system (CNS) affecting about one out of 700 young adults in Switzerland. Histological data and results from animal experiments indicate that T lymphocytes, 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 (Sospedra and Martin, 2004).

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 immunised with myelin antigens, thus 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 caused by adoptive transfer of MOG-reactive T cells alone, in rats and nonhuman primates antibodies are additionally required for the induction of demyelination. Likewise, deposits of immunglobulin and complement can be detected in MS lesions, supporting the role of immunoglobulins in MS pathogenesis.

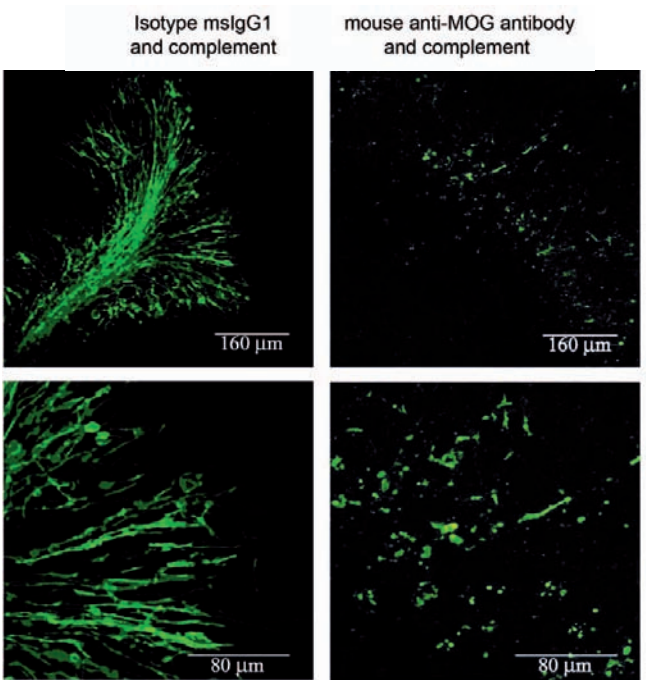

Fig. 1: Cerebellar slices were incubated in the presence of complement with a mouse IgG1-Isotype control (left panels) or with an anti-MOG antibody (right panel). Slices were fixed and stained for myelin basic protein.

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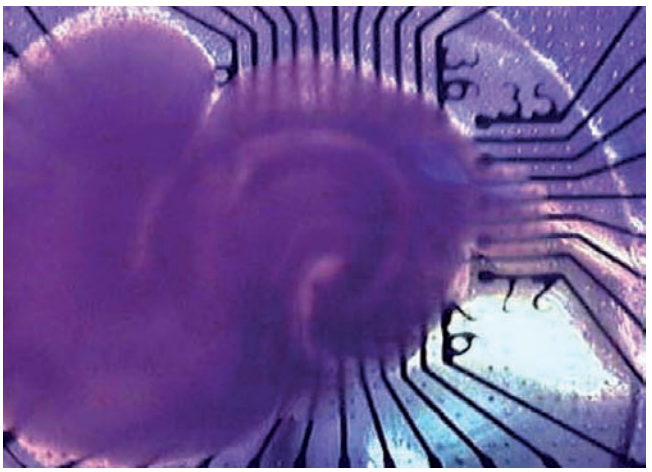

Fig. 2: Hippocampal slices can be placed on top of the electrode. Slices are stimulated in the dentate gyrus and recorded in the CA1 region.

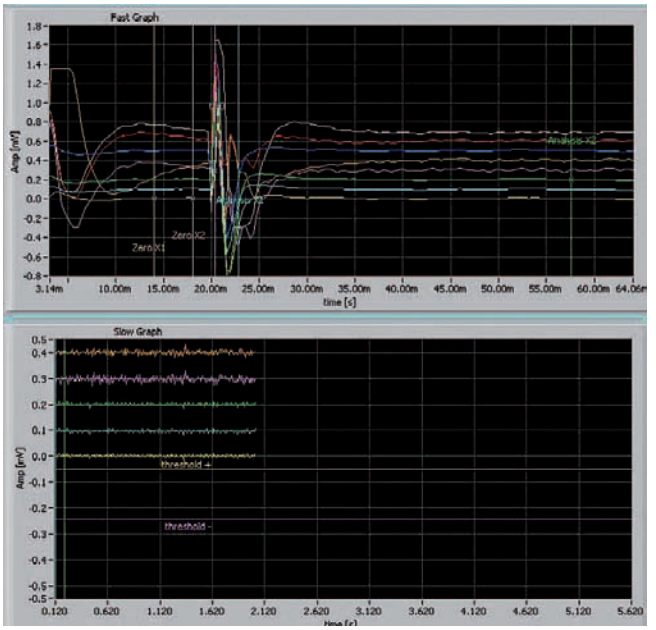

Fig. 3: A typical response pattern of a hippocampal slice.

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 in respect to the induction of CNS damage. 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

Our lab currently focuses on the characterisation of the role of antibodies in MS. We have established methods to amplify immunoglobulin genes from single expanded CSF plasma cells and to express corresponding heavy and light chain immunoglobulin genes as full human recombinant monoclonal antibodies in a eukaryotic expression system. Organotypic slice cultures provide an excellent tool to study the pathophysiological relevance of these antibodies. In order to establish antibody-mediated tissue damage, we used an antibody specific for MOG (myelin oligodendrocyte glycoprotein) in the presence of complement. As shown in Figure 1, the anti-MOG antibody was capable to cause demyelination in organotypic slice cultures (right panel). Complement alone did not alter the integrity of the myelin sheath (left panel). Currently, we are evaluating the effect of the antibodies derived from clonally expanded plasma cells of MS patients mentioned above. To this end, we not only rely on morphological changes, but also on the conductance of electrical stimuli in slice cultures of the hippocampus. In Figure 2, a hippocampal slice is shown on top of multigrid electrodes. Neurons in the dentate gyrus are stimulated and a response is measured in the CA1 region of the hippocampus. Figure 3 shows such a response pattern of hippocampal neurons.

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.
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*. Additionaly, 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.

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Development of *In Vitro* Strategies to Propagate and Characterise Hemotrophic Mycoplasmas

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Keywords: pig, whole blood, vaccination, cell cultures, reconstituted tissue and co-cultures, reduction, replacement

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 splenectomised 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:

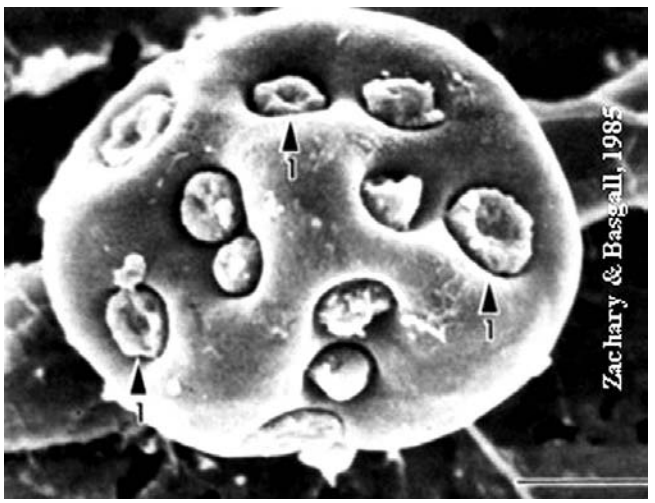

Fig. 1: Electron microscopic picture of *M. suis* in close contact with the host cell (porcine erythrocyte; Zachary and Basgall, 1985).

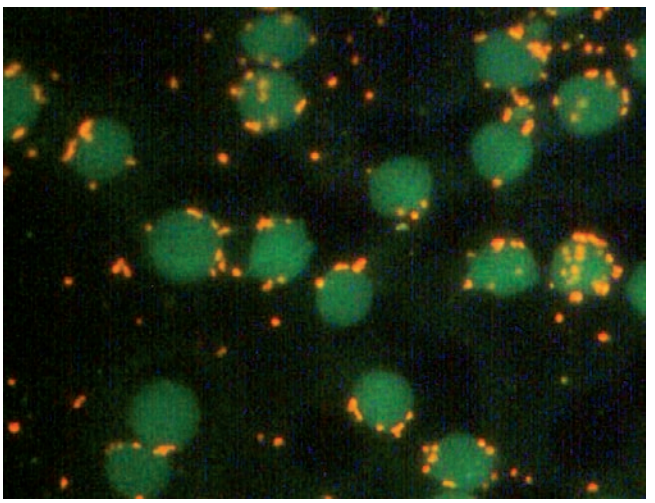

Fig. 2: Acridin orange stained blood smear of an experimentally M. suis infected pig (erythrocytes: labelled green; *M. suis*: labelled orange; Hoelzle, 2007).

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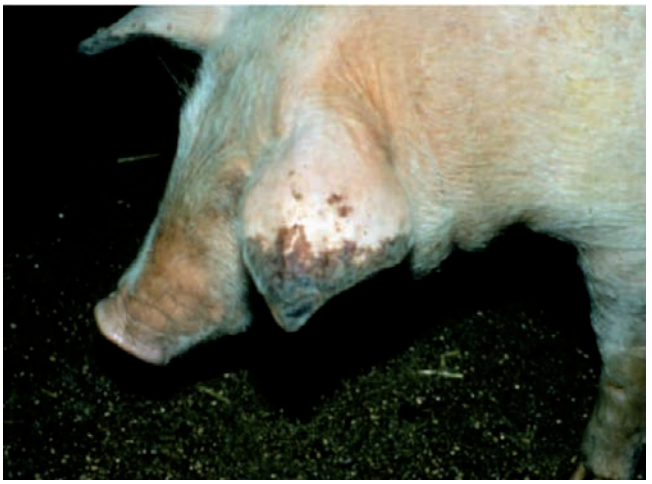

Fig. 3: *M. suis* infected pig showing typical clinical symptoms (acrocyanosis; Hoelzle, 2007).

- Clarification of pathogenetic phenomena in hemoplasma infections.
- Identification of virulence markers.
- Development and improvement of molecular and serological diagnostic assays.
- Realisation 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.

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The Development of an *In Vitro* Intervertebral Disc Organ Culture System

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Keywords: bovine, intervertebral disc, mechanobiology, explants, reduction, replacement

Background and aim

Lower back pain is the most common health problem in individuals between the ages of 20 and 50 (Waddell et al., 1998) with an estimated annual cost as high as $100 billion per year in the US alone. Despite the prevalence of lower back pain, its etiology is largely unknown. There is mounting evidence, however, of a link between degenerated intervertebral discs (IVD) and clinical symptoms.

Several animal 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 (Cassidy et al., 1988); 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: Cole et al. 1985; Hutton et al. 1998; Iatridis et al, 1998; Kaigle et al. 1998; Lotz et al. 1998; Melrose et al. 1992). 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 fulfil the research aims.

Method and results

Bovine coccygeal discs will be harvested from the tails of young cattle (6-8 months old) obtained from the local abattoir. It has already been established that such discs are a suitable model of the human lumbar disc (Oshima et al., 1993). The discs will be cultured in a custom-built chamber similar to that used by Oshima et al. (Oshima et al., 1995). 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

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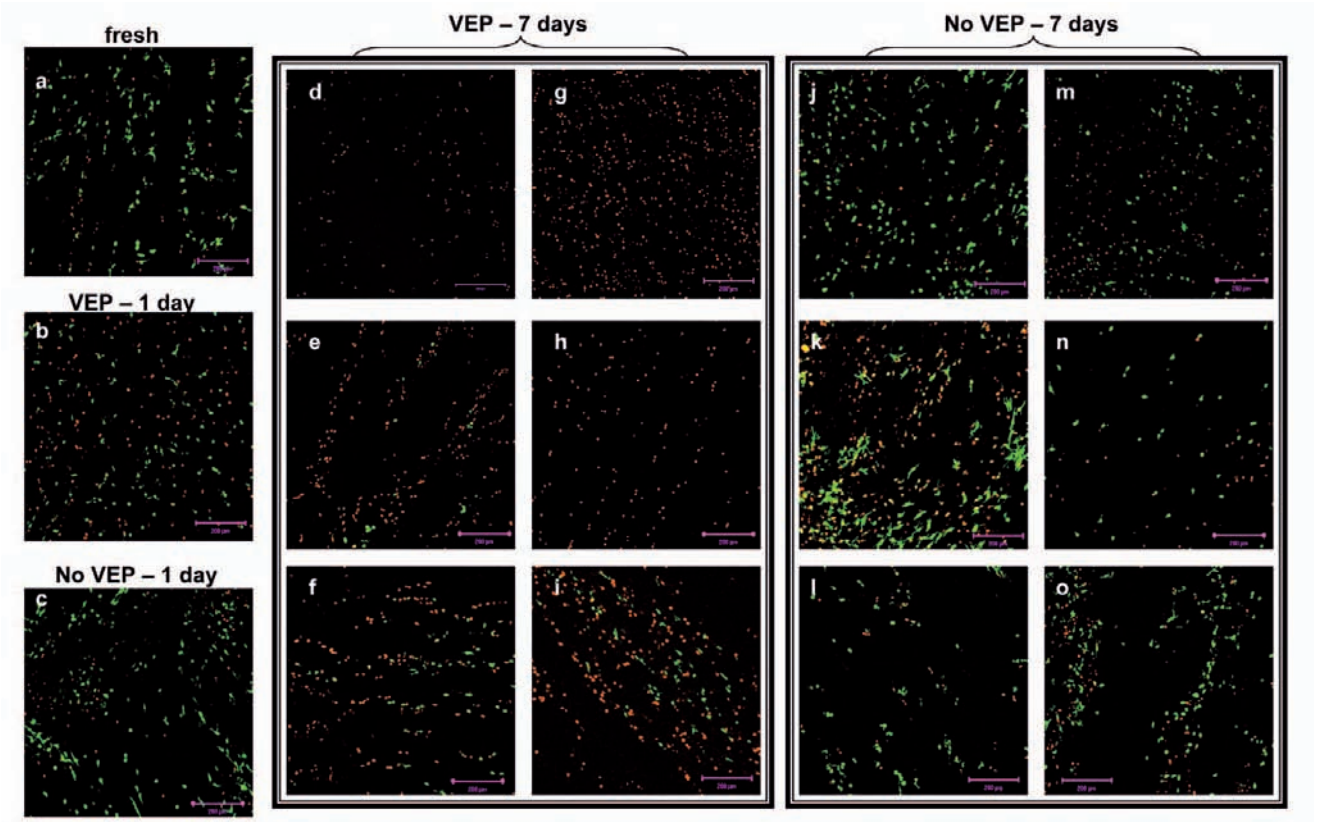

Fig. 1: Nucleus cell viability staining of (A) fresh disc and discs cultured for one (B, C) or seven (D-O) days with (B, D-I) or without (C, J-O) vertebral endplate (VEP).
Live cells fluoresce green, dead cells fluoresce red. Images represent cells in a 50 mm slice of the tissue, starting at a minimum of 50 mm from the surface.

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/DEADR Viability/Cytotoxicity Kit #L-3224, Molecular Probes) of fresh tissue samples. Metabolic activity will be assessed by 35S-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. The first results concerning the establishment of this organ culture system have been recently published (Lee et al., 2004; Lee et al., 2005; Lee et al., 2006; Gantenbein et al., 2006).

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

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Keywords: bacteria, rat, slices, reduction, replacement

Background and aim

Bacterial meningitis remains a devastating disease with high morbidity and mortality. Despite advances in treatment and care, bacterial meningitis holds an in-hospital mortality of 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 unresolved, 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. The research on this subject faces great challenges because of the complexity of harmful processes which involve a variety of microbial factors as well as multiple aspects of the host's inflammatory response.

Brain injury caused by bacterial meningitis prominently affects the cortex and the hippocampus. Cortical damage is associated with areas of focal ischemic necrosis. Hippocampal injury is documented in approx. 75% of patients dying from the disease and in corresponding animal models. This form of injury is characterised by apoptotic cell death of immature neurons, e.g. neuronal stem cells and/or their progeny in the dentate gyrus (Fig. 1). This form of brain damage is associated with long lasting learning deficits. Hippocampal injury is limited to the dentate gyrus, a site of continuous formation of new neurons and therefore potentially well equipped for brain repair.

Research on the pathogenesis of infections of the brain and potential therapeutic approaches supporting brain repair functions largely depends on experimental studies in animals. The grade of suffering (Belastungskategorie) 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 a part of this research by *in vitro* studies. However, due to the multi-factorial pathogenesis of meningitis involving the

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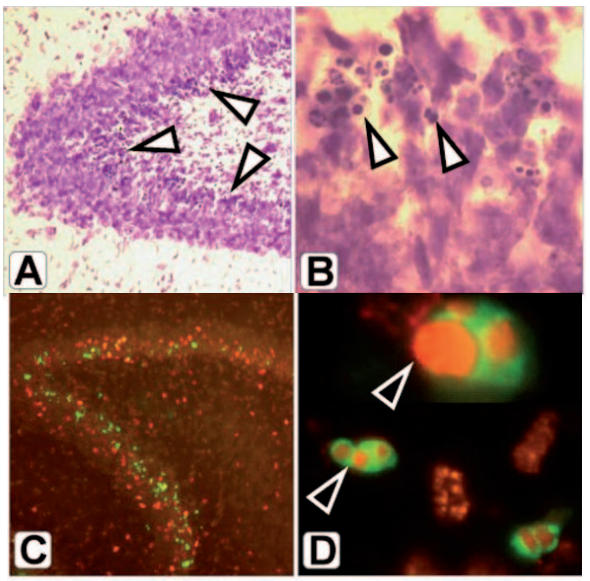

Fig. 1: Brain histology of infant rats with bacterial meningitis .
A) Hippocampal histology of infant rats with pneumococcal meningitis 24 h after infection. Neurons forming apoptotic bodies (arrowhead), a morphological feature characteristic for programmed cell death, are scattered along the inner rim of the dentate gyrus and evenly distributed over the lower and the upper blade (Cresyl violet, x200).
B) Neurons with apoptotic bodies consisting of condensed and fragmented nuclei (arrowheads, x400).
C) Neurons in the subgranular zone of the dentate gyrus labeled with BrdU to identify multiplying cells (brown) and cells undergoing apoptosis by staining for activated caspase-3 (green).
D) Close up of apoptotic cells double positive for BrdU and caspase 3, indicating that they are multiplying progenitor cells or their progeny undergoing apoptosis.

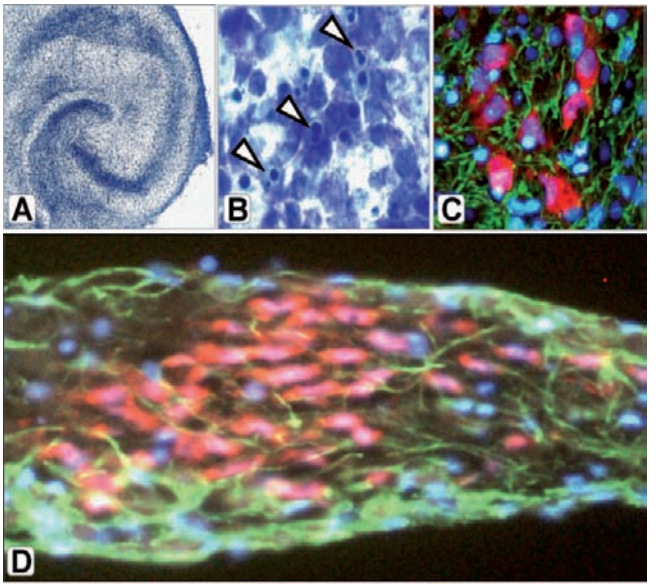

Fig. 2: Architecture and cell-type characterisation and documentation of apoptosis in organotypic slices.
A) The cellular organisation of the hippocampal slice culture system is shown in an overview stained with cresyl violet. Hippocampi are explanted from seven-day-old rats and cut into 400 µm-thick slices. Experiments are started on day 11.
B) After *in vitro* stimulation with pneumococci, apoptosis with formation of characteristic apoptotic bodies (arrows) occurs in neurons of the dentate gyrus.
C and D) The cellular distribution pattern is assessed by cell-type specific markers. Shown here is a horizontal (C) and a vertical (D) section of organotypic hippocampal cultures. Cytoarchitectonic and cell type specific composition of organotypic cultures is shown by the immunohistochemical documentation of astrocytes by GFAP (green) and neurons by NeuN (red). Cellular nuclei are stained by DAPI (blue).

interplay between the susceptible brain cell type, the bacterial pathogen and the host's inflammatory reaction, the majority of the current research activities on meningitis are performed *in vivo*. With this project we plan to establish an *in vitro* model that reproduces important patho-physiological processes of damage and tissue regeneration in infectious diseases of the brain. In the framework of the project we propose:

Aim 1: To reduce and replace studies in animals by developing an *in vitro* system for the study of neuronal brain damage due to bacterial infection. This will be done by differentiating neuronal stem cells into defined developmental stages susceptible for injury, and subsequently challenging them with infectious pathogens and/or inflammatory mediators.

Aim 2: To reduce and replace studies in animals by establishing a co-culture model of neuronal stem cells and organotypic hippocampal cultures for the evaluation of the integrative potential of early neuronal cell lines as transplants into the targeted brain tissue.

Method and results

We have developed an organotypic cell culture system which allows to culture hippocampal or cortical brain tissue in its original architecture and distribution of cell types (Fig. 2). This system is a cornerstone of the proposed co-culture system of organotypic brain tissue with stem or progenitor cells isolated from different origins. This co-culture system will allow us to screen stem cells from different sources e.g. embryonic stem cells from the fetal brain or liver; stem cells of placental origin or stem cells of adult origin, e.g. from neuronal or haematopoetic origin for a potential therapeutic application of neuronal stem cells. Furthermore, this approach will allow us to assess the stage of differentiation that is best suited for integration into the host tissue. This system may lead to a substantial reduction in the number of animals used, since only approaches that prove successful in the *in vitro* system would be considered for further evaluation *in vivo*.

Conclusions and relevance for 3R

The availability of an *in vitro* system would lead to a substantial reduction of animal use by the possibility to screen pathogenetic mechanisms for their relevance and therapeutic approaches for their potential effects prior to conducting studies *in vivo*. The proposed experimental *in vitro* system would allow the reduction or replacement of the following *in vivo* investigations:
i) Testing of 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 differentiated cells, which contribute to their selective vulnerability.
iii) Evaluation of therapeutic approaches able to counteract the selective vulnerability investigated under ii)
iv) Evaluation of therapeutic approaches that involve the grafting of stem cells / neuronal precursors into brain tissue.

Thus, once this system is established, studies screening for pathogenic factors and therapeutic feasibility studies in animals can be substantially replaced by *in vitro* studies.

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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: microcarrier, reduction, replacement

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 sexual stages (oocysts) have to be produced in very young animals (neonatal mice, calves, lambs, piglets). Such infections, which raise serious concerns from the animal protection point of view because of the very early separation of the animals from their mothers, are unfortunately difficult to control clinically, and death due to dehydration is observed regularly. Further, continuous cultivation of the asexual stage of *Cryptosporidium spp.* in conventional 2D *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 2D cultures is to cultivate such parasites in three-dimensional (3D) aggregates of intestinal epithelial cells mimicking their natural environment. Such 3D 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 optimised form of suspension culture that – due to its low-shear and low-turbulence operation – minimises mechanical cell damage and allows cells to aggregate, grow three-dimensionally and to differentiate. In a pioneering study (Nickerson et al., 2001), 3D 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 2D cultures when infected with Salmonella in short time studies. We hypothesise that these particular features of 3D 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

3D aggregates of intestinal epithelial cell lines, either individual or in mixtures, are grown on Cytodex 3 microcarriers (Amersham Bioscience) during 28-32 days in the RCCS (Fig. 1). Evaluation of the quality of the 3D aggregates, in comparison with corresponding 2D cultures, is done by assessing their size, cellular organisation and structure (microvilli formation) by light microscopy of paraffin sectioned 3D aggregates and by immunohistochemical profiling using a panel of commercially available, monoclonal antibodies against epithelial markers (villin, epithelium-specific antigen ESA) and against basement

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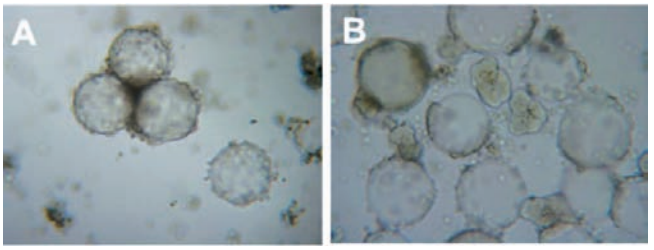

Fig. 1: HCT-8/CCL6 cells growing on Cytodex 3 microcarriers in Rotary Cell Culture System vessels.
A) after 4 days; B) after 21 days of cultivation.

membrane and extracellular matrix components (laminin, fibronectin, collagen IV) as described. Mucus production is demonstrated by histochemical staining with PAS and by using FITC-labelled lectins. 3D cell aggregates are used for short-term infection assays with oocysts of *Cryptosporidium* in 24-well tissue culture plates. Early events of attachment and internalisation of sporozoites will be monitored by using differently labelled (fluorescein, Cy3) monoclonal antibodies directed against Cryptosporidium, with and without cell permeabilisation. Intracellular growth of the parasite will be analysed by transmission electron microscopy in time course studies. Analysis of apoptosis/necrosis following inoculation with Cryptosporidia in 3D aggregates will be done after dissociation into individual cells by treatment with 0.1% EDTA and incubation with FITC-annexin V and propidium iodide and compared to non-infected cells. In addition, 3D cell aggregates will be used for long-term propagation of Cryptosporidium directly in the RCCS vessels (55 ml), and the 3D aggregates will be analysed for intracellular parasite growth by fluorescence microscopy using FITC-labelled mAb, by transmission electron microscopy and by quantitative PCR (qPCR). The viability of oocysts produced in these cultures will be assessed by inoculation of 2D (microslides) HCT-8 cells which are analysed by qPCR.

Conclusions and relevance for 3R

This start-up project should yield a robust protocol for growing 3D aggregates of intestinal epithelial cells which represent a growth environment similar to that between brush border microvilli of epithelial cells and therefore might allow to propagate previously uncultivable organisms and might serve as a relevant model for a wide variety of pathogens (virus, bacteria, parasites) of medical and veterinary importance which infect epithelia of the gastrointestinal, but also the respiratory or urogenital tract. Therefore, our work will contribute to bridge the gap between experiments done with 2D conventional cell cultures with their inherent limitations and those performed in live animals. By providing a better model for what happens *in vivo*, these 3D 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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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, T cell, molecular biology, PCR, reduction, replacement

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 inter-

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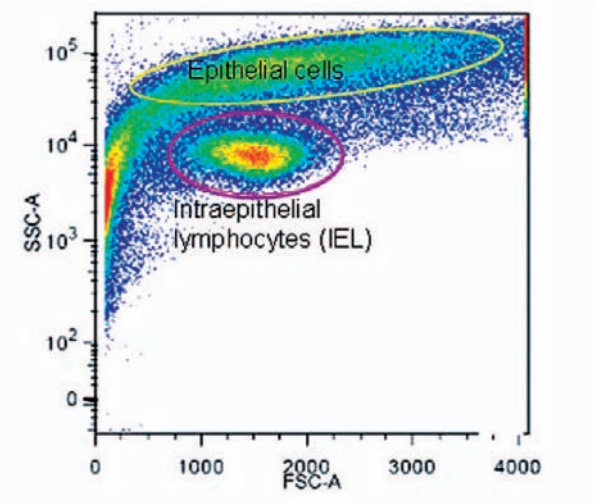

Fig. 1: Scatter blot of the IEL-enriched fraction following elutriation centrifugation. In this preparation 88% of the cells are intraepithelial lymphocytes.

Tab. 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.

	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 IELin 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 %)

play 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 optimise 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 (Tab. 1) and to obtain a highly enriched fraction of IEL (Fig. 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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Development of a Novel Multicellular 3-Dimensional Blood Brain Barrier *In Vitro* Model

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Keywords: rat, astrocytes, brain, epithelia, endothelia, pharmacology, zns, brain disorders, barrier systems in vitro, 3D cell cultures, cell cultures, organ-specific, primary cell cultures, reduction, replacement

Background and aim

The blood brain barrier (BBB) is crucially important for normal brain function, many pathological diseases are characterised by BBB breakdown, such as stroke, Alzheimer's disease, diabetes and multiple sclerosis. Despite this fact, the induction and maintenance of the BBB is still poorly understood. For example, the majority of possible candidates for central nervous system targeting drugs, which have been tested, are inefficient because of poor permeability across the BBB. At present, the majority of studies on BBB integrity and injury are performed *in vivo*. Current *in vitro* BBB models are limited, because these models do not accurately reflect the cellular interactions that occur *in vivo*, as they overlook the 3-dimensional structure of blood vessels and in particular the presence of pericytes that play an instrumental role in the formation of the BBB.

It is the aim of this project to establish a cell culture system that contains all three major cell types that form the BBB *in vivo*: endothelial cells, astrocytes and pericytes. We want to reduce and replace animal experiments with *in vitro* testing and refine current *in vitro* BBB models, by developing a new system that represents a more complete model for BBB formation and maintenance. Development of this system will reduce and partly replace the use of animals in BBB experimentation and aid identification and refinement of new therapeutic targets and protective strategies.

Method and results

Our *in vitro* culture should closely mimic the structure and interaction of cells comprising the BBB *in vivo* (Fig. 1). We have started to develop an astrocyte/endothelial 3-dimensional cell culture system. These cultures consist of cells mixed in a ratio of 1:1 and suspended in a collagen matrix that solidifies at room temperature and is then overlaid with media. After 24 hours in a 3-dimensional culture, the endothelial cells begin to rearrange and form tube-like structures, which the astrocyte end feet contact (Fig. 2). To set up the new system we will first introduce pericytes into our culture system. We then intend to:

1) Observe growth and maintenance of pericytes alone in collagen.
2) Observe growth of pericytes in the presence of either, endothelial or astrocytic cells.
3) Ascertain the optimal ratio of endothelial cell:astrocyte:pericyte to obtain the most stable multicellular 3D cultures *in vitro*. Preliminary results suggest our current 2-cell model remains stable for approximately 6 days. We would like to adapt and prolong this for up to at least 2 weeks, to enable subsequent chronic experiments to be performed. We shall also try matrigel and other matrices as an alternative substrate for cell culturing and compare results. The use of matrigel would be preferable as it is more cost effective.
4) Confirm the BBB phenotype in 3D cultures. Differentiation of the cells and barrier phenotype will also be confirmed by fluorescent microscopy and/or western blotting, this will enable morphological analysis and accurate localisation of the cells within the culture. 3D reconstructions of these images will then be made.

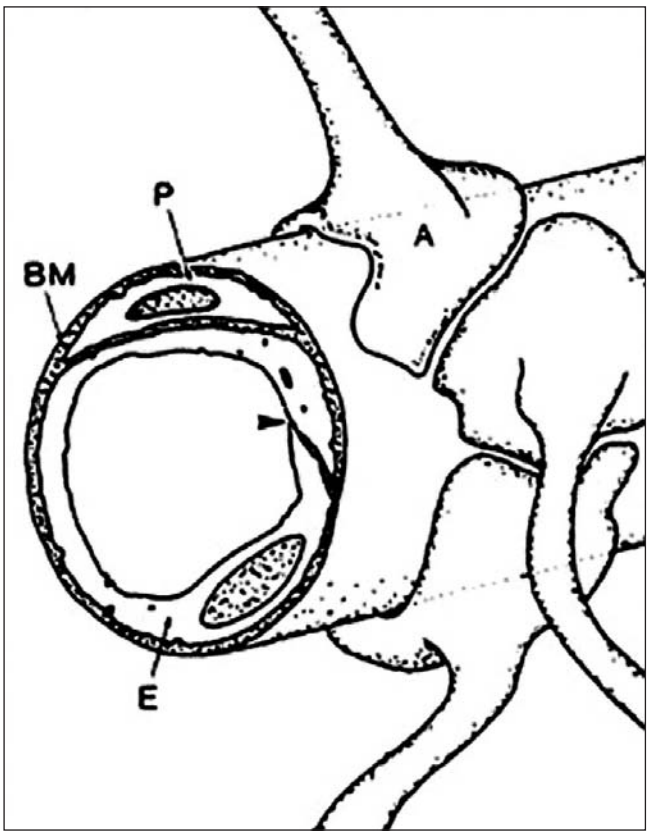

Fig. 1: Cellular relationships at the BBB.
Pericytes (P) are closely associated with capillary endothelial cells (E) and share a common basal lamina (BM). Astrocytic end feet (A) ensheath the capillary (from Abbott, 1989).

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5) Characterise the *in vivo* model with regards to cell movement and rearrangement within the culture using live imaging and confocal microscopy combined with fluorescent tagging of cells and cell-specific molecules.

After the establishment and characterisation of the culture system, changes in gene and protein expression as a result of contact with the other cell types, can be identified and monitored, using molecular biology techniques, FACS analysis sorting on known cell-specific markers, as well as ELISA and RIA techniques to measure the release of different cytokines and growth factors such as VEGF and Epo into the culture media. These methods will lead to identification of proteins expressed in the BBB under normal physiological conditions.

Conclusions and relevance for 3R

Importantly, this model system will provide a unique opportunity to study the BBB and reduce the need for difficult, invasive animal experiments. Given the large number of diseases characterised by BBB breakdown, such a model has wide applications in the medical and pharmaceutical industries, wherein drug and chemical testing could first be carried out exhaustively on the *in vitro* system. Establishment of this model will therefore promote the refinement of potential therapeutic tools and strategies, prior to animal testing and ultimately reduce animal experimentation, consumables and personnel costs. Furthermore, this model system will not only provide information on specific cellular interactions and signals, that promote induction of BBB formation during development, but will be readily manipulated and subjected to different insults such as hypoxia, glucose deprivation and chemical exposure to assist in understanding the breakdown of the BBB. Thus utilisation of this model means BBB research can be more focused and directed to the specific roles of individual cell types, as well as barrier function as a whole, with a minimal use of animal experimentation.

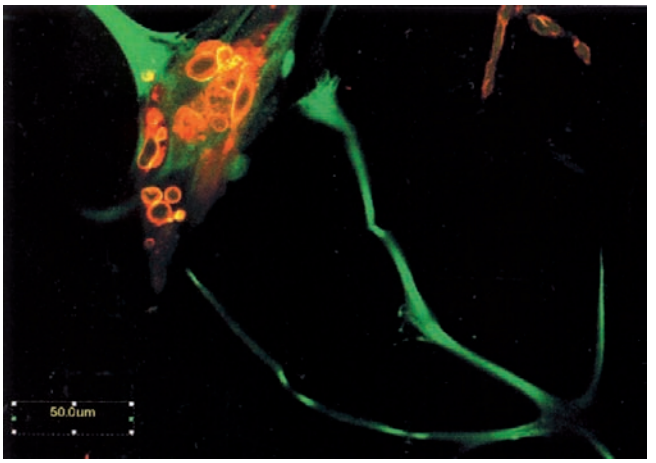

Fig. 2: Current status of 3-dimensional system for *in vitro* BBB model.
Endothelial cells (Labelled with PECAM in red) produce tube-like structures in matrix that are contacted by astrocyte endfeet (GFAP staining in green), Ogunshola, O. (unpublished results).

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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

Background and aim

Vaccines represent the most successful immune-based medical treatments ever discovered and provide long lasting protection against acute infections. The task of limiting adverse reactions by using highly purified antigenic formulas has provided a safer, but nevertheless poor quality of immunogenic therapeutics. Thus, future immune intervention strategies, have a crucial dependence on the identification and characterisation of molecularly defined adjuvants, as well as, on the availability of adequate applicable methods for assessing adjuvanticity.

We would like to develop a system to identify human-compatible adjuvants based on lymphocyte cultures *in vitro*. We have two aims. Firstly, we will assess the capacity of microbial-derived particles and synthetic analogs of enhancing antigen-specific CD4+ T cell responses *in vitro*. Secondly, we will monitor the capacity of compounds endowed with adjuvant

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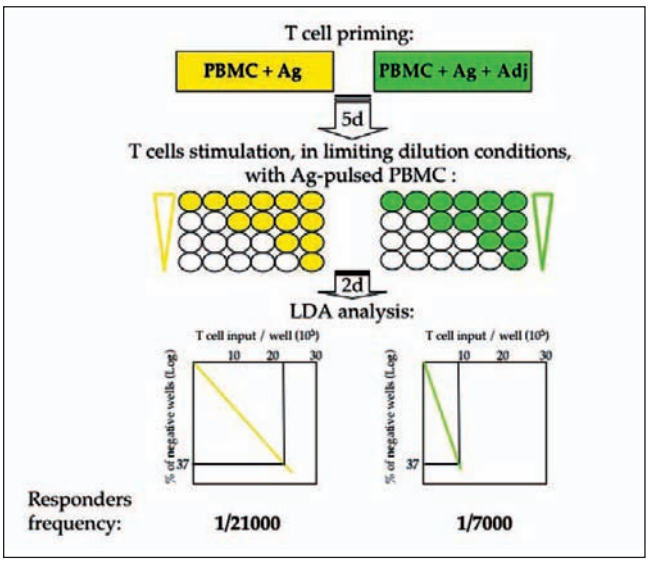

Fig. 1: Adjuvanticity Test I.
Human Peripheral Blood Mononuclear Cells (PBMC) are primed *in vitro* with antigen (Ag) in the presence or absence of adjuvat (Adj). No exogenous cytokines are added to the cultures. After 5 days, cells are seeded at limiting dilutions in a microtiter plate and re-stimulated with autologous Ag-pulsed PBMC. Two days later, T cell proliferation is measured by 3H-thymidine incorporation. The frequency of Ag-specific T cells is finally determined according to the Poisson' s distribution as indicated.

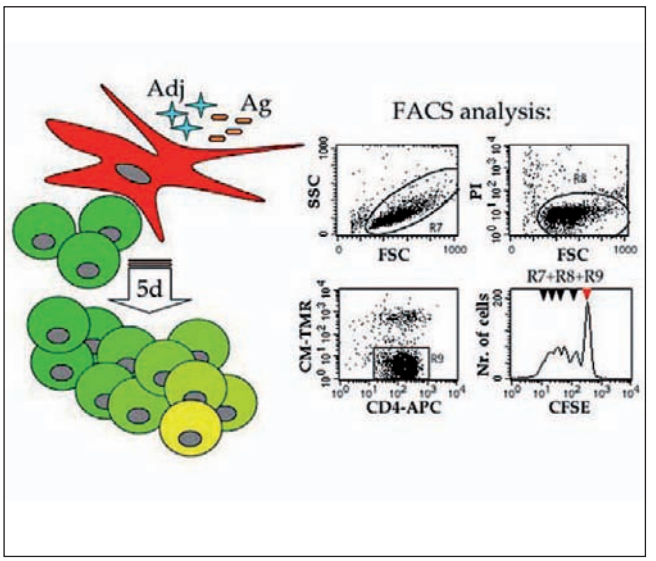

Fig. 2: Adjuvanticity Test II.
Human monocyte-derived Dendritic Cells (DC) are labeled with the red fluorescent dye CM-TMR. Cells are co-cultured with autologous purified CD4+ T lymphocytes, labeled with the green fluorescent dye CFSE, in the presence or absence of antigen and adjuvant. Ag-specific T 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). Data obtained upon PHA stimulation are shown in D; black arrows indicate cell generations; red arrow specifies the non-dividing parent generation.

activity to induce modulation of dendritic cells phenotype and the secretion of soluble factors, in order to identify profiles of activation that correlate to adjuvanticity.

Method and results

Our previous work has shown that di- and tri-palmitoylated bacterial lipopeptide analogs, act as adjuvants for antigen-specific CD8+ T cells reactivity. Based on these results we have developed two types of adjuvanticity tests for assessing the capacity of these compounds to enhance HLA class II-restricted T cell responses. The methods are described in Figure 1 and 2.

Conclusions and relevance for 3R

The assessment of adjuvanticity in animal models has a number of limitations. When applied to large-scale screening of chemical libraries, or to dose-dependent measurements, *in vivo* tests require the employment of high numbers of animals and become very costly procedures. Furthermore, chemicals may induce high toxic effects even if administered at low doses. In this case, animal death, often under painful conditions, hampers any experimental result. It should also be considered that T cell functions are ultimately measured *in vitro*, upon animal sacrifice. Finally, it is somewhat doubtful whether mice or rabbits have sensitivities to various drugs comparable to those of humans.

Our goal is to identify easily detectable immune bio-chemical parameters of adjuvanticity, in order to generate an applicable method for assessing potency and safety of human-suitable adjuvants *in vitro*.

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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, infectious diseases, reduction, replacement, veterinary drugs

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). The economic consequences of such outbreaks are important, mainly due to the stamping out policy and restrictions in 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. Infections with 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 pose a constant threat of reintroducing 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 has suggested that virulence is determined by multiple genetic elements. Simple comparison of genome sequences of strains differing in virulence does not yet permit the prediction of a particular strain phenotype. Again, research in this area is currently pursued with experiments involving pigs. Therefore, the aim of the present study is the reduction and finally the replacement of experimental infections of pigs by a cell culture methodology enabling a prediction of the virulence of a particular CSFV isolate.

Method and results

During the past decade, we have cloned infectious genomes of CSFV strains with defined virulence (Ruggli et al., 1996; Mayer et al., 2003) and have made significant progress in understanding the interaction of CSFV with various primary cell systems of the porcine immune system, representing natural target cells for the virus (Carrasco et al., 2004; Balmelli et al., 2005). Based on these findings, we will systematically compare CSFV strains differing in virulence in terms of virus replication characteristics at different stages of the virus life cycle and in terms of their capacity to influence the activation of cells of the innate immune system.

We will proceed in four steps:

1. Establish a primary porcine cell culture system for virus stock production. Previous work has shown that propagation of CSFV on certain permanent porcine cell lines rapidly selects for adaptive mutants with an attenuated virulence phenotype. Therefore primary cells will be used.

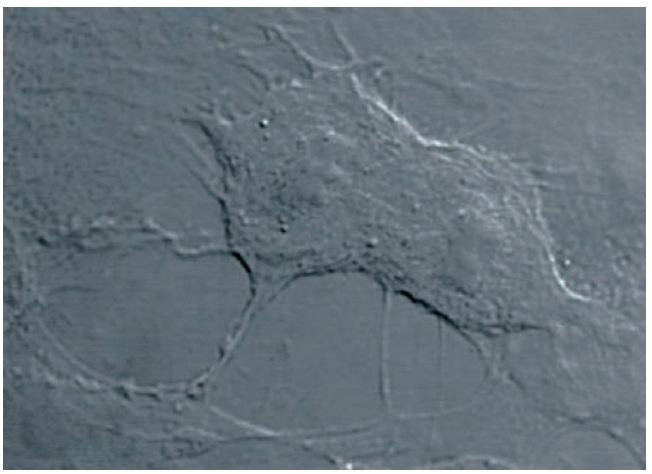

Fig. 1: Porcine endothelial cells visualised under native conditions.

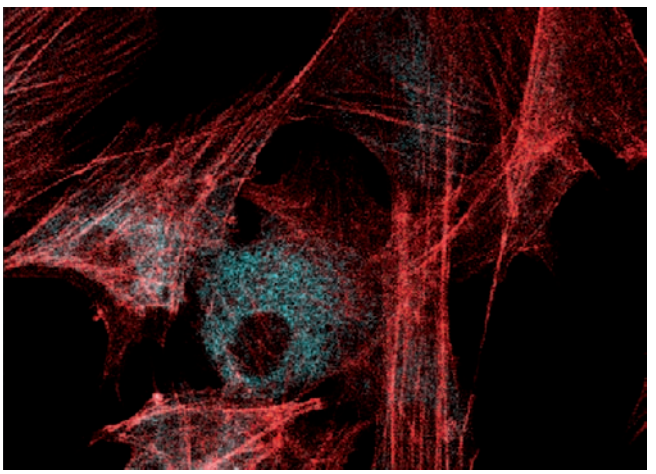

Fig. 2: CSFV-infected primary porcine fibrocytes.
The viral nonstructural protein NS3 detected with a specific monoclonal antibody is visualised in blue. The red-stained microfilaments show the spindle-shaped morphology of the fibrocytes.

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2. Determine the relationship between virulence and *in vitro* virus replication in selected primary porcine cell systems (e.g. endothelial cells, fibrocytes and monocyte-derived dendritic cells; Fig. 1, 2 and 3). We have previously obtained evidence that the ratio of cell-associated to released virus can vary with strains of different virulence (Mittelholzer et al., 2000).
3. Determine the relationship between virulence and *in vitro* parameters of the innate immune response using primary porcine cells (e.g. conventional and plasmacytoid dendritic cells). This is based on our previous work showing that virulence correlates with levels of IFN-α and pro-inflammatory cytokines *in vivo* (Summerfield et al., 2006).
4. Evaluate the accuracy of the *in vitro* model for correlation with virulence.

Conclusions and relevance for 3R

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 for predicting of 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 molecular basis of the pathogenic characteristics of the virus, reducing the number of animal experiments to a minimum. Although cell culture models will only permit a prediction of 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 towards generating safer live virus vaccines.

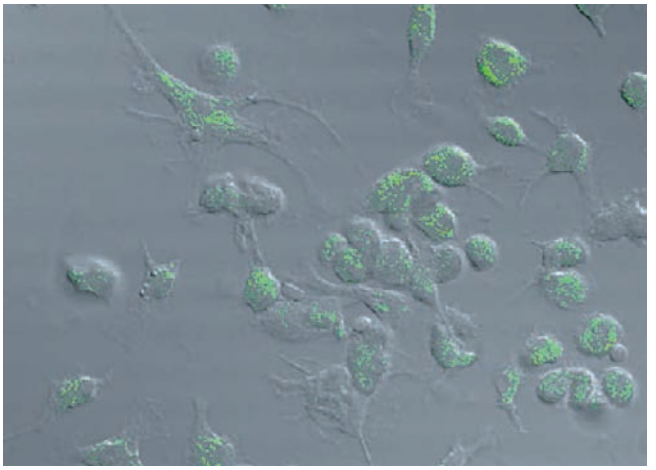

Fig. 3: CSFV-infected porcine monocyte-derived dendritic cells. The viral structural protein E2 is shown in green.

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Information on Serum Free Cell Lines, an Interactive Database

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Keywords: cell cultures, serum free, data base, reduction, refinement, replacement

Background and Aim

Serum is commonly used as a supplement to cell culture media. The most widely used animal serum is derived from foetal 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 foetuses. In terms of the 3Rs, it is desirable to decrease the need for bovine foetuses in the future and to increase 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 toward achieving this.

Method and Results

The interactive database SEFREC (www.sefrec.com), online since April 2006, includes available cell lines, which have been adapted to serum-free and/or protein-free media. In addition to information on 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 database is to encourage change towards serum-free media. All information in the database 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 digitalized in a special data collection program. On conclusion of the official data input, the data is transferred, controlled and compiled in the database. The links to the respective sources of information are activated and an input window is opened for new data. The database is free of charge for registered persons. The selected information can be transferred to a Word or Excel sheet.

The database SEFREC is available at www.sefrec.com. Industry and research groups are invited to place the latest information about their products and keep the database up to date. At the moment, 47 international members present more than 500 serum free media and 32 available serum free cell lines. In the framework of a European network, this database might become the primary neutral source for information on serum free cultures used in the life sciences.

For information on the database and its maintenance please contact Claudio Strebel by e-mail: info@sefrec.com

Conclusions and Relevance for 3R's

Become a member of SEFREC!
The free interactive database is a meaningful way to systematically collect information on cell lines and their serum-free media. Two goals 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 reducing the collection of serum from animals, which is often an animal welfare issue.

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Besides covering the biomedical aspects of animal experimentation, ALTEX is also devoted to the bioethics of the complex relationship between man and animals. Articles published in ALTEX should express a basic concern about the dignity of living creatures. ALTEX is not only aimed at developing a new approach to recognise animals as partners but it also intends to introduce a scientific sight in the discussions on animal experiments. Articles devoted to the social and ethical aspects of this topic will, therefore, be judged according to stringent scientific standards. Manuscripts submitted to ALTEX are evaluated by two reviewers. The evaluation takes into account the scientific merit of a manuscript and the contribution to animal welfare and the 3R-principle.

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