Theme I:
Safety and Efficacy Testing of Chemicals, Pharmaceuticals and Biologicals

Session summaries for Theme I

Session I-1
Potency and Safety Testing of Human Vaccines

Co-Chairs: Coenraad Hendriksen, Netherlands Vaccine Institute, Netherlands
Maria Baca-Estrada, Health Canada, Canada

Animal studies commonly are required to ensure the potency and safety of approved human vaccines prior to their release to the market. As these test are required for every batch of vaccine produced, this can result in the use of a significant number of animals. Recently, there have been major advances in alternative methods that better incorporate the Three Rs into vaccine potency and safety testing strategies. This session addressed approaches to replacing current safety and efficacy testing methods for vaccine evaluation, as well as the opportunities and obstacles and to moving forward with the regulatory acceptance of alternative methods.

The approaches taken to support the development and implementation of the Three R's principles by The European Directorate for the Quality of Medicines and the Finlay Institute in Havana Cuba were discussed. There has been significant progress in the development of alternative potency and safety assays for the quality control of vaccines. Key practical and scientific issues include the availability of reagents, the difficulty of correlating new assays with highly variable *in vivo* assays, the limitations of appropriate samples for validation purposes (e.g. failed/borderline) and the availability of human and financial resources. It was emphasized that, although considerable progress has been made, much room for improvement remains; it was suggested that industry, authorities, and academia need to work together. Implementation of new strategies, such as the consistency approach, are being considered to serve as the basis for the goal of eliminating animal use without compromising quality of safety of these important products. Information was provided on the Vaccines Project: 3Rs and Consistency Approach, which was initiated by the European Partnership for Alternative Approaches to Animal Testing (EPAA).

The alternative test strategies currently under evaluation to replace the histamine sensitization test used for acellular pertussis vaccines were discussed. Industry and regulatory authorities have shown considerable interest, and a number of consultation meetings and collaborative studies have been organized to discuss and evaluate the potential of alternative methods. In particular, two potential *in vitro* assays are currently being evaluated. It was recognized that it is important to move forward in a coordinated and harmonized fashion in order to define suitable protocols and reagents before proceeding with validation and implementation studies. In addition, it was recommended that research on cell-based assays continue in order to improve sensitivity and/or decrease variability. Alternative methods to replace the Limulus-amebocyte lysate assay with the monocyte activation test also were discussed. The Limulus-amebocyte lysate assay raises a number of issues, given that the Horseshoe-crab is on the threatened species list. The monocyte activation test is based on the ability of monocytes to secrete a number of cytokines upon stimulation with immunostimulants, including endotoxin and other pyrogenic molecules. Improvements in the sensitivity of the monocyte activation test are being explored, however, and promising results were presented.

Understanding the obstacles and opportunities to the implementation of alternative methods by regulatory agencies is critical. The results of two studies in Europe and Canada highlighted the major obstacles to implementation of the alternative methods. These include: risk-averse society, varying regulatory requirements, and issues in comparing new versus existing methods. On the other hand, some of the opportunities for implementation include: ethical considerations, desire for collaborations, and harmonization of regulatory requirements.

Session I-2

Addressing Systems Toxicology

Co-Chairs: Paul Jennings, Innsbruck Medical University, Austria
Hans Raabe, Institute for In Vitro Sciences (IIVS), Boston, USA

Background

Currently most of the *in vitro* assays available for the evaluation of chemicals and new therapeutic entities are relatively simple mono and co-culture models that capture a limited number of potential biological effects for a given xenobiotic. The assembly of complex models that are a) sustainable for repeated dose exposure and b) represent intra and inter-organ interactions is a considerable challenge. Furthermore, the place of such models within existing testing approaches is far from certain, given the lower throughput and nature of information that they can provide. During the session six presentations addressed a wide variety of the diverse aspects of systems toxicology including:

1. Biological Models
   - The use of human tissues and organs (coronary arteries andendocardial muscle strips) for safety pharmacology. **David Bunton**, Biopta Glasgow
   - The use of stabilized 2D epithelial cultures for long-term, repeat exposures. **Paul Jennings**, Innsbruck Medical University
   - The problems associated with moving from 2D to 3D systems. **Roger Curren**, Institute for In vitro Sciences, Gaithersburg, USA
   - The identification of cell specific functionally based stress indicators – for example claudin expression in renal proximal tubule epithelial cells. **Paul Jennings**
     Human multiorgan chips. **Uwe Marx**, Techische Universität Berlin
2. *In silico*, QSAR and read across. **Ovanes Mekenyan**, Lab. of Mathematical Chemistry, Bourgas, Bulgaria.
3. *In vitro-in vivo* extrapolations, biokinetics and the importance of the quantification of available compound for extrapolation to *in vivo* dose. **Bas Blaauboer**, Institute for Risk Assessment Sciences, Utrecht.

Summary

While all of these various aspects of systems toxicology are still being developed, there is a need to integrate different organ systems with mechanistic biomarker analysis, functional monitoring and biokinetic analysis. This integration may be virtual, via computational models, or actual, via the use of multiorgan chips (MOCs). It is conceivable that MOCs allowing perfusion through different organ systems will be possible in the near future. Importantly, developments in *in silico* approaches, such as QSAR and read across, are and will continue to be essential complementary tools to these biological approaches.

Session I-3

Biological and Biotechnology-Based Therapeutics

Co-Chairs: Laura Andrews, Genzyme Corporation, USA
John Devine, BASi, USA

The pharmaceutical industry is increasingly developing biological-based and biotechnology-based therapeutics, which present unique challenges during development and safety testing. As the activity of the biologic therapeutic is frequently species- specific, the selection of appropriate animal models or surrogates is crucial to generating useful data. Some animal test systems have been refined to alleviate the inter-species issues, and alternative *in vitro* strategies have been identified that could significantly reduce or eliminate testing.

Aim: To discuss and disseminate the problems associated with, and the solutions to, the development and safety testing of biological-based products.

Kathryn Chapman: *Minimizing Non-Human Primate Use in Monoclonal Antibody (mAb) Development*
This presentation reviewed the development of mAbs and the expected increase in products, resulting in an increase in NHP use. A survey was conducted to determine how drug development companies and CRO's are designing studies for NHP use. Recommendations for consideration were presented.
- Two studies for mAb safety assessment as opposed to traditional three-study approach (1 month, 3 month, 6 month). Proposed one for IND enabling and one for registration
- Consider rodent as tox species if rodent is as relevant as NHP (don't default to NHP)
- Keep group sizes smaller (3+3) with recovery small (2+2). Consider fewer recovery animals with recovery in high dose and control only.
- Use fewer dose groups (2+ control instead of traditional 3+ control).

Laura Andrews: *Consideration of alternative approaches for the purpose of reducing animal numbers in the preclinical development of biotherapeutics*
Review of the challenges associated with development of biologics with an emphasis on evaluating the science behind study design. Recommendations for alternative methods include:
- Transgenic animals
- Homologous proteins
- Adapted study design
- Alternative endpoints (imaging, dried blood spot analysis, continuous analytical work in the same animal)
- Inclusion of safety pharmacology on long term studies (and not a stand-alone study)

Ying Huang: *pre-preIND approach to reduction of animal use in translational research and product development*
A review of the application of the 3R's with respect to OCTGT regulated products was given. Application of the 3R's was suggested by considering:
- Hybrid studies (Proof of concept + Tox + product fate in relevant animal model)
- Mimic the clinical design
- Use adequate groups and numbers of animals in groups

Opportunities for replacement were considered by implementing:
- *In vitro* studies in human derived material
- Computer modeling
- Imaging
- Systems biology considerations
- Biomarker evaluation

Encouragement of the pre preIND interaction that is a non-binding discussion to resolve issues and chart a path forward. Early communication can result in study refinement and animal reduction.

Cyrille Krul: *Better prediction of immunogenicity of biopharmaceuticals in humans, is it possible?*
Reviewed immunogenicity concepts and suggested alternative should be investigated for immunogenicity prediction to reduce animal use. Two suggestions:
- Immunogenicity testing in mini pigs. As the mini pig is an accepted toxicology model, use of this model for predictive immunogenicity was evaluated. Results were shown to be comparable to NHP and human studies.
- Development of a predictive "toolbox." This is a risk-based model using historical data from human clinical trials. The database will be a useful comparative tool for immunogenicity assessments.

Pat Ryan: *In vitro MABEL approach for nonclinical safety assessment of MEDI-565 (MT111), a novel CEA/CD3-bispecific single-chain BiTE antibody*
Review of the biology behind bispecific molecules. Analysis of the development pathway for this Bi-specific showed no cross reactivity with any species (even after surrogate generation). A development program was conducted on *in vitro* assays and a MABEL (minimum anticipated biological effect level) was determined from the most sensitive assay (cell lysis). This approach resulted in entry into a clinical trial at low dose (10 X below the MABEL).

Christoph Giese: *Pharmaceutical testing of follicle stimulation hormone (FSH): a new cell-based assay for the replacement of the Steelman-Pohley in vivo assay*

A presentation of the current Steelman-Pohley assay was given. Various aspects of the new *in vitro* cell based assay were reviewed, together with pro's and con's of the *in vitro* vs. *in vivo* assay. The use of the *in vitro* assay could significantly reduce the animal numbers required for FSH (and FSH-like compounds) for evaluation. At this time further backing and support is required for pursuing this assay as a replacement to the *in vivo* study.

Session I-4

Validation and 3R Strategies for Assessment of Endocrine-Active Substances

Co-Chairs: Warren Casey, NICEATM/NIEHS, USA
Susanne Bremer, ECVAM, Italy

This session focused on the validation of *in vitro* test methods that are applicable to the OECD Conceptual framework for the testing and assessment of potential endocrine disrupters, the US EPAs Endocrine Disruptor Screening Program (EDSP), and the EU-Community Strategy for Endocrine Disrupters. These programs were presented, along with the validation status of the test methods. Alternative approaches to validation and new concepts for their regulatory acceptance, such as performance-based test guidelines, also were discussed. The speakers noted that regulatory requirements for indentifying potential endocrine active substances are expensive, time consuming, and require large numbers of animals. Tremendous effort has gone into developing and evaluating alternative methods and approaches that are designed to address regulatory needs. Some tests have undergone formal validation exercises or in-house validations by industrial users and are successfully used in tiered strategies for priority setting during product development. The agencies' reluctance to embrace alternative screening strategies for product registration remains a significant barrier to their use. Therefore, mechanisms that facilitate regulatory consideration of new technologies and screening strategies are needed to ensure that sound science is translated into a tangible reduction in animal usage.

Session I-5

Nanotoxicology and the 3Rs

Co-Chairs: Alison Elder, University of Rochester, USA
Mark Lafranconi, Procter and Gamble, USA

Developing alternative approaches for evaluating nanomaterial toxicity has presented researchers with numerous challenges. Investigators must confront difficult questions about bioavailability and biodistribution, which are affected not only by the material's chemical properties but also by its size and shape. Direct application of existing alternative methods, such as commonly used *in vitro* cytotoxicity or genotoxicity assays, can be confounded by analytical interference and by the uncertainties regarding delivered dose when working with nanomaterials.

In addition, most attempts to develop alternatives start with a firm understanding of the underlying mechanisms of action and have the benefit of a rich history of *in vivo* information to serve as milestones and to guide the development of alternatives. With nanomaterials this history is missing.

The session led off with **Thomas Hartung** of the Johns Hopkins Center for Alternatives to Animal Testing (CAAT) who, in addition to making a powerful argument that traditional testing methods will not be practical or sufficient for evaluating nanomaterials, provided a systematic review of alternative methods under consideration for nanomaterials, complete with an analysis of strengths and weaknesses of each. He also emphasized the need to define mechanisms of action by which nanomaterials produce effects in cells and tissues. These concepts are reviewed in a recent publication (Hartung and Sabbioni, 2011).

Abigail Jacobs of the US FDA followed with a discussion of what the agency is facing with respect to drugs that are formulated to be nanosized to achieve unique properties or to improve bioavailability, as well as drugs that contain nanomaterials as inactive components. She concluded that the standard alternative approaches to safety assessment have worked well in the FDA experience. She also emphasized the importance of evaluating nanomaterial bioavailability in products that are submitted for approval.

Mathias Forjan reported on the use of simulated lung models that mimic the mechanical ventilation of the lung. As this model is developed further and validated for use with nanomaterials, it may have relevance for characterizing nanoparticle deposition.

Song Huang from Epithelix discussed the application of a "3-dimensional" cell culture model of the human airway epithelium that is comprised of ciliated epithelial, goblet, and basal cells and which can be kept in continuous air-liquid interface culture for at least one year. This model system lends itself well to repeated nanomaterial exposure studies and could be a tool for moving beyond the limitations typically encountered by tissue constructs with respect to short-term viability.

Pat Hayden of Mat Tek Corporation reported on the successful use of 3D constructs of skin (EpiDerm) and lung (EpiAirway) for evaluating nanomaterials. The 3D constructs contain the major barrier elements important in determining the eventual bioavailability of administered nanomaterials.

Conclusions

- Alternative methods will be essential for evaluating the increasing number of nanomaterials already on the market and or under development
- The diversity of commercial and biomedical applications for this class of materials makes the evaluation of their safety complex. The establishment of mechanisms of action under realistic dosing conditions should be a priority
- 3D cell culture models appear particularly well suited for evaluating nanomaterials
- The biggest challenges for alternative approaches are in adequately defining material physicochemical properties that relate to biological response outcomes, delivered and retained doses and disposition, and establishing validated extrapolations to human exposures for risk assessment purposes.

Reference

Hartung, T. and Sabbioni, E. (2011). Alternative *in vitro* assays in nanomaterial toxicology. *WIREs Nanomed. Nanobiotechnol.*, in press.

Session 1-6

Advances in Animal Alternatives in Ecotoxicology

Co-Chairs: Michelle Embry, ILSI Health and Environment Sciences Institute
Scott Belanger, The Proctor and Gamble Company

Aquatic vertebrates, particularly fish and amphibians, are used in a wide array of applications to assess environmental and human safety associated with exposure to industrial and agrochemicals, pharmaceuticals, biocides, and effluents. They are commonly used as *in situ* indicators of the status of natural and perturbed ecosystems. Because of these wide uses and the central role of fish in environmental risk assessment, aquatic vertebrates are amongst the most commonly tested groups. One goal of environmental risk assessment is to protect the survival and propagation of this incredibly diverse group of organisms (fish are the most diverse of the classes of vertebrates). It is no surprise then that aquatic vertebrates are well considered in animal welfare legislation globally and that much research has gone forward to develop alternative approaches for their use in research and environmental risk assessment. This session highlighted advances in alternative methods for environmental risk assessment with a focus on fish. The talks drew upon all three Rs – refinement, reduction, and replacement and considered acute and chronic toxicity, effluent toxicity, bioaccumulation, and endocrine disruption.

Dr **Kristin Schirmer** (Professor of Environmental Toxicology, Swiss Federal Institute of Aquatic Science and Technology, EAWAG, Switzerland) opened the session with an invited lecture titled "Fish cell lines as alternatives to fish toxicity tests." Dr Schirmer discussed the advances in establishing and validating cell culture assays as alternatives to fish tests, as initially suggested almost 40 years ago, as a desired and urgent societal goal. Novel fish cell culture models have been established with a particular focus on cell lines and strategies have been developed to overcome common limitations in the application of cell culture assays as substitutes for fish. These advances have pulled *in vitro* assays into the discussion as outright replacements for standard fish acute toxicity tests, especially when effort is given to understand the bioavailability of chemicals in the assays themselves.

A thorough quantitative review of *in vitro* endocrine disrupting assays was given by Dr **Stefan Scholz**, Helmholtz Centre for Environmental Research – UFZ, Leipzig, Germany in his talk "Quantitative and comparative analysis of alternatives to *in vivo* tests for endocrine disrupting chemicals (EDCs) in fish and amphibians." Data from 1995 to the present were collected related to the detection/testing of estrogen-, androgen-, and thyroid-active chemicals in cell lines, primary cells, fish/frog embryos, yeast, bacteria, cell free systems, and "omics" technologies. Analyses were performed to determine the strengths and limitations of each assay and present conclusions regarding chemical specificity, sensitivity, and correlation with *in vivo* data. The data suggest that a combination of different alternative tests, including endpoints such as receptor binding and hormone synthesis, appear promising to ultimately replace current screening assays for EDCs based on animal tests.

Dr **Dan Villenueve**, USEPA ORD-Duluth, Minnesota, USA presented a forward looking view on "Adverse outcome pathways (AOPs) and extrapolation tools to advance the Three Rs in ecotoxicology". AOPs have emerged in environmental toxicology as a construct to logically associate key molecular initiating events during chemical exposure to toxicity pathways and ultimately ecologically meaningful responses such as population declines. These approaches identify the needs for mechanistic assays to drive "smart testing" when needed or help prioritize assay development.

Traditional fish use in ecotoxicology would be thought of mainly for product testing and assessment of physiological and behavioral responses for individual chemicals. Dr. **Phil Dorn**, Shell Oil Company, Houston, Texas, USA noted that assessment of wastewater effluents is another demand on fish testing. Efforts to reduce fish use in a project sponsored by HESI were described in a presentation titled "What reductions in fish use can be made employing alternatives for wastewater effluent assessment?" Based on estimates from several participating industries it is now becoming understood that fish testing with effluents may actually outpace that for chemicals and new approaches are in need of development.

Assessment of chemical bioaccumulation by fish is a critical endpoint for many chemical management programs. Dr **Bob Hoke**, Dupont, Newark, Delaware, USA, presented "Product stewardship – incorporating the 3Rs while improving bioaccumulation assessments." Product stewardship is an important process for evaluating risks and driving continuous improvements throughout a chemical or product life cycle. Important progress in the evaluation of the bioaccumulation potential of chemicals has been made in the last 5 years. These efforts have been incorporated into corporate product stewardship efforts and have helped improve bioaccumulation assessments while limiting unnecessary use of fish for testing.

The last speaker of the session was Dr **Gary Ankley**, USEPA ORD-Duluth, Minnesota, USA. Dr. Ankley provided an overview of an important program in development at the OECD in "Harmonizing and Optimizing Fish Testing Methods: The OECD Fish Framework Project." The Fish Framework has a goal of integrating over a dozen fish testing guidelines that cover all aspects from acute toxicity to full life cycle testing for endocrine disruption. By developing such guidance, member nations can logically move to address regulatory needs while minimizing testing. Opportunities for animal alternative approaches at each stage are considered and many are already embedded, such as use of QSARs, limit testing, and use of *in vitro* methods for screening.

This well attended session provided highlights and perspectives on meaningful progress for alternatives to traditional *in vivo* fish testing. Large opportunities remain for future scientific advances to ensure environmental protection goals are met through broad implementation of 3R principles.

Session I-7

Potency and Safety Testing of Veterinary Vaccines

Co-Chairs: Marlies Halder, European Commission, Joint Research Centre, Institute for Health and Consumer Protection, European Centre for the Validation of Alternative Methods, Italy
Oksana Yarosh, Canadian Food Inspection Agency, Canada

Animal studies are often required to ensure the potency and safety of approved veterinary vaccines prior to their release to the market. As these tests are required for every batch of vaccine produced, this can result in the use of a significant number of animals. Recently, there have been major advances in alternative methods that better incorporate the Three Rs into vaccine potency and safety testing strategies. This session highlighted some of these approaches and how they can reduce or replace current testing methods.

Hans Draayer (Pfizer Animal Health, USA) reported on the regulatory requirements for safety testing of veterinary vaccines in North America. Batch (serial) release testing can be a combination of testing in target animals and/or laboratory animals (mice, guinea pig). It was emphasized that it might not be necessary to carry out these tests on a regular basis but only for a given number of batches if a number of factors would be considered, e.g., consistency of production process (GMP or equivalent), safety profile of the vaccine during development, historical results of the *in vivo* batch safety test, and additional *in vitro* testing. Batch safety tests in mice and guinea pigs were deleted from European Pharmacopoeia monographs about 15 years ago due to the lack of relevance for the safety of the vaccine. Other regulatory agencies should reconsider their requirements for the batch safety tests in laboratory animals. VICH is currently drafting a guidance document on harmonizing the criteria for waiving the target animal batch safety test.

Jodie Kulpa-Eddy (United States Department of Agriculture, USA) presented the successful development and validation of an antigen capture ELISA for serial release potency testing of four Leptospira serovars. The *in vitro* assay is approved for use in the USA and reference bacterins are available from the USDA. It replaces the hamster vaccination-challenge test, which uses large numbers of animals, induces pain and distress, is lengthy, costly, and requires laboratory staff to handle infectious material. The global use of this ELISA should be explored.

Armando Heriazon (Novartis Animal Health, Canada) gave an overview on the challenges associated with the development of potency tests for fish vaccines and highlighted the differences between the fish immune system and those of mammals and birds. At present, potency testing is based on vaccination-challenge tests using large numbers of fish. Possible alternatives could be *in vivo*-based models using zebrafish or rodents, or serum antibody quantification. Preference should be given, however, to following the principles of the consistency of production approach, i.e., identify critical parameters of the vaccine and the manufacturing process, develop and validate the necessary *in vitro* and analytical methods to measure the critical parameters, and by that reduce as far as possible the use of fish for the batch release.

Heike Behrensdorf-Nicol (Paul-Ehrlich-Institut, Germany) reported on the current status of the development of a combined *in vitro* assay for detection of residual tetanus toxin in inactivated tetanus vaccines for human and veterinary use. Most of the assays developed consider only the proteolytic activity of the tetanus toxin, whereas the combined assay captures the receptor binding and synaptobrevin cleaving features, thus avoiding false-positive results. At present, the test for residual toxicity is carried out in guinea pigs; the combined *in vitro* assay is a promising replacement method.

Session I-8

Safety Testing for Chemically-Induced Eye Injuries: Recent Three Rs Advances

Co-Chairs: Rodger Curren, Institute for In Vitro Sciences, Inc., USA
Nathalie Alépée, L'Oréal Research & Innovation, France

The theme of Session I-8 was the application of new approaches to understanding the mechanisms of ocular injury and to addressing the needs of the regulatory community. The session started with a review by co-chair Rodger Curren of milestones in ocular irritation testing over the last 35 years. Although there were few, if any, changes in the regulatory requirements for ocular testing in animals during the first 20 years of that period, the use of non-animal tests within companies grew substantially. Finally, as regulators and industry began to work more closely together over the last decade, regulatory acceptance of *in vitro* methods has increased at a significant rate.

Dr **Roger Beuerman**, the invited speaker, continued the session by presenting "*Chemical Injuries and the Corneal Response*." He described his experience in the clinic with human corneal injuries and the recovery process, which is very dependent upon the initial degree of damage to the cornea. As indicated, the most frequent injuries are from encounters with extreme pH conditions (<4 or >10). He also discussed the impact of damage to other ocular tissues, especially the limbal area of the cornea, which normally is the source of new cells to repair corneal epithelial damage, and the corneal endothelium, which is incapable (in humans) of regeneration if it is damaged. Additionally, damage to the conjunctiva can lead to extreme dry eye. To understand the outcome for restoration of vision, he concluded, it is critical to understand the access of chemicals to the cells and cellular structure for both awareness and avoidance of potential eye injury.

Next, Dr **Russel Walters** presented an *in silico* approach, developed at Johnson and Johnson, to predict the response of the human eye to surfactant solutions. This combination of physical chemistry data and surfactant theory can be correlated with the results of clinical studies of approximately 20,000 human eyes and more than 170,000 endpoints. This extensive database has allowed subsets of clinical volunteers to be examined for different responses in redness and stinging, and it should eventually allow accurate predictions of human ocular response to new surfactant formulations.

Dr **James Jester** presented techniques for measuring corneal depth of injury. Earlier studies, e.g., in the Low-Volume Eye Test or the ex vivo rabbit eye assay, had shown that the ability of experimental animals to recover from ocular injury was very dependent on the depth and area of the initial injury. In the context of a Colipa project, Dr Jester showed how these measurements could be made in *ex vivo* systems, especially in excised bovine corneas. He presented some preliminary data showing that it may be possible to differentiate GHS Category 1 irritants from less damaging GHS Category 2 ocular irritants – a big step forward in providing a completely non-animal approach to ocular irritation testing.

Dr **Jack Fowle** presented an approach to *in vitro* ocular irritation assessment of anti-microbial cleaning products, which is now in use by the U.S. EPA. Dr Fowle first described the US EPA's programs for incorporating new and more efficient testing strategies into their regulatory assessment of new products and chemicals. He then reviewed one of the first non-animal approaches for ocular irritation (currently a pilot program), which involves the use of up to three *in vitro* assays (EpiOcular™, BCOP, and Cytosensor), depending on the type of material to be tested. Several anti-microbial cleaning products already have been registered using this approach.

Finally **Nathalie Alépée**, session co-chair, reviewed the highlights of the session and suggested that further progress in non-animal ocular irritation methods will occur only as research on mechanisms moves forward, as was discussed in the session. As acknowledged, alternative assays have been extensively researched, and some have undergone formal validation. To date, however, there is no single *in vitro* assay that has been validated as a full replacement for the Draize rabbit eye test. In light of the deadlines established in the EU Cosmetics Directive and the regulation for safety assessment on chemicals in the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH), the applicability, predictive capacity, and the prediction model of the test methods need further evaluation. One person or one company cannot do it alone. Only by working together – industry, regulators, and academia – can our non-animal testing goals be reached.

Session I-9

Methods for Reproductive and Developmental Toxicity

Co-Chairs: Barbara Hales, McGill University, Canada
Robert Chapin, Pfizer, USA

Reproductive and developmental toxicity has been an active research area for the past decade as researchers have struggled to find an alternative to the *in vivo* reproductive and developmental toxicity studies.

Dr **George Daston** served as keynote speaker for the session, presenting a tiered approach to assessing the possible risk of a new compound. This often involves a team that includes various kinds of expertise. It starts with an initial substructure search through a database like DEREK. This can identify likely toxic moieties and will highlight precedented toxicities. These can be confirmed or challenged by running the compound through some cell model (high-throughput or not) and performing some version of pathway analysis and comparing the new compound to precedented closely-related structures. Compounds that tickle the same or similar pathways could be expected to have similar *in vivo* toxicities. Dr Daston closed by reminding us that dose matters, even (or perhaps, most especially) *in vitro*, and referring us to a HESI effort that is trying to incorporate concentration into the decision-making process from *in vitro* studies.

Dr **Andrea Seiler** presented some work on the ability of the ECVAM stem cell assay to distinguish among analogues of valproic acid with varying *in vivo* developmental toxicities. The assay appears to do a reassuringly good job with this class of compounds. The researchers continue to explore and optimize the performance of this mouse stem cell assay.

Turning briefly to a representative of pre-fertilization biology (i.e., reproduction, not development), **Agnes Forgacs** then presented regarding a mouse Leydig cell line from their lab and their characterization of these cells. In addition to its possibly usefulness for characterizing endocrine-altering activities of compounds, a cell line might reduce the need to produce primary cultures of Leydig cells isolated from animals. This cell line is capable of synthesizing testosterone *in vitro*, unlike others lines in which progesterone is the product assessed. The murine origin of the line brings its own challenges, as the mouse has some peculiarities in terms of response to estrogens. It was useful to introduce this cell line to a wider audience and to stimulate some thoughts about the use and exploration of the validity of this model.

Finally, the session returned to development with Dr **Jurgen Hescheler**, who presented some work with human embryonic stem cells and their differentiation *in vitro*. Using gene expression, they could follow a differentiation trajectory and determine exposure-induced changes, which set the stage for evaluations across compounds, based on the degree of divergence from the normal trajectory.

Overall, this session not only gave the audience a close look at some specific tools but also provided an overview of a process by which the likely toxicity of a new chemical could be predicted and then assessed in an animal-free way. True to the vision expressed in the *Toxicology in the 21st Century* publication, all the talks highlighted the use of pathways biology and their exposure-induced alterations. Clearly, this is a foundation on which much emerging toxicology rests. The technology and approaches in this area are evolving so fast that it's a challenge to keep up, and there is no better example of this than a conversation that one of the session's co-chairs had after the session had ended. In talking with a stem cell toxicologist and a Zebrafish scientist, the co-chair suggested that the joint use of their models might be more valuable than either alone. They laughed and admitted that they had just submitted a grant to do precisely that. This, of course, is what makes meetings like this exciting and so worthwhile to attend. And it shows that by working together, we make real advances are more likely.

Session I-10

Safety Testing for Carcinogenicity and Genetic Toxicity: Recent Three Rs Advances

Co-Chairs: David Blakey, Health Canada, Canada
Hajime Kojima, JacVAM, Japan

This session offered the opportunity to examine progress with new Three Rs methods and testing paradigms for genotoxicity and carcinogenicity testing. The presentations focused on the use of new technologies, where the methods have already been developed and are undergoing validation, and where they are undergoing review in preparation for adoption as an OECD test guideline. For example, *in vitro* versions of the Muta™ Mouse Transgenic Rodent (TGR) Mutation Assay for hazard identification of chemicals were discussed.

Dr **White** reported that in July 2011, "Transgenic Rodent Somatic and Germ Cell Gene Mutation Assays" was published as OECD test guideline No. 488. Correlated with this assay, new *in vitro* assays have been developed using the FE1 cell lines and primary hepatocytes from Muta™ Mouse, which are cytogenetically stable, metabolically-competent, contain wild-type p53 and yield reproducible responses upon exposure to a variety of mutagenic substances.

An update was given by Dr **Hayashi** on the ongoing international validation study of the *in vivo* Comet Assay, which is being coordinated by JaCVAM. The aim is to validate the *in vivo* comet assay as a potential predictor of carcinogens and as an alternative follow-up assay to the more commonly used *in vivo* rodent UDS assay. The purpose of the first step of the validation study was to examine the extent of reproducibility and variability of assay results among laboratories using coded test chemicals and the positive control EMS. The second step is an ongoing study to investigate the predictive capability of the assay to determine the carcinogenicity of test chemicals using 40 test chemicals with different characteristics in a variety of different chemical classes.

The current situation regarding the ECVAM prevalidation of the cell transformation assay (CTA) was presented by Dr **Phrakonkham**, in view of future OECD activities and Test Guideline (TG) development. Discussion was focused on ECVAM's decision, based on the prevalidation results that priority should be given to development of a single OECD TG for the two SHE assays. The development of a TG for the Balb assay should be further pursued upon availability of additional data and improvement of the statistical method.

Session I-11

Three Rs Approaches to Skin Sensitization

Co-Chairs: Carl Westmoreland, Unilever, UK
Anthony Gaspari, University of Maryland, USA

Session 1-11 focused on reviewing the state-of-the-art with respect to non-animal alternatives in the area of skin sensitization as well as discussing some of the gaps in our current scientific knowledge that, if adequately addressed, may ultimately lead to improved tools for the future. Presentations were made by Dr **Kreysa**, Dr **Aeby**, Dr **Gaspari**, Dr **Roggen**, and Dr **Yarmush**. Initially, an overview was given of the current status of alternatives in this area based on a recent review carried out on behalf of the European Commission (Adler et al., 2011). There was also discussion of an expert consortium review of this report. Overall, the panel that reviewed the report concluded that the replacement of animal methods (e.g., the local lymph node assay) for use in skin sensitization risk assessments by a panel of robust, validated *in vitro* (or *in silico*) methods would not be completed by the 2013 deadline that is looming in the European Union.

Many methods are available that model (to some degree) events that represent parts of the pathway responsible for the induction of skin sensitization following human skin exposure. Some of these methods are more established than others and three (HCLAT (Ashikaga et al., 2010), DPRA (Gerberick et al., 2007) and MUSST (Ade et al., 2006) are currently being assessed by ECVAM. It is generally accepted that one single *in vitro* assay will not be the way forward for non-animal approaches to skin sensitization and that a 'toolbox' of mechanistically-based assays will be needed in the future. Adler et al. (2011) predicted that the scientific ability to inform all skin sensitization decisions with such toolboxes completely in the absence of animal data should be feasible in another 7-9 years. The challenge of validation of methods for use in such a toolbox was discussed as well as the need to understand how data from different tests may ultimately be integrated to allow decisions on human safety. The session also reviewed the development and evaluation of less mature assays and methodologies that are being developed as part of the Colipa research program (Aeby et al., 2010) and the Sens-it-iv EU FP7 program (www.sens-it-iv.eu) where it was highlighted that, although considerable progress has been made with regard to assays related to some aspects of the skin sensitization process (e.g., protein binding, dendritic cell activation), there are also still gaps in the scientific knowledge of some parts of the process (e.g., skin bioavailability/skin metabolism, sensitizer-induced T-cell responses) that must be addressed if the "toolbox" approach is to be ultimately successful. The science in this area is not a static field and it is essential that as new understanding and insights continue to emerge in this field, they are incorporated into our thinking for 3Rs approaches to skin sensitization. For example, more fundamental aspects of the roles of T-cells were also discussed during the session, where the role of natural killer cells and cell-cell interactions with keratinocytes in the skin was outlined, which led to some interesting discussion on whether these new scientific findings might lead to the development of more tools for the 'toolbox' to allow this part of the skin sensitization process to be modeled.

References

Ade, N., Martinozzi-Tessier, S., Pallardy, M., and Rousset, F. (2006). Activation of U937 cells by contact sensitizers: CD86 expression is independent of apoptosis. *J. Immunotoxicol. 3*, 189-197.

Aeby, P., Ashikaga, T., Bessou-Touya, S., et al. (2010). Identifying and characterizing chemical skin sensitizers without animal testing: Colipa's research and method development program. *Toxicol. In Vitro 24*, 1465-1473.

Adler, S., Basketter, D., Creton, S., et al. (2011). Alternative (non-animal) methods for cosmetic testing: current status and future prospects-2010. *Arch. Toxicol. 85*, 367-485.

Ashikaga, T., Sakaguchi, H., Sono, S., et al. (2010). A comparative evaluation of in vitro skin sensitisation tests: the human cell-line activation test (h-CLAT) versus the local lymph node assay (LLNA). *ATLA 38*, 275-284.

Gerberick, G. F., Vassallo, J. D., Foertsch, L. M., et al. (2007). Quantification of chemical peptide reactivity for screening contact allergens: a classification tree model approach. *Toxicol. Sci. 97*, 417-427.

Session I-12

Epigenetics and its Increasing Relevance in Toxicology

Co-Chairs: Mathieu Vinken, Vrije Universiteit Brussel, Belgium
Moshe Szyf, McGill University, Canada

Epigenetics refers to the heritable changes in the phenotype or gene expression patterns caused by mechanisms other than changes in the underlying DNA sequence. In the last decade, it has become clear that the emerging field of epigenetics is of significant relevance for both the study and practice of toxicology. At the research level, current efforts are focused towards the elucidation of the involvement of chemical-induced epigenetic changes in adverse health effects, as well as to the exploitation of epigenetics, particularly in the area of *in vitro* modeling. At the regulatory level, a major challenge lies ahead in encompassing epigenetic information generated through toxicity testing in current chemical safety assessment exercises. The current session tried to cover several aspects of epigenetics, including its impact and applications, in a toxicological context.

The session was introduced by Dr **Mathieu Vinken** (Department of Toxicology, Vrije Universiteit Brussel, Belgium), whereby major determinants of the epigenome, including DNA methylation, histone modifications and regulation by non-coding RNA species, were briefly discussed. Furthermore, it was illustrated how several external factors, for instance of chemical or physical nature, can interfere with the epigenetic machinery and thereby trigger toxicity. Animal models currently used for studying these epimutagens were also presented.

In the first talk by Prof. **Tamara Vanhaecke** (Department of Toxicology, Vrije Universiteit Brussel, Belgium), it was discussed how epigenetic features can be studied *in vitro*. More specifically, it was presented how epigenetics can be exploited as a tool to stabilize liver-based *in vitro* models, *in casu* primary hepatocyte cultures. The latter are prone to dedifferentiation and hence are of rather limited use for toxicologists. Using a combination of inhibitors of histone deacetylases and DNA methyltransferases, as well as through interfering with microRNA expression, the hepatocellular dedifferentiation process can be counteracted. This opens new perspectives for the establishment of long-term primary hepatocyte culture systems and thereby significantly contributes to the implementation of the 3Rs principle into toxicology.

In the second presentation, Prof. **Moshe Szyf** (Department of Pharmacology and Therapeutics, McGill University, Montréal, Canada) discussed the challenges that our new understanding of the dynamic epigenome pose to our traditional methods for identifying agents that have a long-term deleterious impact on health. DNA methylation patterns are generated by an innate program during gestation but are attuned to both physical and social environments *in utero* and throughout life. DNA function and health could be stably altered by exposure to environmental agents without changing the sequence, just by changing the state of DNA methylation. Our current screening tests do not detect agents that have long-range impact on the phenotype without altering the genotype. The realization that long-range damage could be caused without changing the DNA sequence has important implications on the way we assess the safety of chemicals, drugs, and food and broadens the scope of definition of toxic agents.

The subsequent talk by Dr **Barbara Stefanska** (Department of Pharmacology and Therapeutics, McGill University, Montréal, Canada) elaborated on the role of the loss of DNA methylation that is a hallmark of cancer, particularly focusing on hepatocellular carcinoma (HCC), the most common type of liver cancer. HCC is triggered by several environmental factors including hepatitis B and C infection, alcohol, aflatoxin contaminated food, carcinogens and chronic methyl-deficient diet, suggesting the involvement of epigenetic mechanisms in development of the disease. One of the changes in the epigenome mediated by environmental exposures is promoter hypomethylation that results in activation of a distinct set of genes that are essential for cancer progression and metastasis. These genes are clustered together across the genome, indicating a high-level organization. Depletion of methylated DNA-binding domain protein 2 (MBD2), one of the components of the DNA demethylation machinery, leads to suppression of cancer cell growth and invasiveness along with reversing hypomethylation and induction of genes that are activated in tumors. These effects are cancer-specific and suggest that MBD2 may be a novel therapeutic candidate for remodeling the epigenome in liver cancer.

Session I-13

Toxicity Testing in the 21st Century

Chair: Robert Kavlock, National Center for Computational Toxicology, US EPA, NC, USA

The Tox21 Community consists of a group of federal programs and centers in the United States who have collaborated since 2008 on the research, development, validation, and translation of new and innovative test methods which characterize key steps in toxicity pathways. Session I-13 at the WC8 provided an opportunity for discussion on progress to date in meeting the Tox21 goals, focusing on the strategies for chemical and assay selection, workflows for data management and analysis, and follow-up studies of novel findings.

Session I-14

Comparing the Challenges of Implementing New Non-Animal Methods in the US and Europe

Chair: Bas Blaauboer, ESTIV, Utrecht, Netherlands

The European Society for Toxicology In Vitro (ESTIV) and the newly created American Society for Cellular and Computational Toxicology (ASCCT) used the opportunity presented by WC8 to host a combined session to compare and contrast the challenges of developing and implementing new alternative (non-animal) methods in the US and Europe.

Session I-15

Progression of Three Rs in Shellfish Toxin Testing

Chair: Ngaire Dennison, UK Home Office

Background

There are a number of different types of marine biotoxins that can contaminate shellfish if they feed on certain toxic phytoplankton (algae). In order to protect the health of consumers worldwide, there are regulations that require monitoring of the levels of these toxins in shellfish destined for human consumption. Mouse Bioassays (MBAs) have been used routinely for this testing. Whilst some countries have replaced some or all MBA testing, hurdles remain with respect to acceptance and use of alternative methods. Presentations were given that highlighted some of the remaining problems and provided information on a flexible, high-throughput non-animal method.

Recommendations

1. DG SANCO should be made aware of the concerns with respect to the prescriptive nature of the new Food Hygiene regulations and they should consider redrafting this based on performance criteria in line with Codex Alimentaris recommendations which discuss the principles of tiered testing (screening/confirmatory and reference) and the standards of validation outlined in European Commission Decision 2002/657 EC.
2. Better collaboration where there has been a significant outbreak of toxicity in order to collect a large biomass of contaminated shellfish to generate toxin standards/reference materials.
3. Scientific publications and presentations relating to new and modified alternative methods for detection of marine biotoxins should honestly and openly discuss limitations of methods and technologies.
4. Funding should be targeted more towards validation and implementation of currently developed "hopeful" tests rather than development of new ones at this time.
5. Training of laboratories to improve performance and confidence, preferably in more than one of the methods available, should also be a focus and funding supplied for this.
6. The source of monoclonal antibodies, where used, should be checked to ensure that production of these is not being performed using the ascites method in mice, as more refined options are available.

Presentations

Dr **Katrina Campbell**: *Evolving from the mouse to the opto-electronic mouse for phycotoxin analysis in shellfish*

There are an extensive variety of marine biotoxins currently being detected in seafood produce. These toxins were originally classified based on the acute effects seen in intoxicated individuals. Three of the toxin groups were therefore named paralytic (PSP), amnesic (ASP) and diarrhetic (DSP) shellfish poisoning toxins. In recent years, there has been an increase in the frequency of occurrence of toxin episodes. Further analogues of existing toxins and new, more potent toxins are now emerging in previously uncontaminated marine environments. The possible reasons for this include changes in the climate of the marine environment caused by increases in temperature, pollution and ocean acidification, but the expansion of the shellfish industry globally, leading to increased monitoring and improvements in detection technologies, has also had a significant effect.

The mouse bioassays (MBAs) employed to detect the PSP and DSP have been developed based on the physiochemical properties of toxins. The detection level is based on their acute toxicity rather than potential chronic effects. As more is known about marine toxins, particularly the effects of long-term exposure, food safety authorities are increasingly looking at reducing the acceptable levels of these toxins in seafood. There are a number of alternative methods to the MBAs reported in the literature which can be classified into three groups: functional, analytical and biochemical. Each of these methods has its own advantages and disadvantages, but if the mouse bioassay is to be completely replaced worldwide combinations of these methods will be required.

Research at Queen's University, funded by the EU (FP6 BioCop and Detectox and FP7 Conffidence), has focused on the development of antibody-based surface plasmon resonance (SPR) biosensor screening assays. Single, semi-quantitative SPR screening assays have been developed for domoic, okadaic and a broad spectrum of PSP toxins. Each assay has undergone single laboratory validation and has been compared to existing methods (Traynor et al., 2006; Stewart et al., 2009; Campbell et al., 2010). For PSPs a pilot interlaboratory study has been conducted (van den Top et al., 2011). To improve accuracy in the measure, antibodies have been constructed where the cross-reactivity profile matches the toxicity of the known analogues of the parent toxin.

Generally for marine toxins, each analytical method is specific for a particular toxin and its chemical analogues, with each group of toxins requiring separate analysis. An ideal scenario for the monitoring of phycotoxins in shellfish would be to evolve multiple toxin detection onto a single analytical platform as an optoelectronic mouse. Further research at Queen' University has developed a micro-fluidic immobilization device and prototype multiplex SPR biosensor designed for the detection of up to 16 molecular binding interactions in a 4 line by 4 channel array on a single chip. This dual system was evaluated in its ability to be fit-for-purpose for the simultaneous detection of three key phycotoxin groups. Domoic acid, okadaic acid and saxitoxin calibration curves in shellfish were achieved in separate flow channels with detection limits designed on achieving detection at and below the regulatory action levels (Campbell et al., 2011). This detection system exhibits enormous potential

for multiple phycotoxin screening as alternative to the mouse bioassay with the additional benefit of being able to distinguish between toxin families on a single analysis. The technology also has the capacity to add additional single detection assays onto the multiplex format, for example those currently being developed for emerging toxins: tetrodotoxin, palytoxin and brevetoxin. Investigations are ongoing into low cost portable devices that can be employed more readily for end product testing on site at the farms and processing sites for improved Hazard Analysis and Critical Control Point (HACCP) management and improved quality assurance.

SPR technology has been displayed as a highly promising analytical tool. This technology offers rapid real time detection requiring minimal toxin standards, which is crucial because of limited standard availability.

A further effort is underway in the replacement of animals by efforts to replace antibodies in these and other tests with synthetic binders such as aptamers.

Dr **Mardas Daneshian**: *Regulatory and methodological shortcomings in assessment of marine biotoxins in fish and shellfish*
The consumption of shellfish and finfish is increasing worldwide and therefore it is becoming increasingly important that monitoring programs for marine toxins target the right toxins and can detect these at relevant levels. A recent European Food Standard Agency (EFSA) report (EFSA, 2008) promoted the use of an LCMS method for detection of some groups of marine toxins. This method has been shown to discriminate between toxin analogues but it does not directly measure toxicity.

In November 2010, CAAT-EU hosted an expert working group in order to identify whether current testing strategies provided sufficient protection for public health and, if not, what specific issues were of concern. It was concluded that current regulations are not sufficiently robust.

A number of toxins that have no proven link to ill effects in humans or other animals when ingested orally are included in the European Food Hygiene Regulations, because they cause death in MBAs using an intraperitoneal route of injection. Conversely, toxins which are known to cause serious illness and death in people, such as tetrodotoxin, brevetoxin and cyclic imines, and which have been demonstrated to be present in fish or shellfish harvested from Mediterranean waters, are not currently regulated. The regulations should be amended to properly reflect the risks posed by marine biotoxins and monitoring strategies should be adjusted accordingly.

There are some concerns that analytical methods may miss the presence of such toxins the methods are not set up for their detection. Functional methods may be better able to detect unexpected or unknown toxins. Combinations of analytical, biochemical, and functional assays and those based on human cell test systems may lead to more robust protection of human health.

A report of the conclusions from the working group is being compiled for publication. Recommendations from this will include:

1. Establishment of an European registry of marine biotoxin intoxication incidents
2. Training of medical staff to recognize and treat such intoxications
3. A request to EFSA for consideration of revision of their recommendations to address further scientific concerns
4. Provision of reference materials and toxin standards for the development, validation and implementation of high through-put, non-animal methods
5. Establishment of LCMS methods for hazard monitoring

Dr **Ngaire Dennison**: *Removing the mouse from shellfish toxin testing – Fifteen years of the Three Rs*
Around 98% of UK shellfish samples are now monitored for toxins using analytical methods. Arriving at this position took over fifteen years and a number of "two Rs" (refinement and reduction) strategies were used increasingly whilst alternative methods were being developed and validated. These included shortened duration of assays, the use of clinical endpoints, the use of 2 rather than 3 mice per sample and qualitative pre-screening to eliminate the need for MBA testing. Such approaches reduce the current suffering of animals until alternatives replace their use.

Many barriers remain in place to complete replacement of the MBA, including lack of reference materials, disagreements as to the best *in vitro* methods, resistance to change and differing national attitudes. The aims of the discussion session subsequent to the presentations given were to identify some of these barriers and suggest possible remedies.

Discussion summary
A number of different approaches and methods are likely to be needed to satisfy the diverse levels of production and consumption of shellfish in different countries and the varying prevalence of marine biotoxins. One example of this is within the available HPLC methods for the detection of PSP. The pre-column (Lawrence et al., 2005; Turner et al., 2010) method is more cost- and time-efficient when testing samples in which the presence of toxins is expected to be low, whereas the post-column (Van de Riet et al., 2009) method is preferable if toxin presence is expected. The cost per sample for any test will depend on throughput and availability of specific resources. Delays in results can cause inaccuracies in the results of tests – for example if transport, extract and testing of samples takes several days, the levels of toxin may be significantly higher by the time the results are reported if there is an algal bloom. In such circumstances, the speed of the test to allow the most up-to-date picture of toxicity is important. One strategy will not be appropriate for all and it is important that this is recognized and that Regulations reflect this situation.

Although it is positive that regulators are looking at introducing alternatives, barriers still exist in terms of reluctance and inertia of regulators to accept new methods. Inclusion of non-animal methods in the European Regulations was viewed as a move forward in terms of replacement of animals, but raised concerns from some present that the limitations imposed by specifying specific methods might backfire in the future if human illness occurred due to non-detection of emerging toxins. There were differing opinions as to whether MBAs have a better ability to predict new toxins than alternative methods. Which-

ever method is used, extraction of the toxin is essential to allow detection. Failure of replacement methods to detect new toxins could create pressure to return to animal testing.It was considered that one overly prescriptive Regulation had been replaced by another one. It was proposed that one specific method should not be enforced for all monitoring, but that other validated alternative methods could and should be employed, as appropriate. Such methods must be validated to an acceptable standard. An appropriate approach might be that used for veterinary drug residues (European Commission, 2002). A combination of methods involving a screening approach, which can be either performed on-site at farms as an end product test prior to harvesting or in the laboratory, providing a preliminary yes or no toxicity and whereby a positive result for toxicity should be followed up by a confirmatory approach to identify and quantify with certainty and assuredly the toxins present appears to be the way forward. Better drafting of Regulations is essential.

Validation criteria can vary between regulators and this is extremely unhelpful. Issues arise as to which specie(s) of shellfish tests should be validated for before acceptance due to matrix variation. Generally, if methods have been validated for the species for which most tests are performed they should be able to be accepted, at least for this species, to allow faster progress towards alternatives. Validation of methods should not routinely be against MBAs but via the use of standards wherever possible.

The availability of toxin standards is variable. Although most are accessible to some extent, there is insufficient availability despite some funding in this area and this needs to be addressed. Collection of large amounts of biomass from toxic incidents would improve the situation with respect to standards. The use of naturally incurred materials is likely to be more viable than generating synthetic materials. The source of standards was discussed, as, until recently, there was only one major source. The advantages and disadvantages of a single versus multiple sources of standards were discussed and the group was split as to which was the most secure and cost effective way forward.

There were variable opinions as to whether sufficient collaboration was occurring. Collaboration within scientific fields, for example between analytical laboratories, was often good, but knowledge and acceptance across disciplines of different types of test was often poor. It was considered critically important that more honesty relating to what a test could genuinely do well and did poorly, or not at all, would be very helpful. More funding should be made available to ensure that promising tests are validated and implemented with the training of regulatory laboratories, rather than concentrating on test development.

The issue of the source of antibodies, in particular monoclonal antibodies, for alternative tests was raised by one member of the audience, who had profound concerns that ascites production in mice was still used in at least one European country for antibody production for a currently available commercial "alternative" test system. Those present confirmed that monoclonal antibody production no longer requires this technique and that, once generated in animals using non-ascites methods, the use of immortalization in cell lines should then prevent the need for further animal use.

Acknowledgements

The chair and speakers would like to acknowledge the helpful comments and feedback from the audience during the discussion session and would particularly like to thank Prof. Chris Elliott of Queen's University Belfast for his input, particularly with respect to validation approaches.

References

Campbell, K., Haughey, S. A., van den Top, H., et al. (2010). Single laboratory validation of a surface plasmon resonance biosensor screening method for paralytic shellfish poisoning toxins. *Anal. Chem. 82*, 2977-2988.

Campbell, K., McGrath, T., Sjölander, S., et al. (2011). Use of a novel micro-fluidic device to create arrays for multiplex analysis of large and small molecular weight compounds by surface plasmon resonance. *Biosens. Bioelectron. 26*, 3029-3036.

EFSA – European Food Safety Authority (2008). Marine biotoxins in shellfish – okadaic acid and analogues, Scientific opinion of the panel on contaminants in the food chain. *EFSA Journal 589*, 1-62. http://www.efsa.europa.eu/en/scdocs/doc/589.pdf

European Commission (2002). Regulation(EC) of No 657/2002 of the European parliament and of the council 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results.

Traynor, I. M., Plumpton, L., Fodey, T. L., et al. (2006). Immunobiosensor detection of domoic acid as a screening test in bivalve molluscs: comparison with liquid chromatography based analysis. *J. AOAC Int. 89*, 868-872.

Lawrence, J. F., Niedzwiadek, B., and Menard, C. (2005). Quantitative determination of paralytical shellfish poisoning in shellfish using prechromatographic oxidation and liquid chromatography with fluorescence detection: collaborative study. *J. AOAC Int. 88*, 1714-1732.

Stewart, L. D., Hess, P., Connolly, L., and Elliott, C. T. (2009) Development and single-laboratory validation of a pseudo-functional biosensor immunoassay for the detection of the okadaic acid group of toxins. *Anal. Chem. 81*, 10208-10214.

Turner, A. D., Hatfield, R. G., Rapkova-Dhanji, M., et al. (2010). Single-laboratory validation of a refined AOAC HPLC method 2005.06 for oysters, cockles, and clams in U.K. shellfish. *J. AOAC Int. 93*, 1482-1493.

van den Top, H., Haughey, S. A., Vilariño, et al. (2011). Surface plasmon resonance biosensor screening method for paralytic shellfish poisoning toxins: A pilot interlaboratory study. *Anal. Chem. 82*, 2977-2988.

van de Riet, J. M., Gibbs, R. S., Chou, F. W., et al. (2009). Liquid chromatographic post-column oxidation method for analysis of paralytic shellfish toxins in mussels, clams, scallops and oysters: single-laboratory validation. *J. AOAC Int. 92*, 1690-1704.

Session I-16

Alternatives for Potency Testing for Rabies

Co-Chairs: Marlies Halder, European Centre for Validation of Alternative Methods, Italy
Oksana Yarosh, Canadian Centre for Veterinary Biologics, Canada

Batch potency testing of rabies vaccines for human and veterinary use is based on a multi-dilution vaccination-challenge assay that requires large numbers of mice (up to 170/batch), inflicts severe pain and distress (intracerebral injection with virulent rabies virus for the challenge, at least 50% of the mice develop rabies) and is highly variable. The session addressed possibilities and recent achievements in using alternative methods for batch potency of rabies vaccines.

Marie-Jeanne Schifflers (Utrecht University, The Netherlands) set the scene by reporting on the drivers and barriers to the acceptance and use of 3R methods for the quality control of veterinary vaccines. She concluded that the momentum for a change has been created on various levels: by regulators, who developed and initiated the validation of a serological method for veterinary vaccines (see below), and by manufacturers expressing their willingness to share data.

Lukas Bruckner (Institute of Virology and Immunoprophylaxis, Switzerland) presented the stages in the development of a serological method (determination of antibodies in vaccinated mice) and its successful validation involving 13 official control laboratories and manufacturers from Europe and North America under the framework of the EDQM Biological Standardisation Programme. This single-dilution serum neutralization test is already used by European official control laboratories for the batch release. Its regulatory acceptance, i.e., inclusion into the European Pharmacopoeia monograph on veterinary rabies vaccines, is in progress.

Elisabeth Kamphuis (Paul-Ehrlich-Institut, Germany) described the ongoing activities to further develop the qualitative single-dilution serum neutralization test into a quantitative multiple-dilution assay, which would better meet manufacturers' requirements. Recently, work has started to explore whether the serum neutralization assay could also be used for the potency testing of human rabies vaccines. Modifications might be necessary since the current *in vivo* tests for human and veterinary rabies vaccines differ with respect to vaccination scheme and the age of the mice.

There was broad agreement that the current *in vivo* vaccination-challenge assay should be replaced. Stakeholders should continue to collaborate and undertake efforts to harmonize possible alternative approaches at a global level. Antigen quantification assays had not been discussed during the session; their possible use and suitability to fully replace *in vivo* methods should be further explored.

Session I-17

Update on New In Vitro Models for Detection and Potency Assessment of Botulinum Neurotoxin

Co-Chairs: Warren Casey, NTP Interagency Center for the Evaluation of Alternative Methods, USA
Martin Stephens, The Humane Society of the United States

NICEATM and ICCVAM organized an international workshop on alternative methods to refine, reduce, and replace the mouse LD_{50} assay for botulinum toxin testing and published the workshop recommendations for advancing alternatives in a 2008 report. The workshop report included priority research and development efforts necessary to develop *in vitro* replacement alternatives. With subsequent Federal government and industry support, several innovative *in vitro* assays have been developed to potentially replace the mouse LD_{50} assay for both diagnostic and potency testing for botulinum neurotoxin testing. This session provided an overview of these new *in vitro* methods and discussed applications where they are expected to reduce and replace animal use. There have been significant advancements in the field of *in vitro* alternatives to the Mouse LD_{50} assay for assessing potency of botulinum neurotoixn since the ICCVAM workshop which focused on this issue, and an international Expert Working Group on the replacement of the LD_{50} mouse has recently been established. Although significant challenges still face *in vitro* alternatives, the availability of multiple technologies coupled with the technical flexibility afforded by product-specific validation indicate that these obstacles should be surmountable for most testing applications. The recent FDA approval of a replacement method by Allergan provides hope that many additional alternative methods will soon follow.

Session I-18

Report on the ICCVAM International Workshop on Vaccines

The NICEATM-ICCVAM International Workshop on Alternative Methods to Reduce, Refine, and Replace the Use of Animals in Vaccine Potency and Safety Testing: State of the Science and Future Directions

William Stokes, NICEATM/NIEHS/NIH, USA

Few medical interventions have had a greater impact on human and animal health than vaccines. Immunization efforts have resulted in the global eradication of smallpox, and the elimination of polio, measles, and rubella in the Americas. Vaccines improve animal health and welfare by preventing and controlling infectious diseases, as well as enabling efficient production of food animals to feed the burgeoning human population. Prior to the release of post-licensing production lots of vaccine, regulatory authorities require testing to ensure potency and safety. However, such testing currently involves large numbers of animals, and many experience significant unrelieved pain and distress. This session provided an overview of the recommendations from a recent international workshop that addressed how to apply new advances in science and technology to further reduce, refine, and replace animal use for human and veterinary vaccine potency and safety testing.

Dr **William Stokes** introduced the session by providing an overview of the recent *International Workshop on Alternative Methods to Reduce, Refine, and Replace the Use of Animals in Vaccine Potency and Safety Testing: State of the Science and Future Directions*. The workshop was organized by the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) in collaboration with its international partners ECVAM, JaCVAM and Health Canada. The workshop was held at the National Institutes of Health in Bethesda, Maryland, USA, on September 14-16, 2010. Nearly 200 scientists from 13 countries participated.

The goals of the workshop were to:

- Identify and promote the implementation of currently available and accepted alternative methods that can reduce, refine, and replace the use of animals in vaccine potency and safety testing
- Review the state of the science of alternative methods and identify knowledge and data gaps that need to be addressed
- Identify and prioritize research, development, and validation efforts necessary to address these gaps and to advance alternative methods that can ensure continued protection of human and animal health.

Dr **Richard McFarland** discussed specific recommendations by workshop participants to advance 3Rs methods for human vaccine potency and safety testing. Diphtheria and tetanus toxoids, pertussis, rabies, anthrax, inactivated polio, and combination vaccines were identified as the highest priority vaccines for further activities because they use large numbers of animals and involve significant pain and distress during their testing. Further research into the specific mechanisms of vaccine protection and identifying clinically relevant immunological markers were considered necessary to successfully implement *in vitro* alternatives. Workshop participants also agreed that broader acceptance and use of alternative methods would require 1) improved access to information, 2) increased global communication among regulatory authorities, research institutions, and vaccine manufacturers, and 3) international harmonization of testing requirements.

Dr **Kulpa-Eddy** summarized the recommendations from workshop participants to advance 3Rs methods for veterinary vaccine potency and safety testing. The identified highest priority vaccines were Rabies, *Clostridium sp*., and *Leptospira sp*. vaccines because their testing requires large numbers of animals and involves significant pain and distress. Vaccine challenge testing procedures that require live viruses and bacteria hazardous to laboratory workers, livestock, pets, and wildlife also were considered high priorities. Collaborations between human and veterinary researchers working on vaccines for the same or similar organisms were recommended to leverage scientific resources and expedite progress. Implementation of the workshop recommendations are expected to advance alternative methods for vaccine potency and safety testing that will benefit animal welfare while ensuring continued protection of animal and human health.

Session I-19

Toxicity Testing Strategies: Progress in Skin Sensitization Testing

A COLIPA supported session

Co-Chairs: Monique Marrec-Fairley, The European Cosmetics Association (COLIPA), Belgium
Pierre Aeby, The European Cosmetics Association (COLIPA), Switzerland

Colipa participates in an international research effort to explore the processes governing the induction of skin sensitization and the development of new *in vitro*/*in silico* test methods.

During this session, five companies (Beiersdorf, Shiseido, Kao, L'Oréal, and Unilever) presented their current approaches to developing non-animal testing strategies for skin sensitization risk assessment. The main messages conveyed during these presentations are:

– Some non-animal test methods are currently in late stage method development or under evaluation – termed "non-animal toolbox."
– The majority of non-animal toolbox test methods have been shown to be successful in predicting sensitizer potential (hazard identification).
– The Cosmetic Industry currently applies a range of approaches to reduce the need for animal test data to support skin sensitization risk assessment decision-making, e.g., theoretical chemistry, read-across, TTC approaches. These approaches now are being enriched by the integration of the data obtained through *in vitro* test methods.
– Several speakers demonstrated progress in improving sensitizer potential predictions (sensitizer vs. non-sensitizers) through the integration of datasets from multiple non-animal test methods. However, the ability to reliably predict sensitizer potency information to inform quantitative risk assessment decisions and the ability to apply exposure information (i.e., skin bioavailability, metabolism) remain key gaps.