



## Session 5.9

# Biologicals: Progress and new approaches

### Lecture

## Contributions of the European official medicines control laboratories network and biological standardisation programme to 3Rs

*Marie-Emmanuelle Behr-Gross, Karl-Heinz Buchheit, Arnoldus Daas, Catherine Milne, Guy Rautmann and Jean-Marc Spieser*

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In 1991 a contractual co-operation in harmonising medicines control started between the European Union and the European Directorate for the Quality of Medicines (EDQM)-Council of Europe (CoE): the EDQM has been charged of co-ordinating a network of national official medicines control laboratories (OMCL) and a research programme referred to as Biological Standardisation Programme (BSP). In line with the CoE convention on the protection of animals, the BSP establishes European Pharmacopoeia (Ph. Eur.) standards for biomedicines quality control with a special emphasis on alternative methods for the 3Rs. Sixteen projects on vaccines and one on blood products have been initiated in this field. The programme, run in the spirit of international harmonisation, involves the OMCL network, public and private sector medicines control laboratories in Europe, the Americas, Asia and Australia and non-European

standardisation bodies. Completed projects on Newcastle disease and clostridial veterinary vaccines and on diphtheria and tetanus human vaccines led to new Ph. Eur. general methods and standards thus showing that the BSP promotes regulatory acceptance of alternatives. Further studies deal with botulinum toxin, vaccines for human use (inactivated poliomyelitis virus, hepatitis A, hepatitis B and pertussis) and tetanus immunoglobulin. For the future the programme hopes to benefit from synergies between fundamental, medical and pharmaceutical sciences experts for promoting animal welfare aspects in control whilst guaranteeing quality, safety and efficacy to biomedicines potential users. To prompt interactions, the development of a particular BSP project in the field of vaccines control will be presented in Berlin with a critical review of key steps emphasising all specific and global implications.

**Poster**

## The use of Mono Mac 6 cells as indicators of endotoxin contamination in the quality control of injectable products

*Cristiane Caldeira, Izabela da Costa Gimenes, João C. B. Rolim de Freitas and Octavio A. F. Presgrave\**

National Institute of Quality Control in Health, Department of Pharmacology and Toxicology, Rio de Janeiro, Brazil

**Introduction:** The rabbit pyrogen test is used for detecting contaminations of injectable products. Although broadly accepted, both ethical and economic considerations call for a replacement by *in vitro* methods. Although LAL is to be considered as a possible replacement to rabbit test, it has the limitation of detecting only endotoxins. The test systems using human whole blood and cell line Mono Mac 6 (MM6) have been proposed for detecting pyrogenic contamination. Although the human whole blood assay has greater relevance to the *in vivo* situation, it is not yet widely accepted. The aim of this study was to use the MM6 as an indicator of the presence of endotoxin due to its feasibility of processing and low variability.

**Methods:** The MM6 was provided by Dr. Ziegler-Heitbrock and Dr. Stephen Poole. The cells were incubated in the presence

of different LPS concentrations overnight. After this period supernatant was collected and interleukins 1b and 6 release were determined by ELISA.

**Results:** MM6 presented a good dose-response relationship with linear region between 0.06 and 1.0 EU/ml. Our results showed that MM6 was able to distinguish the threshold pyrogenic concentration (5 UE/ml) from the negative control. Linear regression was used ( $r=0.889$ ).

**Conclusion:** These results suggest that this *in vitro* test can detect endotoxin with high sensitivity and it is able to detect different pyrogen levels. MM6 seems to be a good alternative for replacing the rabbit test in cases of ethical problems on using human beings as donors of whole blood.

**Poster**

## A new method for determining *in vitro* potency of hepatitis B in combined vaccines

*Mario L. Chovel Cuervo, Ivette Abreu Nicot, Ana L. Sterling, Mabel García Rodríguez and Orlando R. Pérez*

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Although some manufacturers have developed *in vitro* potency tests for monitoring consistency of hepatitis B vaccines, the *in vivo* test remains as the most suitable for the evaluation of hepatitis B component in combined vaccines. Nonetheless, the Cuban National Control Laboratory has evaluated an in-house method that allows to get reliable hepatitis B potency results in vaccine combinations. The aim of this paper was to evaluate the potential interferences of the rest of components on hepatitis B vaccines and set up an *in vitro* potency test for lot release of combined vaccines. We evaluated combined vaccines from different manufacturers and compared the results regarding mono-valent vaccines. At the same time, we prepared some experimental vaccine formulations in order to discriminate

potential interferences on the hepatitis B component, including the adjuvant effect. In all cases we performed a neutralisation ELISA using Hepanostika anti-HbsAg kit. It was shown that there's no significant interference on hepatitis B in combined vaccines, although the results were consistently lower than mono-valent vaccines. This most likely arises from a complex antigen mimicking effect of Bordetella pertussis whole cells conforming combined vaccines. In spite of this, all combined vaccines successfully passed the specification defined for our *in vitro* test of hepatitis B vaccine. Besides, we got a relatively significant correlation between our *in vitro* and the *in vivo* potency test. Hence, we have available a reliable, fast and accurate test for lot release of hepatitis B in combined vaccines.



## Poster

# Current status of alternative methods for vaccines in Cuba

Mario L. Chovel Cuervo

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Traditionally the *in vivo* potency tests have had an important role in the quality control of vaccines. However, this tendency has dramatically changed in the world. Nowadays the main trend is the development of alternative methods based on the principle of 3R. There are several previous facts to the development of alternatives methods. One of them is the development of an *in vitro* potency assay for the recombinant hepatitis B vaccine, which was already recognised by WHO. In Cuba there have been some interesting approaches in alternative toxicology. The aim of this lecture is to show the current status of the develop-

ment and implementation of alternative methods in Cuba for vaccines. In our country we have successfully implemented some alternative methods (*in vitro*) for routine quality control tests of vaccines like hepatitis B, DT, DTP, rabies and *Haemophilus influenzae* type b. Some of these methods have been developed in our laboratories (in house techniques) and all of them have been correlated in regard to the *in vivo* assays. At the present time, these methods are being used by the National Control Laboratory and the vaccine manufacturers.

## Poster

# Workshop regarding pain and distress associated with polyclonal antibody production: Discussion and recommendations

Kathleen Conlee

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The Humane Society of the United States (HSUS), as part of its Pain and Distress Campaign, held a workshop of international experts in August 2002 in order to develop recommendations for minimisation of pain and distress associated with polyclonal antibody (Pab) production. A group of twelve experts in the fields of antibody production, animal welfare, *in vitro* alternatives, animal protection and/or regulatory compliance participated in the roundtable discussion. Several aspects of Pab production were considered, including: determination of appropriate adjuvants; optimal volume of adjuvant, number of injection sites, and route of immunisation; use of booster injections; availability of alternatives; and measurement of animal welfare. Recommendations were made on each of these topics in regards to minimising pain and distress. General recommendations addressed outsourcing to reputable Pab suppliers; improving training of personnel; improving pain and distress assessment

via score sheets; harmonising guidelines internationally; minimising the number of animals used when possible; and including relevant Pab production information in published papers. Specific recommendations included using the chicken egg yolk technique as a refinement and reduction procedure; choosing an adjuvant that produces high antibody yield while minimising pain and distress; using the smallest volume of adjuvant possible; determining appropriate use of booster injections; and discouraging use of intramuscular, intraperitoneal, intrasplenic, intravenous and footpad injections while allowing use of subcutaneous and intradermal injections. Finally, areas that require additional research were discussed; such as proper pain and distress assessment; formulation of new adjuvants; and determining the influence that pain, distress and environmental enrichment may have on Pab production.

**Poster****Alternative method for potency test of hepatitis B vaccine**

*Catia Costa, Jaline Costa, Eduardo Pereira and Maria Paixão*  
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The hepatitis B vaccines have been available since 1982 and billion doses have been used. Approximately 100 countries according to World Health Organization policy, have added hepatitis B vaccination to their routine immunisation program. The methodologies for production and control these vaccines are based on WHO's recommendations and the European Pharmacopoeia. Historically, potency test to release vaccine consists of an immunological assay in animals. Frequently, these *in vivo* tests required a large numbers of animals for potency assays. This test involves the immunisation of groups of mice with diluted test and reference vaccines. The *in vitro* method

based on the quantification of hepatitis B surface antigen. Dilutions of test and reference vaccines are assayed in a parallel line to quantify the antigen in each preparation. We tested vaccine's batch from four different manufacturers. The number of the mice used to *in vivo* assays was 9,000 approximately. After introduction of the *in vitro* assay at INCQS this number reduced gradually. In 2004 the *in vivo* assay was replaced completely. The development in alternatives methods to replace the use of laboratory animal improved animal welfare, economic, safety and scientific considerations.

**Poster****Rational vaccine design: Rendering black-box animal potency testing obsolete**

*Sadhana Dhruvakumar*

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Every batch of vaccines is tested for potency and safety, currently consuming approximately 10 million animals per year (an estimated 10% of all animal use in biomedical experimentation globally). Animal potency testing involves a pathogenic challenge, and thus causes much suffering. Animals can be entirely replaced in potency testing by physico-chemical methods of quantifying antigens (the part of the vaccine that stimulates the protective response) if they are known, but for most currently used vaccines which were developed empirically, the protective antigens are not known. This is problematic because poorly characterised vaccines are difficult to test in ways other than black-box animal studies, but animal potency tests are often unpredictable due to species differences as well as non-biological routes of exposure (e.g., rabies inoculation through intracerebral

injection in the NIH test). Antigen quantification systems have been devised for some older vaccines, but resources may be better spent focusing on new vaccines and novel methods of vaccine development. As researchers deduce the sequences and structures of pathogenic proteins and develop a detailed knowledge of their roles, they can purposefully design vaccines with defined components in order to maximise effectiveness and minimise safety concerns. Computational immunology can help predict which epitopes are likely to be the best targets. Fortunately, rational vaccine design by its nature goes hand-in-hand with non-animal testing since designing vaccines to contain defined antigens enables the direct measurement of antigenic content, thus rendering the use of animals in potency testing obsolete.



## Poster

# ECVAM Key Area Biologicals: Summary of activities

Marlies Halder

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The Key Area Biologicals covers ECVAM's activities related to reduction, refinement and replacement of animal tests stipulated for the quality control of immunobiologicals, hormones, blood products and other related products, as well as for pyrogenicity and shellfish toxin testing. ECVAM has established a Steering Group on Biologicals and Task Forces for Pyrogenicity and Shellfish Toxin Testing.

Activities on immunobiologicals and hormones resulted in the regulatory acceptance of several alternative methods for the quality control of various products and deletion of animal tests for routine quality control. ECVAM continues its activities by funding validation studies, commenting on European Pharmacopoeia monographs/EU guidelines and organising workshops. Thus, one workshop held in 2005 was focused on biochemical methods for the quality control of toxoid vaccines

and another is planned on consistency of production approach for less well-defined vaccines.

Regarding pyrogenicity testing, various *in vitro* methods based on fresh and cryopreserved human monocytoïd cells were validated in a 5th Framework Programme project during 2000-2003 and in a catch-up validation study during 2004. They are currently peer reviewed by ECVAM's Scientific Advisory Committee.

Co-operation with DG SANCO on replacement of the mouse bioassay for shellfish toxin testing started in 2004 and a workshop was held in early 2005 (see also poster/oral presentation Hess et al). ECVAM is currently collaborating with the Community Reference Laboratory (Vigo, Spain) on the validation of a functional assay for detection of diarrhetic shellfish toxins.

## Poster

# International validation of novel pyrogen tests based on human monocytoïd cells

Thomas Hartung<sup>1</sup> and Stefanie Schindler<sup>2</sup>

<sup>1</sup>ECVAM, Institute for Health and Consumer Protection, Joint Research Center, Ispra, Italy;

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Parenteral medicines are required to be tested for pyrogens (fever-causing agents) in one of two animal-based tests: the rabbit pyrogen test and the bacterial endotoxin test. Understanding of the human fever reaction has led to novel non-animal alternative tests based on *in vitro* activation of human monocytoïd cells in response to pyrogens. Using 13 prototypic drugs, clean or contaminated with pyrogens, we have validated blindly six novel pyrogen tests in ten laboratories. Compared with the rabbit test, the new tests have a lower limit of detection and are more accurate as well as cost and time efficient. In contrast to the bacterial endotoxin test, all tests are able to detect Gram-positive pyrogens. The validation

process showed that at least four of the tests meet quality criteria for pyrogen detection. From two of these methods, the development of successful cryopreservation procedures led to the validation of fresh and cryopreserved human whole blood or isolated PBMCs in four laboratories using the same protocol. The tests reached >90% sensitivity and specificity.

The here validated *in vitro* pyrogen tests overcome several shortcomings of animal-based pyrogen tests. Our data suggest that animal testing could be completely replaced by these evidence-based pyrogen tests and highlight their potential to further improve drug safety.

**Poster****International validation of novel pyrogen tests based on human monocytoïd cells**

Sebastian Hoffmann<sup>1</sup>, Anja Peterbauer<sup>2</sup>, Stefanie Schindler<sup>3</sup>, Stefan Fennrich<sup>3</sup>, Stephen Poole<sup>4</sup>, Yogesh Mistry<sup>4</sup>, Thomas Montag-Lessing<sup>5</sup>, Ingo Spreitzer<sup>5</sup>, Bettina Loeschner<sup>5</sup>, Mirjam van Aalderen<sup>6</sup>, Rogier Bos<sup>6</sup>, Martin Gommer<sup>6</sup>, Ria Nibbeling<sup>6</sup>, Gabriele Werner-Felmayer<sup>7</sup>, Petra Loitzl<sup>7</sup>, Thomas Jungi<sup>8</sup>, Marija Brcic<sup>8</sup>, Peter Bruegger<sup>9</sup>, Esther Frey<sup>9</sup>, Gerard Bowe<sup>1</sup>, Juan Casado<sup>1</sup>, Sandra Coecke<sup>1</sup>, Jan de Lange<sup>1</sup>, Bente Mogster<sup>10</sup>, Lisbeth Naess<sup>10</sup>, Ingeborg Aaberge<sup>10</sup>, Albrecht Wendel<sup>3</sup> and Thomas Hartung<sup>3</sup>

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It is a requirement that parenteral medicines be tested for pyrogens (fever causing agents) using one of two animal-based tests: the rabbit pyrogen test and the bacterial endotoxin test. Understanding the human fever reaction has led to novel non-animal alternative tests based on *in vitro* activation of human monocytoïd cells in response to pyrogens. Using 13 prototypic drugs, clean or contaminated with pyrogens, we have validated blindly six novel pyrogen tests in ten laboratories. Compared with the rabbit test, the new tests have a lower limit of detection

and are more accurate as well as cost and time efficient. In contrast to the bacterial endotoxin test, all tests are able to detect Gram-positive pyrogens. The validation process showed that at least four of the tests meet quality criteria for pyrogen detection. These validated *in vitro* pyrogen tests overcome several shortcomings of animal-based pyrogen tests. Our data suggest that animal testing could be completely replaced by these evidence-based pyrogen tests and highlight their potential to further improve drug safety.

**Lecture****Achieving the 3Rs in the manufacture and testing of veterinary vaccines – opportunities and challenges**

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<sup>2</sup> Animal Research Review Panel, NSW Department of Primary Industries, Sydney, Australia

In New South Wales, in 2002, 18% of the laboratory animals used for research on human or animal biology and health were used in regulatory product testing where the procedures involved death as an endpoint. Testing is mandatory in the production of biological products, mainly livestock vaccines produced for local use and export. In New Zealand 20% of animals were used in research for commercial purposes including 6% subjected to manipulations in the severe and very severe categories, primarily for regulatory testing. In the Netherlands 15% of laboratory animals used in research were involved in vaccine production. Variations between countries will reflect the types of vaccines produced and the specific testing requirements for each. Before LD<sub>50</sub> and similar tests may proceed in New South Wales, the law requires Ministerial concurrence upon the recommendation of

the NSW Animal Research Review Panel, which monitors legislation regulating the use of animals in research and teaching. Through consultation with industry, significant refinements and some reductions in the use of animals have been achieved. The development of *in vitro* alternatives and phase-out of *in vivo* tests may be difficult, but success will bring substantial animal welfare payoffs, together with improved efficiencies for this industry. However, where animal based tests are used, the requirements of regulatory testing are often at odds with implementation of the 3Rs and further reductions are unlikely without an international effort to identify opportunities and strategies for progressively replacing animals in the regulatory testing of veterinary and other biological products.



## Poster

# Development of an *in vitro* assay for testing the safety of tetanus vaccines

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The characteristic spastic paralysis associated with tetanus infections is caused by a powerful neurotoxin (tetanus toxin) produced by *Clostridium tetani*. This toxin is a zinc-dependent metalloprotease which specifically cleaves synaptobrevin, a key molecule in neurotransmitter release.

Tetanus vaccines are prepared from purified tetanus toxin by chemical inactivation. According to the European Pharmacopoeia, safety testing is required for every batch of tetanus toxoid (inactivated tetanus toxin) to ensure the absence of residual toxin activity, and to exclude reversion to toxicity. At the present state, only *in vivo* methods exist for these safety tests. We are currently developing an *in vitro* assay as a fast and reliable alternative to these safety tests in animals.

Our method is based on the detection of the proteolytic activity of tetanus toxin in an ELISA format. For this purpose,

recombinant synaptobrevin is immobilised and incubated with the test samples. If the samples contain any active toxin, cleavage of synaptobrevin occurs and can be quantified using an antibody which specifically recognises the cleavage product. With this assay, we are able to detect low amounts of purified tetanus toxin (current sensitivity in the range of the LD<sub>50</sub>). Furthermore, we show that the assay allows us to detect active toxin in tetanus toxoids (spiked samples).

Our present studies concentrate on further increasing the sensitivity of the test in order to achieve an even closer approximation to *in vivo* conditions. We are confident that this novel method will represent a useful tool for the safety testing of tetanus vaccines.

## Poster

# The comparative effect between high diluted *Calendula officinalis* in 6CH dinamisation, placebo and culture medium on growth of cultured mammalian cells. Preliminary tests

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We have tested the *in vitro* effect from *Calendula officinalis* on duplication of 3T3 murine fibroblast compared with placebo and standardised normal culture medium. It is already known the antiseptic, tissue repair, antiinflammatory and cicatrisation properties from *Calendula officinalis*. This herb belongs to the family from the vulnerable plants. Its constitution has the three most important groups: the flavonoids, the volatile oils and the triterpenos.

The utilisation of the cell culture technics has often been demonstrated to achieve similar results to the once compared *in vivo*, and allow us to explain the intrinsic potential from some drugs. Murine fibroblasts 3T3 were cultivated with standard culture medium DMEM, supplemented with 10% fetal bovine serum (FBS). The medium was changed to DMEM without FBS, to get the G0 cell point. After 24 hours the medium was

replaced with the medium to be tested: 1. normal culture medium supplemented with 10% fetal bovine serum, 2. the same medium with homeopathic *Calendula officinalis* 6CH, diluted in purified water added at the concentration 1/25, 3. the same medium with dinamised purified water added at the concentration 1/25. The cells were stained with Trypan Blue to exclude the died cells and counted in a haemocytometric chamber.

There was an increase in the cell quantity in the presence of the *Calendula officinalis* 6CH and the placebo. Farther investigations for a longer time period should be done, with higher dinamisations, higher concentrations, different medicaments and other cell types.

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## Lecture

# United States Department of Agriculture "3Rs" initiatives in veterinary biologics

Jodie Kulpa-Eddy

USDA-APHIS-Animal Care, Riverdale, Maryland, USA

This presentation will consist of a brief overview of the U.S. system of regulatory oversight of veterinary biologics (vaccines, bacterins, and diagnostic test kits intended for use in animals). A historical perspective will be given on the impact of alternative test methods on the use of animals for regulatory test requirements.

Examples will be provided regarding the USDA's current initiatives to fulfil its strategic goal of significantly refining, replacing and reducing animal testing of veterinary biologics. This will include updates on information presented at the Fourth World Congress on Alternatives and Animal Use in the Life Sciences, held in New Orleans, Louisiana (USA) in 2002.

## Poster

# *In vitro* models to study biological activity of toxoid vaccines

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Although substantial progress has been achieved in reduction and refinement of the use of laboratory animals in quality control of conventional produced vaccines (like diphtheria and tetanus), still large numbers of animals are required, particularly for potency testing. Application of the "consistency approach" using *in vitro* tests, bring replacement of *in vivo* potency tests for toxoid vaccines within reach. Consistency in structure and conformation of the toxoid antigens can be monitored using (new) chemical techniques. However, additional to these chemical tests, functional tests will be needed to confirm the biological activity of the vaccine (antigen). The poster deals with a project at NVI in which the biological activity of vaccine antigens is studied by *in vitro* immune response models.

Tetanus is used as a model antigen. Tetanus toxoid batches of different quality were *in vitro* compared for their immunogenicity (cytokine production, cell proliferation, cell stimulation etc.) using murine spleen cells, porcine PBMC's and human PBMC's.

Based on the cytokine profiles that were induced after *in vitro* stimulation, tetanus toxoid batches of different quality could be distinguished. The *in vitro* immune response models are considered a promising tool to demonstrate consistency in biological activity of toxoid antigens. Application of these models, combined with chemical tests, will finally result in replacement of large scale *in vivo* potency tests of toxoid vaccines.



## Lecture

# Current approaches to animal testing in regulation of biologics and vaccines by U.S. FDA/CBER

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Current laws administered by FDA – including the Federal Food, Drug and Cosmetic (FD&C) Act, and the Public Health Service (PHS) Act – are intended to ensure product safety and effectiveness, thereby protecting the health of the U.S. consumer. These laws place responsibility on FDA's Center for Biologics Evaluation and Research (CBER) to ensure that the products it regulates, such as vaccines, blood products, tissues, and somatic cell and gene therapies, are safe and effective. Currently, animal testing by manufacturers seeking to market these products is often necessary to establish product safety prior to human exposure. CBER supports and adheres to the provisions of applicable laws, regulations, and policies governing animal testing, including the Animal Welfare Act, the ICCVAM Authorization Act of 2000, and the Public Health Service Policy on Humane Care and Use of Laboratory Animals. Moreover, in

all cases when animal testing is used, CBER advocates that research and testing using animals derive the maximum amount of useful scientific information from the minimum number of animals, while employing the most humane methods available. CBER recognises that emerging areas of biopharmaceuticals provide an opportunity to incorporate innovative testing strategies into the product development and manufacturing paradigms. While committed to its primary mission to protect the public health, CBER suggests and encourages the development, validation and use of alternative testing methods that refine, reduce or replace animal testing. CBER advocates the use of validated non-whole animal techniques, which may include *in vitro* methodologies (e.g. tissue culture, cell-based assays), biochemical assays, or emerging proteomic and genomic methodologies.

## Lecture

# Alternatives for potency testing of toxoid vaccines: A realistic option?

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**Introduction:** The most critical step in the production of diphtheria vaccines is the inactivation of the toxin by formaldehyde. Diphtheria toxoid is produced during this inactivation process through partly unknown, chemical modifications of the toxin. Consequently, diphtheria vaccines are difficult to characterise and the quality of the toxoids is routinely determined with potency and safety tests. We have developed a series of physico-chemical and immunochemical tests for monitoring product quality.

**Methods:** Diphtheria toxin was treated with increasing formaldehyde concentrations resulting in toxoid products varying in potency and residual toxicity. Differences in the quality of the experimental toxoids were also assessed with the following *in vitro* techniques: electrophoresis, primary amino group determination, fluorescence spectroscopy, circular dichroism and

biosensor analysis. Subsequently the methods were used to analyse routine toxoid samples from different manufacturers.

**Results:** The results obtained with experimental toxoids correlated well with the potency and safety tests. Further assessment of the methods with routine samples showed that one test (circular dichroism) needs further evaluation and for another assay (electrophoresis) adaptation of criteria were necessary.

**Discussion:** The methods developed are excellent for demonstration of comparability of toxoid batches. This opens the possibility to limit the number of animal tests to one per bulk instead of testing the bulk every time it is used to prepare a batch of final lot. In principle the set of *in vitro* analyses can replace the classical *in vivo* tests completely, provided that the validity of these tests is demonstrated in extensive validation studies and regulatory acceptance is obtained.

**Poster**

## Using HET-CAM for testing mucosal irritation potential of vaccines and vaccine adjuvants

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The Centre for Toxicology and Biomedicine (TOXIMED) in Santiago de Cuba has among its tasks the toxicity testing of vaccine components and vaccine formulations, using traditional methods (*in vivo*) as well as alternatives (*in vitro*). The vaccine application by mucosal via is of high-priority interest in the contemporary vaccinology; however, the pre-clinical study *in vivo* of the mucosal irritation potential of a vaccine components or its formulation is a difficult procedure, not only for the way done but also for the interpretation of the results. On the other hand, the assay may cause suffering to the laboratory animals and there is not a standardised or internationally validated method

that allows its routinary use. The fact that any ocular irritating substance may be also irritating for other mucosa led to consider the possible usefulness of the hen's egg test on chorioallantoic membrane (HET-CAM) to evaluate the mucosal irritation potential of vaccines and adjuvants. Seven substances used as adjuvants or candidates to adjuvants and four vaccine formulations were tested. The values obtained led to classify them as no irritating. New evidences of how useful the HET-CAM is to determine the mucosal irritation potential of vaccines and vaccine adjuvants are given in this work.

**Poster**

## The use of cytokine release (whole blood assay) for detecting pyrogens in anti-venom sera

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**Introduction:** Pyrogen is one of the most important problem of injectable products. The rabbit assay is still used, mainly for products like hyperimmune sera (e.g. anti-venom sera) and vaccines, since these products strongly interfere in the LAL reaction. The use of animal in experimentation has been severely criticised during the last years. In order to reduce the number of animals subjected to pain and distress, many alternative assays have been studied. Although LAL is to be considered as a possible replacement to rabbit test, it has the limitation of detecting only endotoxins. The whole blood assay was developed for replacing the *in vivo* pyrogen test since it can detect all types of pyrogens. Brazil has a large production of anti-venom sera and it represents about 70% of pyrogen tests performed at INCQS.

The number of rabbits used reaches more than 1,000 per year. So, the cytokine release assay could be useful for replacing rabbits for detecting pyrogens in this kind of products.

**Methods:** Human whole blood was incubated with hyperimmune sera overnight. After this period supernatant was collected and cytokines (IL-1b and IL-6) release was measured by ELISA.

**Results:** When tested undiluted, hyperimmune sera showed strong cytotoxicity with no cytokine release. When diluted 1:10 in order to avoid cell death, cytokine release presented a good dose-response curve and distinguished the rabbit threshold pyrogenic dose (5 EU/ml) from a non-pyrogenic dose (2,5 EU/ml).

**Conclusion:** The whole blood assay can be used for detecting pyrogen contamination in hyperimmune sera diluted 1:10.



## Poster

# Program to reduce animal testing for EPA registrations of antimicrobial products – A novel approach to animal alternatives

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In the US, antimicrobial cleaning products are registered as pesticides with the US EPA. To support registration, an assessment of dermal and eye irritation must be completed. Normally required for such assessments are data from the Draize eye and Draize skin irritation tests. For antimicrobial cleaning products, much research has been done to develop non-animal alternatives to these tests, and today these alternatives are routinely used by many to make safety and labelling decisions. However, because these tests have not undergone formal validation, there are barriers to their acceptance by the EPA. Although the database is sufficient to support use of these alternatives for antimicrobial cleaning products, there are data gaps for some materials outside this specific category, which inhibits validation for all formulations. Therefore the companies that manufacture antimicrobial

cleaning products have embarked on a novel, modular program with the US EPA to obtain acceptance of alternative methods specifically for this limited category of products – antimicrobial cleaning products. These companies have come together to define the specific formulation types that are to be included in this evaluation and to provide all the animal and non-animal data necessary to demonstrate the predictability of these assays for the purpose of determining appropriate cautionary labelling. This information will be assembled and submitted for a technical review by an expert panel organised by ICCVAM. A favourable review by this panel would lead to new EPA guidelines, allowing the use of these alternative approaches for these specific formulations.

## Lecture

# International validation of pyrogen tests based on fresh and cryopreserved human primary blood cells

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Pyrogens as fever-inducing agents can be a major health hazard in parenterally applied drugs. For the control of these contaminants, pyrogen testing for batch release is required by Pharmacopoeias. This has been done either by the *in vivo* rabbit pyrogen test (since 1942) or the limulus amoebocyte lysate test (LAL), since 1976. A new approach are cell-based assays employing *in vitro* cultivation of human immune cells which respond e.g. with cytokine production (IL-1, IL-6) upon contact to pyrogens. 6 variants of these assays have recently been validated in a collaborative international study. From two of these methods, the development of successful cryopreservation meth-

ods promises to make standardised immunoreactive primary human blood cells available for widespread use. Furthermore, the pre-testing of donors for infectious agents such as HIV or hepatitis has made it possible to develop a safe and standardised reagent for pyrogen testing. Using altogether 13 drugs, we have validated here two pyrogen tests based on fresh and cryopreserved human whole blood or isolated PBMCs in four laboratories. The tests reached >90% sensitivity and specificity. In contrast to the LAL, the tests are capable of detecting non-endotoxin pyrogens derived from Gram-positive bacteria or fungi.



## Lecture

# Animal usage in quality control tests for the release of immunological veterinary medicinal products in the United Kingdom

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Animals and animal derived starting materials are used routinely in the production and quality control of immunological veterinary medicinal products (IVMPs). Quality control (QC) tests are necessary to provide assurance that each batch of an IVMP is safe and efficacious before it is released onto the market. The Veterinary Medicines Directorate (VMD) has recently conducted a study to investigate the extent of animal usage in QC tests for the release of IVMPs onto the UK market in 2003 and 2004. This has identified a number of areas where efforts could be made in reducing or eliminating some animal tests on IVMPs.

The project investigated the number of batches of authorised IVMPs released onto the UK market, the in-process and final product QC tests performed on each batch for release purposes and the number of animals used to conduct these tests.

In 2003, the VMD released 1101 batches of IVMPs onto the UK market for 221 products. A total of 31,047 animals were used in QC tests in 2003 and 26,160 in 2004. The QC tests, in which the majority of animals are utilised, were the batch

potency tests and safety tests, accounting for 52.1% (16,175) and 20.9% (6,480) respectively of animals in 2003. Other significant animal tests include extraneous agents testing of avian vaccines (10%, 3,111) and absence of toxicity of clostridial vaccines (15.4%, 4,773).

Under the new provisions of the European Pharmacopoeia it is now possible for manufacturers to remove the final product batch safety test, subject to agreement of the competent authority and providing data on a sufficient number of consecutive batches to support the safety of the product. The number of animals used in batch safety tests for IVMPs will continue to be monitored to assess the progress in reducing animal usage. Furthermore, the amended monograph for the final product test for extraneous agents in live avian vaccines should result in a similar reduction of animal usage.

To reduce animal usage in final product QC tests for potency, further research and investment is needed to develop satisfactory *in vitro* alternatives to the *in vivo* tests used for most inactivated vaccines.

## Poster

# The 3Rs – A breeder's perspective

*Emma Whiting*

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Reduction, refinement and replacement, as put forward by Russell and Burch, are partial solutions to finding alternatives to the use of laboratory animals in research, education and testing. As a commercial breeder we believe that harvesting body fluids and tissues from laboratory animals that no longer have a commercial value is an ethical way of achieving both reduction and replacement.

This presentation describes how as a commercial breeding and supplying establishment we have implemented a policy for the minimisation of overproduction and wastage of animals. It also describes how this policy has allowed us to successfully implement the 3Rs, by the use of unused and unsold animals for the

harvesting of biological samples. To achieve this one of our main concerns was making use of the animals we had available from our own breeding colonies to supply biological samples such as blood products, body fluids, tissues and danders. In doing this we believed that it would lead to a reduction in the number of animals being bred for the same purpose at other establishments. Similarly, in the time that we have been supplying such products our expertise has improved so that we are now more efficient and are able to obtain the same amount of material from fewer animals. In addition, our techniques have been refined to improve not only the quality of the products, but also our efficiency and more importantly animal welfare.

**Poster*****In vitro* evaluation of the release profile of a papain-incorporated non-cytotoxic polymeric matrix**

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Drug delivery systems for topical administration in therapeutics treatments have attracted attention due to advantages such as elimination of the first passage effect, reduction of adverse effects and ease of interruption in cases of intolerance. Papain is a proteolytic enzyme widely used in dermatology and cosmetology in wounds treatment, peelings and skin whitening. Toxicity studies of papain were done and are related in the literature. In addition, efficacy and assurance of the enzyme were evaluated *in vitro* by using keratinocytes cell cultures. Papain is usually incorporated in common base vehicles, such as emulsions and gels. Such vehicles, however, require more than one application

a day, which reduces patient compliance and consequently reduces treatment efficacy. In this research we report the development of a biological system for controlled release of papain based on elastomer silicone polymeric matrix. The polymer was assessed non-cytotoxic by using *in vitro* Neutral Red method with NCTC cell cultures. The delivery properties of the papain-containing membranes were evaluated *in vitro* over 28 hours period by analysing the amount of released enzyme using Specific Substrate Dosage Test. Preliminary results indicate the possibility of this biological system control the release of papain during one day.