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

Session I-1: Potency and safety testing of human vaccines

Session I-1: Oral presentations

I-1-107

EDQM's Three Rs activities in the field of quality control of vaccines

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The EDQM (European Directorate for the Quality of Medicines & Health Care, Council of Europe) is a standard setting body involved in ensuring the quality of medicines, including vaccines for human and veterinary use. This is done through the elaboration and publication of the European Pharmacopoeia (Ph. Eur.). In addition, the EDQM runs the Biological Standardisation Programme (BSP) which elaborates reference standards and validates methods, including Three Rs approaches for the quality control of biologicals. Furthermore the EDQM is the secretariat of the European network of public control laboratories that are involved in the official control authority batch release of human and veterinary vaccines. It is through this process that the public control laboratories use animals for the quality control of vaccines.

The Three Rs activities of EDQM are based on the "European Convention for the protection of vertebrate animals used for experimental and other scientific purposes", which was published by the Council of Europe in 1986 and which represents the first international legal text in this field.

A highlight of EDQM activities in the application of Three Rs principles will be presented including insight on challenges for development and implementation of alternative methods. Examples will include projects with successful introduction of alternative assays for the assessment of the potency of human and veterinary vaccines. An overview of future plans in this field will also be presented.

I-1-048

Development of 3Rs alternatives for determining potency and toxicity of vaccines in Cuba: Current challenges and research projects in progress

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Vaccines have been traditionally released for immunization purposes once a set of relevant quality control parameters have been controlled. To do so, classical animal potency and toxicity tests have played a determinant role. Over the last decade, efforts have been made in Cuba to introduce and develop Three Rs alternatives for potency and toxicity testing of vaccines. Significant progress has been made for some vaccines such as the potency of Hepatitis B (neutralization ELISA), the toxicity/potency of Diphtheria (guinea-pig serology-Vero cell) and the potency of Tetanus (mouse serology-ELISA). The remarkable reduction in the number of animals used, the refinement of the procedures and the potential for the full replacement of the challenge potency tests and some other animal assays have been considered as ways of implementing Three Rs alternatives for vaccines. The Finlay Institute is the major manufacturer of vaccines in Cuba and it is undoubtedly interested in this field. We aim to provide an overview of the state of the science and to show the progress we have made in the introduction/development of Three Rs alternatives for the evaluation of vaccines, as well as the upcoming research projects and those in progress. The Finlay Institute, along with some other Cuban institutions, are planning to introduce some of the most updated Three Rs approaches for classical animal tests for Potency and Toxicity tests of vaccines, including *in vitro* methods (ELISA, cell culture assays, biochemical and immunological functional tests), serology and consistency approach.

I-1-167

Three Rs acceptance and implementation: obstacles and opportunities for new technology

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The development, production and quality control of vaccines is characterized by extensive laboratory animal use and a high frequency of procedures inflicting severe pain and distress to the animals involved. Alternatives to traditional animal methods do exist which follow the Three Rs principles of reduction, replacement and refinement of animal use. However, despite progress made over the last decades to adopt Three Rs methods into vaccine testing and the existence of legislation such as European Directive 2010/63/EU, which requires the adoption of Three Rs alternatives when and where possible, the acceptance and use of Three Rs methods moves forward at a slow pace.

The authors have identified the factors which support or challenge the acceptance and use of Three Rs methods, as well as the motivations behind these factors. First, we describe the factors involved in the acceptance of Three Rs methods in vaccine quality control as identified through a Canadian case study. These factors are compared with factors identified through a second study, in the broader context of regulatory testing in Europe. We use the concept of technology transitions and technology paradigms to explain the mechanisms of acceptance and use of Three Rs models in regulating the risks of products such as vaccines. Recognition and understanding of these mechanisms will provide regulatory authorities and industry stakeholders with a basis for practical discussions on how to integrate scientifically sound alternatives into regulatory testing.

I-1-672

The consistency approach in lot release testing of vaccines

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Vaccine lot release testing is characterised by extensive use of laboratory animals, particularly to demonstrate product safety and potency. Successes have been achieved in replacing existing animal models by Three R methods; cell-based and serology or humane endpoints to remove lethality parameters. However, progress is tedious, time consuming and costly.

A new paradigm in lot release testing of established vaccines (e.g. Tetanus and Diphtheria toxoid) is the consistency approach. This approach starts from the idea that subsequent lots of vaccine produced can be compared to an earlier (reference) lot (clinical-, historical batch) with a thoroughly tested and well defined profile of safety and efficacy/potency. Consistency for lot release has come within reach since vaccine starting material is better characterised (quality by design), production processes have been optimised and standardised, a tight protocol for in-process testing has been implemented, quality monitoring systems such as GMP, QA and pharmacovigilance are now state-of-the-art and, last but not least, new physicochemical and immunochemical techniques have become available.

Consistency testing may lead to a significant reduction in animal use, since a narrow set of animal tests performed on each final lot, with potentially limited power to predict vaccine behaviour in the target populations, may be replaced by a battery of meaningful physicochemical-, immunochemical- and eventually *in vitro* functional tests with enhanced capacity to measure equivalence with batches of proven safety and efficacy.

The paradigm of consistency is an interesting strategy for vaccine manufacturers as it might allow for a reduction in testing costs and a shortening of the testing period. The concept of consistency testing was recently adopted by the European Partnership on Alternative Approaches to Animal Testing (EPAA) as a promising strategy to animal reduction.

This presentation will provide an introduction of the approach and discuss advantages and limitations. An outlook will be given of remaining research questions and implementation strategies.

I-1-148

Alternative safety testing strategy for acellular pertussis vaccines

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The mouse histamine sensitization test (HIST) is the currently accepted regulatory method used to monitor for residual pertussis toxin (PTx) activity in acellular pertussis vaccines. This lethal end-point test is technically challenging and raises ethical concerns, thereby making the development of alternative methods highly desirable. Various *in vitro* assays have been developed which each monitor different biochemical or cellular functions of PTx. These include PTx binding activity, enzymatic activity, and its ability to agglutinate or induce a signal transduction event in cultured cells. As the mechanism of PTx toxicity is complex, a panel of these *in vitro* assays will most likely be required as an alternative to the HIST. Several scientific meetings have been held recently to discuss how to proceed with the validation and adoption of these alternative assays. Major questions to be addressed include the sensitivity and specificity required of each assay, identification of assay limitations, effects of vaccine formulation on assay outcome, and the relation of *in vitro* data to HIST. This presentation will provide a review of the outcomes from these scientific meetings and the potential path forward to adopt these alternative assays to meet regulatory requirements.

I-1-326

Alternatives to animal use for the LAL-assay

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The Limulus Amebocyte Lysate (LAL)-assay is frequently performed to quantify endotoxin (LPS), the most effective pyrogen (fever inducing substance) known. Due to its persistence despite the use of cleaning/inactivation procedures, the impact of LPS on the pharma, life sciences and medical devices sectors is enormous. The LAL-assay has replaced the Rabbit Pyrogen Test (direct animal test) for most products, and is obligatory for many source materials, intermediates and final products. However, the "alternative" LAL is an "indirect animal test": the *Limulus* lysate is obtained from wild populations of horseshoe crabs (mainly *Limulus polyphemus*). To harvest the hemolymph, the crabs are captured, partially bled and released. Despite immense efforts by the LAL-industry, the mortality caused by this procedure is approximately 10-15%. In 2009, 512,552 horseshoe crabs were caught, bled, and released in the US, of which approximately 60,642 died (official estimation). All horseshoe-crab species are endangered (IUCN Red List), mainly due to loss of habitats, pollution (oil spills, etc.) and commercial use. The US *Limulus* population is controlled by fishery management plans. The Asian *Tachypleus* population (especially China) is threatened severely. The still growing demand for lysate cannot be satisfied by these wild populations, which could result in a major drug safety problem. The Three Rs could be applied immediately, in particular replacement and reduction alternative methods (validated alternatives like the monocyte activation Test / MAT, recombinant LAL-assays) and refinement (reduced lysate volume). These opportunities will be discussed in the presentation. Furthermore, we describe a more sensitive and accelerated MAT-version to make a feasible alternative available.

Session I-1: Poster presentations

I-1-060

Monocyte activation test (MAT) reliably detects pyrogens in parenteral formulations of human serum albumin

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The monocyte activation test (MAT) was performed to detect pyrogens in human serum albumin (HSA), and results were compared with those obtained with the rabbit test and Limulus assay (LAL). All batches were contaminated with (1,3)-β-glucans as assessed by conventional LAL, however endotoxin-specific LAL was not suitable to test HSA due to unacceptable endotoxin recoveries in the interference test. Three batches failed the rabbit test and were clearly detected with the MAT using IL-1β and IL-6 response as readout. Experiments combining polymyxin B and the MAT demonstrated that pyrogenic batches were contaminated with endotoxins, but the endotoxin-specific LAL failed to detect one of them, thus LAL did not offer a high security level to test pyrogens in HSA. (1,3)-β-glucan enhanced the IL-6 response to endotoxin, but not IL-1β. In addition, endotoxin concentrations obtained with IL-6 readout were usually higher than with IL-1β, likely related to a direct IL-6 response of monocytes to (1,3)-β-glucans. Contaminating (1,3)-β-glucans produced no pyrogenic reactions in rabbits. Hence, IL-1β readout resembles better the typical pyrogenic response. Nevertheless, IL-6 can be a useful readout to assess glucan contamination and its immunemodulating effect, which are potentially deleterious and not necessarily evidenced through a febrile reaction. The MAT correlates with the rabbit test while providing a higher safety level for pyrogenicity testing in HSA and probably other therapeutic proteins, so it can be a useful alternative method to detect any pyrogenic contamination in these products.

I-1-106

Comparison of the bioactivity for pertussis toxin by the histamine sensitization test and *in vitro* assays

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Pertussis toxin (PT) in its detoxified form is an important antigenic component of acellular pertussis vaccines. The specific toxicity test for acellular pertussis vaccines, the histamine sensitizing (HIST) assay, is still the only assay used, and involves many experimental animals. Currently, the enzyme coupled-HPLC assay and carbohydrate binding assay for pertussis toxin have been developed for potential replacement of the current *in vivo* HIST. In this study, we compared the bioactivity for pertussis toxin by the HIST and the *in vitro* assay. Although some questions still need to be answered in relation to the development of suitable replacements for *in vivo* tests of pertussis vaccines, the prospects for further improvements are promising.

I-1-138

Endotoxin and non-endotoxin pyrogens trigger inflammatory cytokine release in the Monocyte-Activation Test with cryopreserved human blood

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Pyrogen tests for products of the pharmaceutical industry so far require the utilization of animal-based test methods, the Rabbit Pyrogen Test (RPT) or the Limulus Amoebocyte Lysate (LAL) test. As the LAL test is limited to endotoxins, the RPT must be used for the detection of non-endotoxin pyrogens. With the development of the Monocyte-Activation Test (MAT) using cryopreserved human blood, there is now a convenient, validated *in vitro* alternative. In 2010 the European Pharmacopoeia declared that "the MAT is suitable after a product-specific validation as a replacement for the rabbit pyrogen test" (Chapter 2.6.30). Here we show that the MAT with cryopreserved whole human blood is a useful tool to study details of the fever reaction pathway in the innate human immune response. Different inflammatory cytokines are induced and regulated by a number of ligands for Toll-like receptors (TLRs) or intracellular nucleotide-binding oligomerization domain (NOD) proteins. Whereas TLR 4 is known to be activated by endotoxin, TLR 2 is a receptor for non-endotoxin bacterial cell wall components and TLR 7 and 8 show specificity for nucleic acid pyrogens. The cytokine induction with a collection of well characterized pyrogens thus reflects the effects expected for the human body. In contrast to the animal model the MAT allows differentiation between the activities of the substances and allows the kinetics of the reactions to be analyzed individually. IL-1β and IL-6 were found to be equally useful for a sensitive and specific readout of the inflammatory reaction.

I-1-149

Characterization of binding assay components used to detect residual pertussis toxin in vaccine preparations

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We previously described a modified binding assay for the detection of residual pertussis toxin (PTx) in acellular pertussis vaccines as a potential alternative to the mouse histamine sensitization test (HIST). The aim of the current study is to further understand two critical steps of the binding assay which confer specificity for PTx: 1) the use of fetuin to capture the PTx and; 2) the monoclonal antibody (7F2) used to detect the captured PTx. Enzymatic HPLC, CHO cell agglutination, inhibition binding assays, and surface plasmon resonance (SPR) were used to characterize the binding properties of PTx and chemically-inactivated pertussis toxoid (PTd) to 7F2, fetuin, and asialofetuin. 7F2 did not affect PTx enzymatic activity, but did inhibit both PTx-induced cell agglutination and fetuin binding activities. However, inhibition of agglutination required a far lower 7F2:PTx ratio than inhibition of fetuin binding, suggesting a divergence between cellular response and binding activities. SPR studies showed that PTx binds strongly to both 7F2 and fetuin with B50 of 2.3 and 2.0 μg/ml, respectively. Interestingly, fetuin and asialofetuin both inhibited the binding of 7F2 to PTx, suggesting a structural link between the binding domains of PTx subunits S2/S3 and the non-binding S4 subunit, which is recognized by 7F2. Despite this apparent competition between fetuin and 7F2 for PTx binding, the use of both agents provides very high sensitivity in the detection of residual PTx in vaccine samples which contain high concentrations of PTd.

I-1-222

Alternate *in vitro* methods for detection of pertussis toxin in component pertussis vaccines

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Current requirements for component pertussis vaccines include testing for residual pertussis toxin (PTx). Histamine sensitization tests in mice, currently used for this purpose, are problematic due to the high variability, ethical concerns and issues with non-specificity.

Alternate *in vitro* methods that measure the binding and enzymatic activities of PTx have been proposed as replacement tests. Based on these methods, we have developed and validated an enzyme-linked immunosorbent assay (ELISA) method for the carbohydrate-binding activity of the B-oligomer of PTx and a high-pressure liquid chromatography (HPLC) method for the ADP-ribosyltransferase activity of the A-protomer in a pertussis combination vaccine. The ELISA was validated as a limit test and as such LOD, LOQ, specificity and robustness were assessed. The method was specific for detection of PTx, with an LOD of 0.004 μg/ml and an LOQ of 0.008 μg/ml.

The multi-step gradient elution HPLC method was converted to isocratic separation employing a temperature controlled, solvent-saver octadecyl silane column, which resulted in enhanced peak resolution, specificity and 70% lower acetonitrile consumption. Method parameters validated included system suitability, specificity, accuracy, linearity, range, LOQ, repeatability, intermediate precision, robustness and stability of test solutions. An r2 = 1.00 was obtained for the linear regression of area response versus PTx spiking concentration in a mock vaccine sample from 1 μg/ml (LOQ) to 30 μg/ml. CVs <7% and <8% were obtained for repeatability and intermediate precision.

A significant positive relationship between HIST activity (either dermal or lethal endpoint) and ELISA binding activity was determined by statistical modeling.

I-1-229

Interferon genes regulated by pertussis toxin: potential for an *in vitro* pertussis vaccine safety assay

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Pertussis vaccines have proven very effective in decreasing the incidence of whooping cough. Since these vaccines are routinely administered to healthy infants and contain inactivated bacterial components, tests to ensure their safety are of critical importance. The histamine sensitization test (HIST) is currently the standard assay to test for the absence of active pertussis toxin (PTx). HIST is a lethal animal test that is difficult to standardize, therefore, replacement of the HIST is a priority. Moreover, the exact mechanism of the test is undefined, and it is not clear whether the assumed underlying mechanism, i.e. PTx-mediated ADP-ribosylation of G proteins, is the only relevant effect of PTx. Therefore, we decided to perform micro-array experiments in a relevant human cell line to analyze PTx-induced quantitative gene expression to gain insight into PTx mechanisms. The selected human EA.hy926 cell line is a hybrid of endothelial and epithelial cells, i.e. cell types that are involved in *in vivo* PTx effects. Unexpectedly, exposure of EA.hy926 cells to 250 ng/ml PTx differentially regulated only a limited number of genes with modest changes. Gene set enrichment analysis revealed that most affected genes were within interferon signaling pathways. We are currently investigating the validity of these pathways by quantitative PCR using other cell lines and sources of pertussis toxin. The ultimate aim is to develop an *in vitro* safety test for pertussis vaccines that does not require the use of experimental animals and that may be more informative than current *in vivo* testing.

I-1-260

Immunological response of MUTZ-3 dendritic cells to the different components of conjugated *Haemophilus influenzae* type B vaccine: potential *in vitro* assay for vaccine immunogenicity

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The whole suite of immune responses to vaccination that occur *in vivo* in humans cannot be tested *in vitro* using a single cell type. Nonetheless, dendritic cells (DC) form an important candidate cell type, since they are pivotal in inducing and orchestrating immune responses. We used the human cell line MUTZ-3, the cell line that most closely resembles *ex vivo* human DC, and compared its response to monocyte-derived DC (moDC). Haemophilus influenzae type B (Hib) vaccine was chosen as model vaccine because its components exert different effects *in vivo*: while the Hib antigen, poly ribosyl phosphate (PRP) fails to induce sufficient protection in children below 2 years of age, conjugation of this sugar antigen to outer membrane protein (OMP) of *Neisseria meningitides*, results in sufficient protection. PRP induced little or no effects on cytokine production and surface marker expression. OMP induced high levels of IL-6, IL-8, IL-12p40, and TNF-α in MUTZ-3 cells, and of IL-6, IL-10, IL-12p40, IL-12p70, IL-23, and TNF-α in moDC. In MUTZ-3 cells decreased expression of CD34, CD209 (DC-SIGN), and CD86 was seen, while CD1a, CD80 and CD83 expression was increased. In moDC decreased expression of CD209 (DC-SIGN) was seen, while CD80 and CD83 expression was increased. Conjugated PRP-OMP induced a considerably smaller response in both cytokine production and surface marker expression than OMP alone. PedVax showed a similar response compared to PRP-OMP. In conclusion, we have developed an assay that is able to measure immunogenicity of the different Hib vaccine components.

I-1-327

The Pertussis-ATP-Test to replace the animal experiments for testing acellular pertussis vaccines

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Pertussis is caused by the Gram-negative bacterium *Bordetella pertussis*. One of its most eminent virulence factors is Pertussis Toxin (PTx). PTx is also of utmost importance as antigen for immunization via so called Acellular Pertussis Vaccines (ACV). To guarantee non-hazardous application for all recipients, the pertussis toxin (PTd) needs to be detoxified. The success of this detoxification has to be controlled to exclude residual or reversed active toxin. The animal test used for that purpose, the Mouse Histamine Sensitisation Assay (HIST) – required by the pharmacopoeias – is hard to standardize, since it is affected by inconsistencies, which make repetitions and accompanying use of high resources of animals inevitable. An alternative testing method is needed.

The so called Pertussis-ATP-Test (PAT), which utilizes PTx enzymatic activity such as NAD-glycohydrolase and ADP-ribosyltransferase, was developed by our group. Inside the cell, PTx transfers ADP-ribose onto an inhibitory G-protein and thereby interferes in the signal transduction pathway. This leads to an increase of cAMP and a decrease of ATP. We implemented decreasing ATP levels caused by PTx as indicator for the activity of the latter. Freshly isolated peripheral blood mononuclear cells (PBMCs) as well as the permanent human lymphocyte cell line Jurkat were used as human indicator cells. Their ATP levels were monitored by luciferin-luciferase mediated bioluminescence.

I-1-328

Improved Monocyte Activation Test (MAT) for accelerated and sensitive pyrogen detection

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Testing for the absence of pyrogenic (fever-inducing) substances is of utmost importance for safe administration of various pharmaceuticals. If administered intravenously, pyrogens can lead to complications with life-threatening consequences within minutes. Therefore, all Pharmacopoeias demand the Rabbit Pyrogen Test (RPT) as standard animal test for pyrogens. This commonly accepted test was established over 50 years ago, and still requires an intensive use of animals. Great efforts during the last 15 years have led to animal-free alternative methods. One of these methods, the Monocyte Activation Test (MAT), uses human monocytes from different sources such as fresh blood, peripheral blood mononuclear cells (PBMCs), monocytic cell lines or cryopreserved human whole blood. The MAT in its diverse variants involves an overnight-incubation followed by detection of pro-inflammatory cytokines (mainly IL-1β, IL-6 or TNF-α) via ELISA. The MAT has been implemented in the European Pharmacopeia. This test provides reliable and reproducible results for many final products, but might be too time-consuming in several cases, e.g. for inline-production-testing. Confronted with the need for a faster and more sensitive test, a variant of the MAT was developed measuring intracellular accumulated cytokines in individual cells by flow cytometry. Here, monocytes in fresh human whole blood are chemically impaired regarding the export of cytokines, and then incubated with samples (e.g. lipopolysaccharide / LPS). The accumulated cytokines are detected intracellularly by fluorescent-labelled antibodies. This method is capable of detecting 6.25 pg/ml LPS within 2 hours with a strong potential to be further improved time- and sensitivity-wise.

I-1-346

Application of 454 pyrosequencing technology on the detection of adventitious agents in vaccines

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Next generation sequencing (NGS) technology, also known as "deep sequencing" is a novel cutting edge technology which has the capacity to discover and determine genetic sequences with high sensitivity. This technology may revolutionize the method for adventitious agents (AVA) detection, which is required for release of commercial products and clinical trial materials. Currently, animals are used in these *in vivo* tests to ensure that the products do not contain any AVA that might be harmful to humans. Each year, animals such as mice, rats, ferrets and guinea pigs are used for vaccine release. Yet, animal tests may not be sensitive enough to identify all AVA in the vaccine materials. A recent report (Victoria et al., 2010) demonstrated that adventitious agents, such as non-pathogenic porcine circovirus (PCV-1), were detected in commercial rotavirus vaccine materials by the deep sequencing technology. However, PCV-1 was not readily detected by the existing *in vivo* tests. Thus, genetic sequencing may be more sensitive than the animal test in AVA detection and this technology may eventually eliminate or substantially reduce the numbers of animals used in these tests. A proof of concept study was conducted to sequence live attenuated influenza vaccine (LAIV) by 454 pyrosequencing and the data will be presented to illustrate the application of 454 pyrosequencing on AVA detection.

Reference

Victoria, J. G. et al. (2010). *J. Virol. 84*, 6033-6040.

I-1-361

Comparison between rabbit pyrogen test (RPT) and human whole blood cytokine release assay (IPT)

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Pyrogenic contamination is the most important problem of injectable products. Since LAL has the disadvantage of detecting only endotoxins, the rabbit is still used, despite interferences. In 2010 the European Pharmacopoeia introduced the Monocyte Activation Test (MAT) as a third method to detect pyrogenic contamination. In the routine of quality control of injectable products, the National Institute of Quality Control in Health performs RPT in large volume parenterals (LVP), biologicals and medical devices. In order to start a comparison between RPT and IPT, some samples were tested by both methods. Thirty LVP and biologicals were tested. Twenty-six samples were non-pyrogenic in both methods while 5 samples needed to repeat the assay with new animals. One anti-rabies serum presented sum of 8 rabbits equal to 4.2°C being pyrogenic in both methods. One Ringer solution presented sum of 8 rabbits of 3.0°C, being negative in RPT and positive in IPT. Two anti-scorpion venom sera presented only one rabbit above 0.5°C and the sum of 8 rabbits was 1.9°C, being also negative in IPT. It shows that IPT is sensitive enough to detect pyrogenic contamination early while in case of RPT it is necessary to repeat the assay, using 8 rabbits at all. It can be also seen that, when the summed temperature is low enough to be considered pyrogenic, IPT showed it was not contaminated, while it was necessary to repeat the assay in case of RPT. These partial results strongly indicate that IPT may, in the future, replace RPT.

I-1-379

The re-use of rabbits in pyrogen testing (RPT) of hyperimmune sera and vaccines contributes to the reduction of animal use

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Rabbits are still used for determining pyrogenicity of samples in the quality control of injectable products, since up to now, there is not a method for replacing this method. In case of biological products it is not recommended to re-use rabbits, due to possible cross-reactions. The National Institute of Quality Control in Health (INCQS) analyzes between 200 and 300 injectable products and medical devices per year, around 50% of which are hyperimmune sera and vaccines. For assaying these products, 1200 rabbits are used per year. For this study, 5 IU/kg LPS-spiked and non-spiked Anti-Bothrops venom, Anti-Rabies and Anti-Tetanus sera and Anti-Miningitidis C vaccine were assayed. Five rabbits per group were used, following the 48 hours-interval administration schedule: I – only spiked sample; II – one non-spiked and one spiked; III – two non-spiked and one spiked; and IV – three non-spiked and one spiked. The result of the last injection of each group (spiked sample) was compared to response of the group I in order to verify if there was any influence of previous non-spiked injections. There was no statistical difference among the four spiked responses for any of the products assayed. It is possible to re-use a rabbit that received non-pyrogenic hyperimmune sera or vaccine up to four times in a one-week period without jeopardizing the animal's response. By re-using rabbits, it is possible to reduce the number of animals used in the Rabbit Pyrogen Assay by 70%.

I-1-443

Development of xMAP technology for the control of multicomponent vaccine bioactivity

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Vaccines are often composed of three, four, five or six different antigens, for example tetanus toxoid (T), diphtheria toxoid (D) and Bordetella pertussis (Bp) proteins. Usually, the vaccine bioactivity is determined using lethal or paralysis challenge on animals for tetanus, dermal or lethal challenge on guinea pigs for diphtheria and a serological method on mice for pertussis. For these antigens, serological methods on guinea pig are now available and referenced in the Ph. Eur. They are based on the antibody detection in serum from immunized animals using a single antigen ELISA. Recently a new technology has been developed: xMAP technology, a multiplex bead-based assay that can analyse simultaneously up to one hundred different analytes in the same sample.

In this context, the objective of our study is to develop this technology for the control of the vaccine bioactivity. The same animals will be used for the analysis of the three vaccine components' bioactivity. Consequently, the animal use will be reduced and refined. The guinea pigs were immunized with a tetravalent vaccine and blood sampled 35 days later. The antibody titers for tetanus, diphtheria and pertussis were determined in the serum using single antigen ELISA and xMAP technology.

Our first results show quite a good correlation between the different methods for the vaccine bioactivity analysis. Tetanus and Diphtheria vaccine titres have been determined and compared with the results obtained by challenge method in our laboratory. In conclusion, this method may be a good alternative method to replace the challenge method.

I-1-462

Identification and characterization of monoclonal antibodies for use in *S. pneumoniae* vaccine characterization assays

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The objective of Sanofi Pasteur's pneumococcal vaccine is to treat *Streptococcus pneumonia*, a leading cause of serious illness in children and adults throughout the world. Since the *S. pneumoniae* vaccine is aimed at inducing a protective antibody response, it is desirable that the potency of the vaccine can be evaluated with monoclonal antibodies (mAbs) that can recognize protective epitopes. To this end, a panel of mAbs against an *S. pneumoniae* protein was extensively screened as potential reagents for assay development. Multiple approaches have been utilized in characterizing these reagents including *in vivo* protection models, surface accessibility assays, Western blots, affinity binding and epitope specificity assessment by ForteBio Octet®. The data demonstrates a progression from the initial screening of mAbs in animal models to *in vitro* testing of the attributes of the applicable protein by using a selected panel of mAbs. The ultimate goal of the project is to effectively assess vaccine integrity and potency by monitoring availability of protective epitopes in *in vitro* assays and replace *in vivo* potency assays.

I-1-504

International validation and evaluation of an alternative to HIST for pertussis containing vaccines

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Pertussis vaccines are commonly used worldwide for the prevention of the disease pertussis (whooping cough). Currently a significant number of animals are routinely used in the lethal challenge histamine sensitisation test (HIST) for control of pertussis toxin presented in pertussis and pertussis based combination vaccines. Thus, there is an urgent need to develop an alternative to the HIST. A refined detection strategy of an *in vitro* test system has been developed to examine both the functional domains of PTx, based on a combination of enzyme coupled-HPLC and carbohydrate-binding assays. Preliminary in-house validation of the developed assay system with the *in vivo* HIST showed that by using a mathematical equation linking the multi-functions of carbohydrate binding and enzymatic activities, there is a good correlation between the *in vitro* and *in vivo* tests. The acceptance of this system as an alternative to the current *in vivo* HIST by regulatory authorities would depend on its transferability between laboratories. An international collaborative study between national control laboratories (NCLs) and manufacturers for method transfer as well as for further validation of this *in vitro* assay system has been carried out. Three types of products representing the major types of ACV products currently in the worldwide market are included in the study and this study involves a total of 17 laboratories from 9 countries including vaccine manufacturers and NCLs.

I-1-592

Implementation of the *In Vitro* Pyrogen Test (IPT) for replacement of the rabbit

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The *In Vitro* Pyrogen Test (IPT) was adopted into the European Pharmacopoeia in 2010 (EP 6.7, chapter 2.6.30, Monocyte Activation test MAT). With this final step, the IPT is a fully validated and, under the name of PyroDetect, commercially available replacement method for the rabbit pyrogen test. As it is now vital to implement the test for routine use and as a service to industry, our laboratory in Tübingen, Germany, will acquire good manufacturing practice (GMP), at the same time building on the already existing GLP system for hemocompatibility. With the creation of a GMP laboratory, a signal is sent to national and international parties to adopt the *in vitro* test instead of the former "gold standard" rabbit pyrogen test, which has been in use for injectables since the 1940's. All in all, the IPT has the capacity to save 200,000 rabbits each year in Europe alone and, unlike the animal test, it is suitable for a broad range of applications.

I-1-675

Characterisation of antigen adsorbed to aluminium salts

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Suspensions of aluminium salts are still the most used vaccine adjuvants. The antigen is adsorbed to the surface of the particles. This hampers characterisation and demonstration of batch comparability as well as effects between adsorbed and non-adsorbed antigen. Nevertheless there are physico-chemical techniques like spectroscopy and calorimetry that allow the characterisation of proteins adsorbed to solid phases. The development and use of physico-chemical techniques to characterise adsorbed antigens, notably diphtheria toxoid, will be presented. It is shown that adsorbed toxoid has a different conformation as compared to non-adsorbed antigen.

I-1-676

Pertussis toxin content of acellular vaccines assessed by *in vitro* cAMP responses

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Current safety testing for pertussis toxin (PT) content of acellular pertussis vaccine batches relies on the histamine sensitisation test (HIST). This *in vivo* test is based on the empirical finding that pertussis toxin sensitises mice for histamine. Studies into the mechanism of this test demonstrated that decreased contractile properties of resistance arteries and increase of blood pressure significantly contribute to PT induced histamine sensitisation. In addition, sensitisation was not limited to histamine but could also be induced by the vasodilating substance sodium nitroprusside. This knowledge was used to develop the functional *in vitro* cAMP assay. The assay is based on a rat vascular smooth muscle cell line and centres on the enzyme adenylate cyclase. This enzyme converts ATP into cAMP and its activity is regulated by specific $G_{inhibitory}$ and $G_{stimulatory}$ proteins.

The majority of the biological effects of PT are attributed to its ability to ADP-ribosylate Ginhibitory proteins. This leads to ineffective proteins unable to inhibit adenylate cyclase. Consequently, intracellular cAMP may increase, especially in the case that stimulatory proteins are active. In our assay, activation of $G_{stimulatory}$ proteins is realised by isoprenaline stimulation. Aim of the study was to determine the sensitivity and the specificity of the cAMP method for PT. Treatment of the cells with different amounts of PT resulted in a dose-dependent cAMP increase. In addition, treatment with other acellular vaccine components including filamentous hemaglutinin, pertactin, fimbriae, $AlPO_4$ and the components diphtheria toxoid, tetanus toxoid and inactivated polio virus did not significantly affect cAMP levels. Current activities focus on optimisation of the assay procedures and identifying and reducing sources of variation. Subsequently we aim for technology transfer. Our ultimate objective is to replace the *in vivo* HIST by a functional *in vitro* method.

I-1-698

The consistency approach for diphtheria and tetanus toxoid vaccines

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Tetanus and diphtheria toxoid vaccines are among the safest and most successful vaccines produced in decades. According to the requirements of the European Pharmacopoeia, the World Health Organization and the US Food and Drug Administration each individual lot of diphtheria and tetanus vaccine should be tested for potency in an animal model based on measuring the vaccine induced protection against a lethal challenge with the subsequent toxin or by bleeding the animals followed by an *in vitro* or *in vivo* antibody titration. In general the potency of both diphtheria and tetanus vaccine are far above the minimum protective level. Diphtheria and tetanus vaccine are produced worldwide and the annual animal usage for potency testing is extensive. In contrast with diphtheria and tetanus vaccine, HiB and HepB vaccines are relatively new vaccines that can ultimately be released on the basis of an *in vitro* characterization test after demonstration of consistency in production. For inactivated poliomyelitis vaccine the *in vivo* potency test can be waved after demonstration of the predictability of the D antigen ELISA.

At the NVI a comparable approach is explored for tetanus and diphtheria vaccine. For various lots of routinely produced D and T vaccines, in process quality indicators, batch release QC results and results of additional testing on intermediate and final products were collected. Based on the historical data of individual lots passing the *in vivo* potency test, pass and fail criteria for each parameter were set.

The results show that in a strict GMP setting and after demonstration of consistency in production the combined results during manufacturing (in process controls) and release testing are good quality indicators and safeguards for product consistency. It is concluded that diphtheria and tetanus vaccines produced by a manufacturer with a demonstrated consistency in production and using a panel of tests to obtain additional quality information, can safely be released on the basis of *in vitro* tests only.