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

Poster Session: Tuesday, September 14, 2010

Abstracts are ordered alphabetically by first author's last name

1 *In Vitro* Antigen ELISA for Quality Control of Combined Vaccine Products
L. Coombes, R. Tierney, D. Sesardic, et al.

2 The Consistency Approach for Quality Control of Vaccines: A Strategy to Improve Quality Control and Implement 3Rs —A Summary of the Recommendations of the ECVAM (European Centre for the Validation of Alternative Methods) and EPAA (European Partnership for Alternative Approaches to Animal Testing) Workshop (held in January 2010 in Brussels, Belgium)
F. De Mattia, J-M. Chapsal, J Descamps, et al.

3 Bridging the Gap Between Validation and Implementation: Replacing Animal Use in Vaccine Batch Potency Testing
S. Dozier, J. Brown, A. Currie

4 Summary of Recommendations of the ECVAM Workshop on Three Rs Approaches in the Production and Quality Control of Fish Vaccines
M. Halder, P. Midtlyng, C. Hendriksen, et al.

5 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
M. Hoefnagel, J. Vermeulen, R. Scheper, et al.

6 An *In Vitro* Biochemical Assay System Alternative to the *In Vivo* Histamine Sensitisation Test for Pertussis Vaccines
Y. Horiuchi, C.-T. Yuen, C. Asokanathan, et al.

7 Application of Proteomic and Spectral Counting Methods for the Characterisation of a Leptospira Vaccine
P. Humphryes, M. Weeks, W. Newell, et al.

8 Alternatives to the Mouse Histamine Sensitization Test: *In Vitro* Consistency Measures of Toxin in Acellular Pertussis Vaccines
R. Isbrucker, F. Cornel, B. Lorbetskie, et al.

9 Opportunities and Challenges for the Three Rs in Vaccine Quality Control:
A Canadian Case Study
M. Long, G. Griffin

1

In Vitro Antigen ELISA for Quality Control of Combined Vaccine Products

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For conventional vaccines containing diphtheria and tetanus components, *in vivo* potency assays are gold standard methods to confirm biological activity. Antigen and adjuvant are the major components contributing to vaccine potency, although their precise contribution to the measured potency of a vaccine is difficult to predict and will be influenced by other factors in addition to the amount of antigen and degree of adsorption. Additional factors likely to affect the measured potency include antigen purity, formulation excipients, and the interaction between one or more components in a combined vaccine product. Consistency of production is recognised as an important feature of vaccine quality control and antigen and adjuvant are critical factors that should be evaluated as part of *in vitro* consistency testing. We have developed a simple and sensitive enzyme-linked immunosorbent assay (ELISA) to quantify diphtheria and tetanus antigens in combined vaccine products and measure the degree of adsorption to adjuvant. This assay has been applied to various combined vaccine final products and is robust, specific, and highly sensitive, with a limit of quantification of approximately 0.001 limit of flocculation units/ml for both diphtheria and tetanus antigens. Compared to *in vivo* potency assays, *in vitro* assays are likely to be better suited for providing information on production consistency and batch-to-batch variation since these *in vitro* assays are often inherently less variable. In routine use as a consistency test for a pentavalent vaccine, the antigen ELISA has a geometric coefficient of variation (GCV) for total diphtheria antigen content of 12% compared to 39% for diphtheria potency (challenge assay, n=22) and a GCV of 7.3% for tetanus antigen content compared to 14.1% for potency (challenge assay, n=22). Whilst the antigen assay cannot be assumed to predict potency of a vaccine and is not a replacement potency test, it is an excellent tool for characterisation of final product vaccines, providing important information on batch-to-batch variation and allowing for more accurate monitoring of trends over time. For well-established vaccines, the antigen assay could be used as part of a panel of *in vitro* tests to provide a more informative product profile and is a key test that should be performed when reduction schemes are implemented for potency testing of diphtheria and tetanus vaccine components.

The Consistency Approach for Quality Control of Vaccines: A Strategy to Improve Quality Control and Implement 3Rs

A Summary of the Recommendations of the ECVAM (European Centre for the Validation of Alternative Methods) and EPAA (European Partnership for Alternative Approaches to Animal Testing) Workshop (held in January 2010 in Brussels, Belgium)

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Current batch (= lot/serial) release testing of established vaccines emphasizes quality control of the final product and is often characterized by extensive use of laboratory animals. The consistency approach is based upon thorough characterisation of the vaccine during development and the principle that the quality of subsequent batches is the consequence of the strict application of a quality system and of a consistent production of batches (typically based on Good Manufacturing Practice). The concept of consistency of production is state-of-the-art for new generation vaccines, where batch release is mainly based on non-animal methods. There is now the opportunity to introduce this approach into established vaccine production, where it has the potential to replace *in vivo* tests with analytical and/or *in vitro* tests designed to demonstrate batch quality. In this way, the highest quality, safety and efficacy standards could be maintained while allowing for a significant reduction in animal use.

The workshop conclusions and recommendations indicate how this approach may be further developed for application to established human and veterinary vaccines and emphasize the continuing need for regulatory coordination and harmonization of testing requirements, and underline the efforts to be undertaken in order to encourage acceptance and implementation of the consistency approach.

3

Bridging the Gap Between Validation and Implementation: Replacing Animal Use in Vaccine Batch Potency Testing

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PETA's Regulatory Testing Division specializes in collaborating with regulators and industry to promote the implementation of scientifically valid alternatives to animal-based tests, including vaccine potency testing methods. As technologically advanced high-throughput techniques are developed that replace, reduce or refine animal use, harmonization of validated protocols between international regulatory authorities is necessary to foster wide-reaching implementation. Even regulatory acceptance does not guarantee that an approved non-animal method will be adopted by manufacturers; therefore interfacing with industry to disseminate information regarding exemptions from *in vivo* regulatory standards is necessary to encourage the use of validated non-animal methods at the point of production. Company and government policies must be in place to take advantage of new scientific advances that reduce the use of animals for vaccine testing. Continued efforts are necessary to ensure that available waiver or exemption processes allowing for the use of replacements for animal-based tests are not inhibited by fees or a lack of regulatory oversight. By engaging with regulators and manufacturers, PETA helps promote the 3Rs approaches to vaccine batch potency testing. This multi-component promotion process is customized to the needs of each project and aims to confirm the acceptability of data from novel methods by regulatory authorities, to distribute information on available and accepted non-animal approaches via stakeholder alerts, to involve the press in publicizing accepted non-animal techniques, and to confirm manufacturer implementation of these methods. This poster will examine a detailed case study of PETA's approach to fostering regulatory and industrial integration of *in vitro* erysipelas vaccine batch potency methods. This poster will also provide examples of additional cases in which the organization was able to facilitate the implementation and distribution of protocols that allow for reduced animal use across multiple regulatory agencies and within the biologicals industry.

Summary of Recommendations of the ECVAM Workshop on Three Rs Approaches in the Production and Quality Control of Fish Vaccines

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Due to the increasing importance of fish vaccines in aqua culture and animal welfare concerns associated with fish vaccine quality control, the European Centre for Validation of Alternative Methods (Institute for Health and Consumer Protection, European Commission Joint Research Centre, Ispra, Italy) organised a workshop on Three Rs Approaches in the Production and Quality Control of Fish Vaccines in 2008. The workshop was attended by experts from academia, regulatory authorities, a scientific animal welfare organisation, and the fish vaccine industry. The main objectives of the workshop were (a) to identify animal tests currently stipulated for the production and quality control of fish vaccines and highlight animal welfare concerns associated with these tests; (b) to identify viable options to replace, reduce, and refine animal use for fish vaccine testing; and (c) to discuss the way forward and give recommendations how these options may be realised without lowering the vaccine quality. The participants in the workshop agreed that efforts should be undertaken to replace the vaccination-challenge batch potency testing with tests based on antigen quantification or antibody response. Regulatory requirements of questionable scientific value and relevance for the quality of fish vaccines as the re-testing of batches produced outside of Europe or the double-dose batch safety test should be reconsidered. As an immediate measure the design of the current animal tests should be evaluated and modified in the light of refinement and reduction, for example, the number of unprotected control fish in vaccination-challenge tests should be reduced to the minimum.

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5

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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Due to various limitations to *in vivo* vaccine efficacy assays, there is need for *in vitro* alternatives. They should preferably originate from the target species, i.e. humans. The efficacy of a vaccine is dependent on its ability to induce an immune response of the appropriate type and sufficient vigor. 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. Cell lines are preferred over *ex vivo* cells for reasons of reproducibility, accessibility, and safety. We therefore used the human cell line MUTZ-3 (1), the cell line that most closely resembles *ex vivo* human DC (2), 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 (3). Effects of PRP, OMP, conjugated PRP-OMP, and adjuvated vaccine (PedVax); on cytokine production and surface marker expression were established. 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, and of IL-6, IL-10, IL-12p40, IL-12p70, IL-23, and TNF-α in MoDC. In MUTZ-3 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.

References

(1) Masterson et al. 2002. Blood 100:701-703.

(2) van Helden et al. 2008. Immunol Lett 117:191-197.

(3) Heath. 1998. Pediatr Infect Dis J 17:S117-122.

6

An *In Vitro* Biochemical Assay System Alternative to the *In Vivo* Histamine Sensitisation Test for Pertussis Vaccines

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Pertussis vaccines are important for the prevention of the disease pertussis (whooping cough). The control of pertussis vaccines like other biological products, requires that they conform to specified standards of safety and efficacy. The histamine sensitisation test (HIST) is currently the official toxicity test for acellular pertussis containing combination vaccines. HIST is a lethal challenge procedure which requires a large number of animals due to large variations in test performance. There is an urgent need to develop alternatives to the HIST. Pertussis toxin (PTx) has the typical A-B type structure of many bacterial toxins, having an enzymatic A-monomer, the S-1 subunit and a binding B-oligomer of subunits S-2 through to S-5. An *in vitro* enzymatic high-pressure liquid chromatography coupled assay to measure the A-monomer and a carbohydrate binding assay to measure the B-oligomer activities were developed. Validation of the developed assay system with the *in vivo* HIST was carried out. Using multiple regression analysis, a mathematical equation linking with the *in vitro* multi-functions of carbohydrate binding and enzymatic activities has been identified for predicting the *in vivo* pertussis toxin activity in HIST. There is a clear correlation between the *in vivo* and *in vitro* results. However, the regression coefficients and constant factors were found to be product-specific, which indicates and emphasizes the importance of validation study for each product. An international collaborative study involving both the vaccine manufacturers and regulatory laboratories is planned to assess the methodology transferability and further validation of the assay system.

Statement: All animal use carried out in this study are in accordance with all applicable animal care and use Home Office regulations in the UK. It has been also approved by local ethical committees.

7

Application of Proteomic and Spectral Counting Methods for the Characterisation of a *Leptospira* Vaccine

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Vaccination is widely used to control the incidence of leptospirosis in companion and food-producing animals, which otherwise would cause substantial losses in the farming community. Annual vaccination is a requirement due to the short-lived efficacy of the current vaccines, and each batch of vaccine produced has to be assessed for potency prior to market release. The current potency test requires the use of a large number of hamsters. Whilst effective, a safer, cheaper, more ethical replacement is desired. The aim of this study was to determine the relative abundance of proteins in *Leptospira canicola* vaccines to aid development of an *in vitro* potency test.

Crude protein was purified from commercially available vaccines in triplicate, using a 5KDa molecular weight cut-off filter. The protein and lipopolysaccharide (LPS) content was assessed using the Bradford and *Limulus* amebocyte lysate assays respectively. Following heat denaturation (95^{o}C, 5 min), protein (100μg) was digested with trypsin (20 μg) and the resulting tryptic peptides separated using strong cation exchange chromatography. Fractionated peptides were then further separated on a reverse-phase high-pressure liquid chromatography chip and loaded directly onto an Agilent 6520 Q-TOF mass spectrometer for peptide mass sequencing. The resulting tryptic peptide ion mass spectra were searched against the *Leptospira copenhageni* proteome (NCBI), using Spectrum Mill, enabling accurate protein identification. The normalised spectrum abundance factor (1) which normalises based on protein length, was calculated for each identified protein using a novel automated program written in R.

Leptospira proteins were detected in vaccines A-D (86 ± 21, 31 ± 4, 19 ± 4 and 30 ± 13 proteins [mean ± 1SD] found respectively). Five proteins were found to be common to each vaccine: Lipoprotein 1, Lipoprotein 2, Flagellin Protein, Cell Wall Hydrolase and a Hypothetical Protein. Of these, the relative abundance of Lipoprotein 1 (determined using the normalized spectral abundance factor) was found to be significantly higher ($p \leq 0.01$) in a batch of vaccine that had passed the *in vivo* potency test, compared to one that had failed.

Based on these findings, we suggest that Lipoprotein 1 is a good potential target upon which to base an *in vitro* potency test. Further we have shown 2-dimensional liquid chromatography/mass spectrometry to be an effective and reliable tool for vaccine analysis which could be utilised for a multitude of other applications. Additional work is necessary to determine the precise amount of Lipoprotein 1 required to confer protective immunity in the host, so that a quantitative *in vitro* test can be developed.

This research was supported by funding from the National Center for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs, UK).

References

(1) Zybailov et al. 2007. Mol Biosyst 3:354-360.

8

Alternatives to the Mouse Histamine Sensitization Test: *In Vitro* Consistency Measures of Toxin in Acellular Pertussis Vaccines

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The mouse histamine sensitization test (HIST) is the currently accepted safety assay for the detection of residual pertussis toxin (PTx) activity in acellular pertussis vaccines. Depending on the protocol used, the HIST is either a lethal endpoint assay or monitors change in body temperature. Although the latter protocol is a humane refinement for HIST, the assay remains technically challenging, has a high false-positive outcome, and both methods require the use of a substantial number of animals in the quality control process for vaccine lot release (1).

Over the past decade a series of *in vitro* assays have been developed which monitor the enzymatic (2) and binding (3) activities of PTx, both of which are required for toxicity. Enzymatic activity is measured using an enzyme-coupled high-pressure liquid chromatography (eHPLC) assay which detects the ADP-ribosylation of a fluorescent substrate. As pertussis toxoid (PTd) generated by a formaldehyde/glutaraldehyde toxoiding process retains a substantial level of ADP-ribosylating activity, this method is insufficient on its own to monitor for residual PTx in vaccines. The binding function of PTx can be measured using an enzyme-linked immunosorbent assay (ELISA) which uses the glycoprotein fetuin to capture the toxin in a microwell plate. Although PTx has a greater binding affinity for fetuin than the pertussis toxoid (PTd), it is insufficient to differentiate between the two proteins in vaccine preparations (4). A modified ELISA has been developed which uses a monoclonal antibody that increases the sensitivity and specificity to PTx with little interference by PTd or other vaccine components. This modified ELISA has a limit of quantitation of 0.5 – 2 ng PTx/ml vaccine, a concentration below what is understood to induce a positive HIST effect.

Although these 2 biochemical assays, along with a biological assay such as CHO cell agglutination, do not measure vaccine safety *per se*, they could provide a series of highly sensitive and quantitative measures to establish additional vaccine specifications and monitor for consistency of vaccine manufacture. Health Canada is currently evaluating the use of these assays as a potential alternative to HIST for those acellular pertussis vaccines which have an established history of safe use.

References

(1) Corbel and Xing. 2004. Expert Rev Vaccines 3:89-101.
(2) Cyr et al. 2001. Biologicals 29:81-95.
(3) Gomez et al. 2006. Anal Biochem. 356:244-253.
(4) Isbrucker et al. 2010. Vaccine 28:2687-2692.

9

Opportunities and Challenges for the Three Rs in Vaccine Quality Control: A Canadian Case Study

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The principles of the Three Rs are acknowledged worldwide as being important for the ethical use of animals in science. As Canada's organization for setting and maintaining standards for the ethical use and care of animals in science, the Canadian Council on Animal Care (CCAC) requires that the Three Rs be considered by Study Directors and researchers when designing their studies, and that Three Rs alternatives be used whenever practicable (1). In the last 20 years, Canada has adopted several reduction and refinement measures for quality control testing and lot release of vaccines (2), but large numbers of animals continue to be used. A case study was performed to identify factors which affect the implementation of the Three Rs in Canada.

Data was obtained through semi-structured interviews performed with stakeholders involved in the production, testing and evaluation of vaccines. Participants were probed on their knowledge of Three Rs methods, the role of *in vitro* data in testing, motivation for test development, and international factors which may affect Canadian vaccine policies. The results show that factors providing opportunities for Three Rs method implementation include high reliability and reproducibility of *in vitro* assays in comparison with highly variable animal methods; interest by both industry and regulators to reduce animal use; and initiatives taken by industry to harmonize methods across countries. Obstacles to the implementation of Three Rs methods include societal expectation to protect the safety of infants and children; the difficulty in validating *in vitro* and cell-based methods against variable and poorly understood *in vivo* methods; and conflicting regulatory requirements between countries.

Our results suggest that both industry and the Canadian government are open to implementing Three Rs alternatives for vaccine quality control, but that the methods adopted must be proven to be reliable and biologically relevant. International collaboration and harmonization of regulatory requirements will be key in furthering the implementation of Three Rs alternatives. Future activities will explore how implementation factors compare between Canada and Europe. Understanding the drivers behind European successes may provide guidance as to how the adoption of Three Rs methods may be further facilitated in Canada.

Prior to initiation of this study, all materials and protocols received ethical review and approval from Institutional Review Board Services.

References

(1) CCAC. 1989. CCAC Policy Statement on: Ethics of Animal Investigation. Ottawa, Canada: CCAC.
(2) Calver. 2002. Dev Biol (Basel) 111: 207-212.

10

Alternative *In Vitro* Methods for Detection of Pertussis Toxin in Component Pertussis Vaccines

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Regulatory authorities require that routine safety testing be performed on component pertussis containing combination vaccines for residual pertussis toxin (PTx) that may be present either due to inadequate detoxification of the PTx or due to reversion of the pertussis toxoid to its native toxin form. Histamine sensitization tests in mice are currently performed for safety testing. The animal assays are problematic due to the high variability, ethical concerns of using large numbers of animals and issues with non-specificity.

Alternate *in vitro* methods that measure the binding and enzymatic activities of PTx have been proposed as replacement tests for the histamine animal assays (1,2). Based on these methods, Sanofi Pasteur Canada has developed and validated an enzyme-linked immunosorbent assay (ELISA) method that measures the carbohydrate-binding activity of the B-oligomer of PTx and an high-pressure liquid chromatography (HPLC) method that measures the enzymatic activity of the A-protomer in an HCPDT-IPV-PRP-T vaccine. The ELISA was validated as a limit test and test parameters assessed during validation were level of detection (LOD), level of quantitation (LOQ), specificity and robustness. The ELISA method was shown to be specific for detection of PTx. The LOD was determined to be 0.004 μg/mL and the LOQ was determined to be 0.008 μg/mL. The assay was found to be robust with respect to small changes in incubations times of samples and reagents and other conditions. The published, multi-step gradient elution HPLC method was converted to an isocratic separation employing a temperature controlled, solvent-saver octadecyl silane column, which resulted in enhanced peak resolution, specificity and 70% lower acetonitrile consumption. This isocratic, column thermostated procedure lends itself to greater robustness during method transfer and routine operation because significant sources of variation are eliminated, such as gradient delay volume, on-line solvent mixing inaccuracy and fluctuation in ambient temperature. The method was validated as a quantitative assay for measurement of ADP-ribosyltransferase activity. The validation parameters tested were system suitability, specificity, accuracy, linearity, range, LOQ, repeatability, intermediate precision, robustness and stability of test solutions. An $r^2 = 1.00$ was obtained for the linear regression plot of analyte area response versus PTx spiking concentration in a mock vaccine sample from 1 μg/mL (LOQ) to 30 μg/mL. CVs $\leq$ 7% and $\leq$ 8% were obtained for repeatability and intermediate precision. These methods may be applied as an *in vitro* test system to replace the animal histamine sensitization (HIST) assay for batch release of acellular pertussis combination vaccines. Correlation studies with the HIST assay are currently under investigation.

Animal Care Statement: All studies involving the care and use of animals were pre-approved by the Sanofi Pasteur Canada Animal Care Committee and conducted under the approved Animal Use Protocol in accordance with the Ontario Animals for Research Act, the guidelines of the Canadian Council on Animal Care and the Sanofi-Aventis Charter on the Humane Care and Use of Laboratory Animals.

References

(1) Gomez et al. 2006. Anal Biochem 356:244-253.
(2) Yuen et al. 2002. Vaccine 21:44-52.

11

Serological Assay for Diphtheria and Tetanus Potency in DTaP-IPV-Hib Vaccine

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Introduction: Sanofi Pasteur Limited has developed and implemented, in collaboration with the National Institute for Biological Standards and Control (U.K.) and Health Canada, a serological assay for batch release potency testing of diphtheria and tetanus toxoids in DTaP-IPV-Hib pediatric vaccine formulation as per the European Pharmacopoeia requirements to replace the lethal challenge assays.

Materials and Methods: Guinea pigs were immunized with multiple doses of either an in-house homologous reference vaccine or a production lot. The resulting sera were tested in validated ELISA methods for antibody to diphtheria and tetanus toxoids. Results were entered into a multidose parallel-line model calculation program or a *t*-test based single dose potency calculation program to obtain a potency estimate.

Results: Six routinely manufactured lots of DTaP-IPV-Hib combination vaccine were included in the verification studies. A three-dilution (1/1.67, 1/5 and 1/15) serological assay for diphtheria and tetanus potency provided similar results to the lethal challenge assay and showed good linearity and parallelism. A single-dose potency assay was shown to be suitable for tetanus potency determination as an alternative to the multidose assay.

Conclusion: The serological assay allows for the use of the same group of animals for testing the potency of both diphtheria and tetanus components, thereby significantly reducing the number of animals required for testing and resulting in a more humane endpoint. Additional benefits of this assay include reduction in the invalidity rate, reduction in cycle time, and significant cost savings.

Animal Care Statement: All studies involving the care and use of animals were pre-approved by the Sanofi Pasteur Ltd. Animal Care Committee and conducted under the approved Animal Use Protocol in accordance with the Ontario Animals for Research Act, the guidelines of the Canadian Council on Animal Care, and the Sanofi-Aventis Charter on the Humane Care and Use of Laboratory Animals.

12

An *In Vitro* Neutralization Assay for PlyD1, a Detoxified Pneumolysin Derivative From *Streptococcus pneumoniae* as an Alternative to the *In Vivo* Challenge Model

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Pneumolysin (PLY) from *Streptococcus pneumoniae* is an important virulence factor that causes a variety of toxic effects including cell lysis and induction of inflammation. Due to its toxicity, a highly detoxified PLY variant PlyD1 was generated as a potential candidate for new pneumococcal vaccines. During preclinical testing, a murine *in vivo* PLY IN challenge model was used following active immunization of mice with PlyD1 in order to demonstrate the protection potential of the candidate vaccine. Although this model was successful in demonstrating a functional immune response against PlyD1, the use of a mouse challenge model was a poor choice for a potency test, due to the inherent variability of the animal model. In addition, in an effort to move away animal testing an alternative *in vitro* neutralization assay was developed. This assay measures the inhibition of hemolysis induced by PLY in the presence of red blood cells. In order to ensure that the assay can replace the *in vivo* challenge model, bridging studies were conducted in which neutralizing and non-neutralizing monoclonal antibodies were evaluated in both *in vivo* and *in vitro* assays. Results have shown that anti-PLY neutralizing monoclonal antibodies that were able to inhibit tissue damage caused by PLY *in vivo* were also able to inhibit lysis of red blood cells *in vitro*, while non-neutralizing monoclonal antibodies had no effect on PLY-induced toxicity *in vivo* and were unable to inhibit hemolysis *in vitro*. Thus, the *in vitro* neutralization assay can be considered as a functional antibody assay that can be further developed into a potency assay for product release.

Animal Care Statement: All animal use was carried out in accordance with all applicable animal care and use laws, regulations, and guidelines, and that the appropriate Institutional Animal Care and Use Committee approved the studies.

13

Factors Influencing the Acceptance and Use of 3R Models for Marketing Authorization and Quality Control of Rabies Vaccines

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Vaccines, like all medicines, are subject to statutory requirements that regulate their production, quality, and use. However, due to their biological origin, vaccines unlike pharmaceuticals are highly complex products and various factors can influence their production. As a result, each batch of the finished product is tested on potency and safety standards. The use of animals in batch release testing is a regulatory obligation and represents around 80% of the total number of animals used in the vaccine industry (1). Over the last decades this heavy reliance on animal experimentation has met serious objections that are ethical, scientific, and economical in nature. The European directive 86/609/EEC, which regulates the protection of animals used for experimental and other scientific purposes, applies a 'no unless' principle and stipulates that alternatives, if available, should be used. In addition, the requirements dealing with the registration and release of vaccines often leave room for authorizing bodies and manufacturers to use the testing method they perceive as most suitable, provided that the method is scientifically sound. Nevertheless, the acceptance of alternative methods for regulatory purposes proves to be a difficult process. This raises the question how regulators and vaccine manufacturers use the discretionary space and which factors influence the acceptance and use of 3R models for regulatory purposes. This PhD research aims at clarifying this process and at defining the main obstacles and drivers to optimize the process of acceptance and use of 3R models for regulatory purposes.

For this purpose several case studies will be conducted. The first case study will examine the barriers and drivers in the area of marketing authorization (MA) and quality control (QC) of vaccines in general and more specifically of rabies vaccines. Special attention will be given to the regulatory acceptance and use of 3R methods to replace the NIH potency test. The case study will consist of literature research and in-depth interviews with representatives from licensing authorities, standardization bodies and vaccine manufacturers, and other relevant stakeholders like animal welfare organisations.

The preliminary findings of this case study and empirical findings of a previous study (2,3) are combined with several theoretical perspectives on technology acceptance and risk regulation. This will result in a technology acceptance model to clarify the acceptance and use of 3R models in this field. The preliminary model presented here consists of several categories of scientific, social and political factors that play a role in the slow acceptance of 3R models for regulatory purposes.

References

(1) European Vaccine Manufacturers. 2008. Animal welfare and vaccine development. Available: http://www.evm-vaccines.org/pdfs/vaccines_and_animal_welfare_fin.pdf.

(2) Schiffelers et al. 2005. Regulatory Animal Testing, Report number: P-UB-2005-10, August 2005.

(3) Schiffelers et al. 2007. Factors stimulating or obstructing the implementation of the 3Rs in the regulatory process. ALTEX 24, 4/07.

14

In Vitro Alternative Test for Residual Toxin Activity and Irreversibility of Tetanus Toxoid: Estimation of Synaptobrevin II Cleavage

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In the frame of the 3Rs concept, many initiatives have been set up to try to replace animal tests, especially safety tests used for the vaccine release control. Tetanus vaccines are prepared from detoxified tetanus neurotoxin (toxoid) and therefore are subject to strict safety control for each individual batch in order to exclude residual toxicity. The safety determination must be performed in compliance with the European Pharmacopoeia. According to the monograph "Tetanus Vaccines for Human Use," the "absence of toxin and irreversibility of toxoid" safety tests have to be performed by injecting the material into guinea pigs, and by the subsequent observation of the injected animals for symptoms of tetanus over a period of 3 weeks.

An *in vitro* alternative method has been developed based on an *in vitro* endopeptidase assay for the detection of active tetanus toxin in toxoid samples. The detection of this activity can be done by several approaches such as quenching of fluorescence (1), western blot (2), and fluorescence (3). Sanofi Pasteur has selected the fluorescence approach combined to high-pressure liquid chromatography (HPLC) and based on the digestion of synaptobrevin-II, a synthetic peptide derived from the sequence of the natural substrate synaptobrevin. If the tested samples contain any active toxin, cleavage of the peptide occurs and is monitored by HPLC, which separates the cleaved fragment from the entire peptide. This *in vitro* assay could be useful for the detection of either insufficiently inactivated or reversed toxoid preparations, and therefore might represent an alternative to the animal toxicity tests.

Our results indicate that tetanus toxoid batches, even in reducing conditions, do not present any endopeptidase activity. Besides, this method displays both high specificity and sensitivity (detection limit ≤ *in vivo* test), a good reproducibility ease of performance, cost-efficiency, and short testing time, along with absence of additional reaction step (4,5,6)

References

(1) Anne et al. 2001. Anal Biochem 291:253-61.
(2) Kegel et al. 2002. Dev Biol (Basel) 111:27-33.
(3) Perpetuo et al. 2002. Biotechnol Appl Biochem. 36:155-61.
(4) Behrensdorf-Nicol et al. 2008. Vaccine 26:3835-41.
(5) Behrensdorf-Nicol et al. 2010. Toxicol In Vitro 24:988-94.
(6) Kegel et al. 2007. Toxicol In Vitro 21:1641-9.

15

Towards a Mechanism-Based Safety Test for Acellular Pertussis Vaccines

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Pertussis toxin (PTx) is the major virulence factor of *Bordetella pertussis* and in its detoxified form is an essential component of acellular pertussis vaccines. Detoxification of PTx is critical since active PTx can cause severe harmful effects. Therefore, regulatory authorities require safety testing to ensure the (virtual) absence of residual toxicity. The histamine sensitization test (HIST) is currently the standard method for evaluating residual PTx activity and is required for batch release testing of pertussis vaccines. This test, however, has major disadvantages of being highly variable and requiring large numbers of animals with (percentage) lethality as read-out. Moreover, the exact mechanisms underlying the test are largely unknown, making its clinical relevance doubtful. Together, these issues imply that replacement of the HIST is a high priority.

Several efforts to develop alternative assays to detect residual PTx activity have been undertaken, but so far without successfully replacing the HIST. The aim of our project is to develop a novel cell-based assay to detect residual PTx activity in vaccines. The assay should reflect the clinically relevant effects of PTx. Furthermore, the assay should be specific, sensitive, and robust for batch-to-batch analysis.

PTx affects cells of different origin, including pancreatic cells (Islets of Langerhans), smooth muscle cells, barrier cells (epithelial and endothelial cells), and a variety of immune cells including neutrophils, macrophages, dendritic cells (DC), and T-cells. The mechanisms of action can be divided into two main pathways: ADP-ribosylation of G_i and G_0 proteins abrogating signaling through G-coupled receptors, and direct receptor-mediated signaling probably involving Toll-like receptor 4, T-cell receptors and possibly other receptors. So far, the development of novel assays has focused on the first pathway, ADP-ribosylation, but it is unclear whether this pathway is the only, or most significant, one in the observed clinical effects of PTx.

We will examine the complete panel of PTx effects using a microarray-based approach. For this purpose, two human cell lines will be exposed to PTx: EA.hy926, a hybrid cell line of human umbilical vein endothelial cells (HUVEC) and A549 airway epithelial cells, and MUTZ-3, a cell line of DC precursors. Both HUVEC and DC have been implicated in the pathological effects of PTx. The first array experiment is due in September 2010.