Alternatives to HIST for acellular pertussis vaccines: progress and challenges in replacement

Report on an international workshop

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ABSTRACT

The 'International Workshop on Alternatives to the Murine Histamine Sensitization Test for Acellular Pertussis Vaccines: Progress and Challenges in the Replacement of HIST' was held on 24 August 2014, in Prague, Czech Republic, as a satellite meeting to the 9th World Congress on Alternatives and Animal Use in the Life Sciences. Participants discussed the progress and challenges associated with the development, validation, and implementation of in vitro assays as replacements for the histamine sensitisation test (HIST) for acellular pertussis vaccines. Discussions focused on the consistency approach, the necessary framework for regulatory acceptance of a harmonised method, and recent international efforts towards the development of in vitro assays to replace the HIST. Workshop participants agreed that acceptable alternatives to the HIST should be based on ADP ribosylation-mediated cell intoxication and therefore that the CHO cell clustering assay, which measures cell intoxication, should be further pursued and developed as a possible replacement for the HIST. Participants also agreed to continue ongoing multinational discussions involving national and international standardisation authorities to reach consensus and to organise collaborative studies in this context for assay characterisation and calibration of reference materials.

KEYWORDS

Pertussis vaccines, histamine sensitisation test, replacement alternative.

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1. INTRODUCTION

Pertussis, commonly known as whooping cough, is a respiratory disease caused by the bacterium *Bordetella pertussis*. The classical presentation of pertussis is characterised by a prolonged paroxysmal cough accompanied by an inspiratory whoop, although the syndrome following the infection varies with age and history of previous exposure or vaccination. Severe symptoms are infrequent in healthy vaccinated persons. Infants, particularly those who have not received the recommended primary vaccination series, are most at risk from complications and in rare cases, death. Pneumonia is the most common complication in all age groups [1].

In the pre-vaccine era, pertussis was a common childhood disease and a major cause of child and infant mortality. Routine childhood vaccination led to a reduction in disease incidence and mortality. For example, the United States disease incidence dropped from an average of 150 reported cases per 100 000 persons between 1922 and 1940, to 0.5 per 100 000 in 1976. However, reported pertussis cases began increasing in the 1980s. While the reasons for this increase are not fully understood, multiple factors may be contributing to the upturn, including waning immunity from childhood pertussis vaccines, enhanced recognition of the disease, mismatch between vaccine strain and circulating strain, better diagnostic testing, and improved reporting [1,2,3]. The World Health Organization (WHO) estimated that, in 2008, about 16 million cases of pertussis occurred worldwide, 95 % of which occurred in developing countries, and 195 000 children died from the disease [4]. Therefore, pertussis continues to be a public health concern.

Two types of pertussis vaccine are available worldwide: whole-cell (wP) vaccines based on killed *B. pertussis* organisms, and acellular pertussis (aP) vaccines. Rather than whole inactivated cells, aP vaccines contain one or more purified pertussis antigens, always including chemically-inactivated pertussis toxin (PTxd), plus filamentous haemagglutinin, pertactin, or fimbria type 2 and type 3. The first aP vaccines were used in Japan in 1981, and aP vaccines have gradually become the dominant type in the industrialised world.

Differences among aP vaccines include the number and quantity of antigen components per dose, the bacterial strain used for primary antigen production, methods of purification and detoxification, incorporated adjuvants, and the use of preservatives [4]. The exact contribution of the different aP antigens to protection is not clear, but all vaccines currently on the market contain PTxd. Regulators worldwide require manufacturers to test each aP vaccine lot to ensure both sufficient chemical inactivation of pertussis toxin (PTx) in the manufacture of PTxd and that PTxd has not reverted back to the active agent.

The standard test for confirming inactivation of PTx in aP vaccines is the histamine sensitisation test (HIST), an *in vivo* lethal challenge assay. The HIST is technically demanding, requires many animals (approximately 60 mice per test), and can cause significant unrelieved pain and distress to the test animals. Therefore, there is widespread interest among vaccine manufacturers and support by regulatory authorities for identification of a valid non-animal alternative to the HIST. Although the recent marked increase in reported pertussis cases in the United States and other countries has stimulated interest in the development of new or improved pertussis vaccines, development and eventual approval of new vaccines presents many challenges [5]. Therefore, the need for testing the residual activity of PTx in aP vaccines will continue into the foreseeable future, and the necessity for a non-animal alternative for the HIST remains a priority in the context of broader efforts to replace, reduce, and refine animal use in research and testing.

With this goal in mind, an international group of experts convened in Prague, Czech Republic, on 24 August 2014, to discuss progress and challenges associated with the development, validation, and implementation of alternatives to HIST. The ‘International Workshop on Alternatives to the Murine Histamine Sensitization Test for Acellular Pertussis Vaccines: Progress and Challenges in the Replacement of HIST’ was convened as a satellite meeting of the 9th World Congress on Alternatives and Animal Use in the Life Sciences.

The workshop objectives were to:

- Discuss the implementation of *in vitro* assays as replacements for the HIST for aP vaccines on the basis of the consistency approach (i.e. the efficient functioning of a Quality System since the product licensing, involving process and testing validation. This approach ensures that the licensed manufacturer produces batches of vaccine that are consistent with those that fulfilled the criteria for quality, safety, and efficacy defined in the marketing authorisation):
  - For licensed/registered products: discuss the importance of the relevance of an in vitro assay to replace the HIST from the product profile of an aP vaccine.
  - For new products: discuss the requirements that need to be met to accept one or more in vitro assays as replacements for the HIST in the product profile for licensing/registration.
- Discuss the necessary framework for regulatory acceptance of a harmonised approach that uses *in vitro* assays instead of the HIST.
- Discuss recent international efforts towards the development of *in vitro* assays to replace the HIST.

2. PARTICIPANTS

This workshop was organised by the International Working Group for Alternatives to the HIST, a consortium of interested stakeholders representing government, industry, and research and regulatory institutions. The authors would also like to acknowledge the input of Ute Anna Rosskopf (WHO), for her help in the design of the programme and the workshop attendees, who, together with the members of the Organising Committee, contributed to a comprehensive and thoughtful discussion.

3. WORKSHOP PRESENTATIONS

3.1. Prague, pertussis, and vaccines

Dr Peter Sebo, Institute of Microbiology of the ASCR, provided a historical perspective of vaccines used in Prague and the surrounding region, noting in particular that Czechoslovakia was the first country to eradicate polio in 1961. Pertussis was a very serious disease in Czechoslovakia in the pre-vaccine era, responsible for 96 % of all deaths of infants under 3 years of age in the period 1949–1957. Manufacturing of wP vaccines started in Czechoslovakia in 1958, and use of wP vaccines kept the disease under control for many years. However, perhaps due to deterioration in the quality of the wP vaccine, pertussis incidence started to increase in the Czech Republic, beginning around 1994. A further increase in pertussis incidence coincided with the adoption of aP vaccines in the country. Recent findings show that aP vaccination does not protect against *B. pertussis* colonisation in a non-human primate model, and that infected non-human primates vaccinated with aP vaccines can transmit pertussis to naive contacts [6]. This led Dr Sebo to recommend the development of either aP vaccines more effective in preventing colonisation or less reactogenic wP vaccines, which are considered more effective in preventing colonisation. Addition of other antigens such as adenylate cyclase toxin to current aP formulations may improve their efficacy.

Dr Roman Prymula, University Hospital Hradec Kralove, discussed the Czech vaccination calendar and pertussis. Czech children are required to receive six doses of pertussis vaccine, the first four as part of a hexavalent combination (DTaP-IPV-HB-Hib) vaccine. Doses of pertussis vaccine are administered at 9 weeks; 3, 4, and 18 months; and 5–6 and 10–11 years of age. The booster at 10–11 years of age with TdaP-IPV combination was added to the protocol in 2009, after 2008 epidemiological data indicated that the highest disease incidence was in adolescents (10–14 years of age). Protocols for pertussis vaccination vary among countries in the European Union, both in the non-pertussis composition of the vaccines used and in vaccination schedules. Vaccine coverage in the Czech Republic is very high (≈ 98 %), but an

increase in pertussis cases occurred beginning around the year 2000. The current pertussis incidence in the Czech Republic is approximately 12 cases per 100 000, relative to the lowest incidence of 0.5 cases per 100 000 in 1993. Mortality has been low throughout this period, but a slight increase appears to coincide with the shift to using aP (≈ 70 % cases after 2007). This increase demands the adoption of new strategies that may include either the development of more effective aP vaccines or the use of alternative immunisation schedules, perhaps alternating doses of aP and wP vaccines.

3.2. The road to Prague 2014

Dr Richard Isbrucker, Health Canada, provided a synopsis of the efforts that led up to the Prague workshop.

In September 2010, the US National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), the US Interagency Coordinating Committee on the Validation of Alternative Methods, and the European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM) sponsored an 'International Workshop on Alternative Methods to Reduce, Refine, and Replace the Use of Animals in Vaccine Potency and Safety Testing' at the National Institutes of Health in Bethesda, Maryland, USA. Among the topics discussed at this workshop were pertussis vaccines and the HIST. Because potential *in vitro* replacement alternatives for the HIST were already well into the development and validation stages, HIST was identified as a priority for developing a replacement [7].

In June 2011 experts convened in Langen, Germany for a 'Workshop on Animal-Free Detection of Pertussis Toxin in Vaccines – Alternatives to HIST'. This workshop, co-sponsored by the Paul Ehrlich Institute, the Netherlands Vaccine Institute and the EDQM, brought together experts working on alternatives to the HIST to compare data from the different *in vitro* protocols under development and to define criteria that alternative methods should meet for regulatory acceptance [8]. However, comparison of assay results between laboratories was not possible due to the differences in the PTx preparations used and in the assay outcomes. Therefore, among the consequences of this workshop was the establishment of an International Working Group on Alternatives to HIST comprised of participants from government, industry and academia. This working group was tasked with establishing a framework for the assessment of alternative methods, identifying a common source of PTx for all laboratories to use, and defining a set of aP vaccines and a protocol for spiking the vaccines with PTx. Creation of the working group also ensured continued discussions among the interested parties.

A satellite meeting to the 8th World Congress on Alternatives and Animal Use was convened in Montreal, Canada, in August 2011 [9]. This meeting further clarified what regulatory authorities might require from the *in vitro* assays intended as alternatives to the HIST and addressed requirements for assay validation and comparability studies. This meeting also provided an opportunity for the working group to meet face-to-face and start organising a collaborative study.

The first collaborative study, conducted in 2012, compared the performance of available *in vitro* cell-based and biochemical methods among multiple laboratories. Twelve laboratories from different countries tested 7 vaccines from 3 different manufacturers (GlaxoSmithKline, Sanofi Pasteur, and Statens Serum Institute). Participating laboratories were provided with a common set of aP vaccines, a PTx reference preparation, a protocol for adding to the vaccines known quantities of PTx (spiking), and desorption methods. As vaccines that fail the HIST are not readily available, spiking with PTx was used as a model to mimic failed batches. Participating laboratories were asked to test the spiked vaccines using their in-house method. The selected vaccines contained different non-pertussis antigens, adjuvants, and PTx detoxified using several chemical procedures. EDQM provided the PTx to each laboratory, and also served as the project co-ordinator to facilitate sample collection and distribution.

Data from Phase 1 of the collaborative study were reviewed during the 'International Workshop on Alternatives to the Murine Histamine Sensitisation Test (HIST) for Acellular Pertussis Vaccines: State of the Science and the Way Forward' in Bethesda, Maryland, in 2012 [10]. After viewing a comprehensive review of the *in vitro* protocols and data generated, participants recommended a second phase of the international collaborative study to assess the use of

the Chinese hamster ovary (CHO) cell clustering method both for calibrating PTx preparations and evaluating modified protocols for testing spiked vaccine samples. As a cell-based assay, the CHO cell assay was considered optimal because it could adequately measure the activity of the entire toxin (i.e. binding, internalisation, and ADP ribosylation of Gi coupled receptors). Phase 2 of the collaborative study was planned to commence in the fall of 2014, with results to be reported during a subsequent workshop in 2015.

3.3. The murine histamine sensitisation test

Dr Blaise Descampe, GlaxoSmithKline, provided a summary of the current HIST protocol used for regulation of aP vaccines.

The HIST was introduced into the Japanese Pharmacopoeia in 1981 (in an alternative version that uses a non-lethal endpoint), and subsequently into the U.S. licensure (1991) and the European Pharmacopoeia (1999). Historically, the test was standardised to detect the histamine sensitising activity present in wP vaccines. The HIST is based on the principle that mice are naturally resistant to the toxic action of histamine but become sensitive to histamine toxicity after injection with *B. pertussis*, which decreases the LD_{50} for histamine up to 300-fold [11–14].

In the current HIST protocol, groups of mice are injected concurrently with a range of PTx doses and the vaccine being tested. Four to five days later, the animals are challenged with histamine. The PTx dilution series is used to determine that animals injected with the test vaccine are adequately sensitive to PTx within historical limits, which are defined by the median histamine dose (HSD_{50}: the dose that sensitises 50 % of a group of mice to histamine-induced lethality).

The primary pathophysiological mechanisms involved in the HIST are PTx cell intoxication and the ribosylation of G proteins by ADP. $G_{\alpha i1}$ and $G_{\alpha i3}$ proteins are considered the critical *in vivo* targets [15]. PTx is an A/B toxin (an active enzymatic protomer and a binding oligomer). The A subunit causes ADP ribosylation of the G proteins in vascular smooth muscle cells and inhibits compensatory mechanisms [16]. B subunits bind to glycoconjugate molecules on the target cell surface and affect cell signaling. Together, these steps result in vasodilation and hypovolaemic shock that cause death [17–18].

The HIST has been used for a variety of purposes in aP vaccine development and manufacturing including in-process testing of detoxified PTx bulk for residual PTx activity, testing of final formulated product for residual PTx activity and reversion to toxicity, for lot release and stability testing purposes, and characterising changes in production process.

One of the challenges in developing a non-animal alternative to the HIST is the fact that multiple versions of the assay are used. The existence of multiple versions of the assay confounds defining the reference test for validation of an alternative, since different manufacturers and/or regulatory authorities may use and approve different versions of the test. The three primary versions of the HIST are defined by the measured endpoint: (1) lethal endpoint limit test, (2) temperature drop endpoint limit test and (3) temperature drop-endpoint quantitative test. While the temperature-based versions of the HIST offer the possibility of a quantitative assay, issues exist that have precluded universal acceptance of a protocol using this endpoint. For example, the mechanism leading to death is more clearly understood than the mechanism leading to temperature decrease. In addition, there is no temperature drop that is unequivocally predictive of imminent death, indicating at which point the mice could be euthanised to prevent unnecessary suffering (Arciniega, personal communication). Other important assay design variables include the mouse characteristics (strain, source, sex, and age), injection route, and length of the sensitisation period [17]. Accurate detection of PTx in aP vaccines is dependent on the test sensitivity, which in turn is highly dependent on the assay design.

Although the different versions of the HIST have a demonstrable history of ensuring the release of safe aP vaccines, a number of reduction and refinement modifications have been proposed, some having been implemented. For example, some manufacturers conduct the HIST only on the final formulated bulk sample, rather than on each intermediate that comprises the bulk, thereby reducing the number of animals used by approximately three-fold. Furthermore, efforts have increased towards developing a non-animal alternative that is at least as reliable as HIST.

The key to a successful alternative is accounting for the mechanisms associated with histamine toxicity, including PTx binding, trafficking and ADP ribosylation (cell intoxication).

3.4. Animal use for HIST and the potential impact of *in vitro* alternatives

Dr Coenraad Hendriksen, IntraVacc, presented an evaluation of global animal use for the HIST, estimated at 100 000 mice each year and the potential impact on animal use of adoption of non-animal alternatives. He addressed 2 primary questions:

- What are the differences in performance and specifications of the HIST?
- What would be the impact of replacement of the HIST by alternatives in terms of animal numbers and animal welfare?

A survey was conducted among pertussis vaccine manufacturers and national control laboratories to identify the regional differences in HIST specifications and acceptance criteria, as well as to provide an estimate of the number of lots tested and the resulting number of animals used. This survey revealed noteworthy differences in HIST study designs that are driven by national regulatory requirements (see Table 1). A key variation is a requirement for either a limit or a quantitative test. Other differences are specific to the protocol components, including characteristics of the mouse used (strain, sex, age, etc.); number of mice per group, including positive and negative controls; vaccine dose; dose of histamine challenge; readout used for the test (lethality or temperature); time interval between vaccination and challenge; and time interval between challenge and readout.

Since the actual number of animals used in each test was not provided, the testing frequency and the regional requirements as noted in Table 1 were used to estimate the annual total number of animals used based on data from 2012–2013 (Hoonakker and Hendriksen, manuscript in preparation). Two important variables in calculating testing frequency and the associated number of animals used are the number of times a HIST was repeated and how often a vaccine is retested. Criteria for test validity and acceptance, which differ among regulatory authorities, are used to determine whether the HIST needs to be repeated or if a vaccine must be retested. Validity criteria are based primarily on the responses in the positive and negative control groups. If validity criteria are not met, the HIST is repeated using the same study design. Acceptance criteria are based on the responses in the animals receiving the vaccine. If the acceptance criteria are not met, the vaccine is retested in HIST using twice as many animals.

Clearly, there is a wide range in numbers of tests, groups, and animals per group used, depending on the relevant regional requirements. Therefore, harmonisation of regulatory requirements would be a logical step towards minimising animal use by standardising a HIST protocol and avoiding duplicative testing. Of course, the ultimate goal is replacing the HIST with a non-animal alternative, the workshop participants therefore turned to a discussion about the best (and fastest) way forward.

4. DISCUSSION

4.1. The purpose of the HIST

The HIST is a mouse bioassay that measures an activity attributed to PTx in wP vaccines and that detects residual PTx and reversion to toxicity of PTxd in aP vaccines. There is no clinical evidence available that would enable definition of an unsafe amount of PTx present in a dose of pertussis vaccine. However, the toxin is capable of inducing physiological changes in animals, therefore, its presence in an abnormally high quantity in wP vaccines would seem to be undesirable, and monitoring its levels for consistency during production is encouraged by the WHO [19]. For aP vaccines, HIST results have been used as either an indicator of vaccine safety or of manufacturing consistency to ensure sufficient inactivation of PTx, although the intended use of the assay varies among regulatory authorities.

While no specific effort is made to detoxify PTx in wP vaccines, the presence of PTx in aP vaccines above the specification may be due to inadequate detoxification or to reversion of the

toxoid to native toxin. The HIST is used to detect PTx in both of the latter instances. In some regulatory systems, HIST is the sole test used to measure residual PTx activity, not only in final bulks of aP vaccines, but also at upstream steps of the manufacturing process. In other regulatory systems, manufacturers have been given the option of using the CHO cell clustering assay instead of the HIST to measure residual PTx activity during the manufacturing of aP vaccines. Likewise, the CHO cell assay is recommended by WHO to monitor the levels of PTx during manufacturing of wP vaccines.

As noted above, there are a number of differences in the HIST protocols accepted by various regulatory authorities for the testing of final bulks of aP vaccines, most notably in the response being measured and the type of analytical procedure used. Another notable difference is in the type of stability testing performed. Some regulators require accelerated testing of every lot of aP vaccine at high temperature to detect reversion of PTx to toxicity. Other regulators accept a history of real-time stability testing of lots at licensing and the establishment of an ongoing stability program with periodical HIST testing of a representative sample of product kept at recommended storage temperature for its dating period.

4.2. The pathophysiological mechanisms of HIST

Since the discovery of the lethal sensitivity of mice to histamine, the HIST has been compared to an anaphylactic shock, although histamine is most probably not an important mediator in mouse anaphylaxis. In anaphylaxis, mast cells release histamine in inordinate amounts. The dilation of blood vessels caused by histamine increases permeability and lowers blood pressure. However, the contribution of histamine to blood pressure appears negligible in passive systemic anaphylaxis in mice due to PTx intoxication. In this case, PTx seems to inhibit a function of epinephrine necessary to maintain the blood volume, possibly by interfering with the vascular compensatory mechanism that regulates the permeability changes produced by histamine [20]. Death from histamine challenge in mice appears to be due to circulatory collapse caused by a loss of blood into the extravascular space for which the animal is unable to compensate. This theory is supported by the observation that mice dying from histamine challenge (and anaphylaxis) can be protected by restoring blood volume with physiological saline [21]. It may be not be far-fetched to suggest that PTx mediates the ADP ribosylation of G proteins involved in the regulation of the vasoconstriction of vascular smooth muscle cells. This may decrease the sensitivity of the cells to contraction-inducing agonists, particularly in the presence of histamine-induced vasodilation. Although no direct evidence points to the participation of any particular cell type in the events leading to the death of mice sensitised to histamine by PTx, it has been shown indirectly that $G_{\alpha i1}$ and $G_{\alpha i3}$ proteins are the critical *in vivo* targets of ADP ribosylation underlying histamine sensitisation elicited by PTx exposure [15]. Additionally, it has been shown that the gene *Hrh1*, which encodes the H1 histamine receptor in mice, controls differential susceptibility to PTx-induced histamine sensitisation [22]. Taken together, the available evidence indicates that the response exhibited by mice to active PTx in pertussis vaccines in the HIST is based on cellular intoxication and the ADP ribosylation of G proteins, most probably of the tissue involved in the vascular compensatory mechanism, such as the vascular smooth muscle.

ADP ribosylation-mediated disturbance of intracellular signaling by PTx is the most important mechanism through which PTx produces clinical effects at physiological concentrations [23] and it is important that any HIST alternative assess this process. However, ADP ribosylation may not be the only factor involved in the clinical effects of PTx. In fact, two signaling pathways exist by which PTx can elicit biological responses, one dependent on ADP ribosylation and the other independent of ADP ribosylation. The ADP ribosylation-independent pathway involves bridging of cell surface receptors via binding to the B oligomer. In this regard, it is important to note that ADP ribosylation-dependent effects appear to occur at very low PTx concentrations, while the ADP ribosylation-independent effects require significantly higher concentrations. This difference is complemented by the fact that the onset of action is slow for ADP ribosylation-dependent effects, while ADP ribosylation-independent effects occur more rapidly [23].

It is believed that HIST or an alternative based on assessing ADP-ribosylation mediated cell intoxication would likely reflect, at least in part, the effect that the inactivating agent has on

the binding sites on the B oligomer to cell receptors. There is at least a theoretical chance that multiplicity of receptor types or cellular signaling routes may be responsible for clinical effects [23].

Given this understanding of the purpose of the HIST and its associated pathophysiological mechanisms, the workshop participants agreed on using the HIST as a reference test method, and discussed a number of challenges associated with the development, validation, and implementation of alternatives to the HIST.

4.3. Comparing the different versions of the HIST

There is widespread appreciation of the differences among the HIST protocols and the regional differences in use of the HIST for manufacturing and regulatory purposes.

The US Food and Drug Administration Center for Biologics Evaluation and Research has licensed several aP vaccines, approving the HIST as a limit test for residual PTx activity that uses a lethal endpoint. The test is designed to show only that residual PTx activity in the vaccine is below an acceptable threshold; it does not quantify the amount of active PTx.

Japanese regulations for wP vaccine include the HIST among several toxicity tests. PTx is measured relative to a reference vaccine, to which a histamine-sensitising unitage (HSU) has been assigned. This quantitation of PTx activity provides another means of comparing pertussis vaccine products, especially when evaluating new manufacturing procedures. Also, quantitation of PTx in terms of HSU in pertussis vaccine-containing products used for clinical trials could contribute to the understanding of the clinical effects of PTx in humans.

Preparation of aP vaccines, which contain larger quantities of PTx per dose than wP vaccines, differs from preparation of wP vaccines in the inclusion of a detoxification step to reduce, in a reliable way, the activity of the toxin to a minimum compatible with its role as an effective antigen. As a consequence of this detoxification step, the HIST with lethal endpoint used for wP vaccines was found to be not sensitive enough to quantitatively measure residual PTx in aP vaccines, because the PTx activity was reduced at least 10-fold.

In 1954, it was reported that mice injected with wP vaccines showed a dose-dependent body temperature reduction after histamine challenge [24]. In Japan, this finding was used to develop a quantitative method to measure residual activity of PTx in aP vaccines using rectal temperature in treated mice instead of fatal sensitisation [25]. Residual PTx activity of a test vaccine was expressed relative to that of the same wP standard to which HSU/mL had been assigned. A dermal temperature method was then developed and found to be consistent with the rectal temperature method [26]. However, while a linear relationship has been demonstrated between the dose of PTx and temperature, the percent mortality is not linear with respect to the dose of PTx. It has also been found that a temperature drop following histamine sensitisation is not a good predictor of mortality and therefore not a likely refinement alternative.

The physiological mechanism that mediates the drop in temperature following the challenge with histamine of PTx-sensitised mice is not as well understood as the mechanism of death following PTx challenge, and no studies exist that establish whether the mechanisms underlying the two endpoints are related. Knockout mice that do not produce histamine do not exhibit the significant temperature decrease observed in normal mice during a passive systemic anaphylaxis reaction. Blood pressure dropped in both the normal and the knockout mice, and intravenous injection of histamine in naive knockouts induced a body temperature decrease similar to that experienced by their normal counterparts [27]. This would suggest a lack of association between the temperature and lethal HIST outcomes if the mechanism underlying the lethal challenge resembles anaphylaxis.

Many of the aP vaccines currently on the market have residual PTx activities that cannot be detected by the HIST due to the high efficiency of the inactivation methods. Therefore, the need for a quantitative method is debatable. Equivalence of the lethal HIST, which is a limit test for control of impurities, and the quantitative non-lethal method has not been established from either a mechanistic or a quantitative standpoint. Therefore, the use of the non-lethal method as a quantitative test for residual PTx activity is limited.

4.4. Sensitivity of HIST for detecting PTx

Detection of PTx in pertussis vaccines depends on the test sensitivity and whether there is a need to measure the limit of sensitivity or level of quantification. Based on the assumption that the HIST can achieve that level of sensitivity, a value of 2 IU (international units)/mL has been used as the required sensitivity for an acceptable alternative to HIST. However this is only a convenient, conventional benchmark supported by data from a collaborative study of the lethal endpoint HIST, which indicated that the highest levels of residual PTx in a borderline acceptable DTaP (diphtheria, tetanus and pertussis) -based combination vaccine may be in the range of about 2 IU/single human dose (SHD) [28].

An accumulation of data indicates that HIST as currently performed (European Pharmacopoeia method) allows detection of 1-1.5 IU of PTx per two SHDs [8]. In Japan a limit of 0.2 HSU per dose has been established, which is equivalent to 1.09 IU of PTx/SHD [28]. In the US, PTx spiked into two pools of DTaP vaccine at 3 IU per 0.5 mL sensitised 8/10 and 6/10 mice, respectively. In a separate study of PTx alone, the HSD10 was calculated to be 7 IU per 0.5 mL [17].

4.5. Mechanistic requirements for HIST alternatives

According to the consistency approach, the purpose of HIST and therefore of any potential replacement is to ensure that all newly manufactured lots of aP vaccines contain a residual PTx activity (or absence thereof) similar to those lots of vaccine submitted in support of licensure that showed acceptable safety in clinical studies. The choice of one alternative assay over another is often based on the extent to which it can measure the same critical aspects that the original assay measures.

4.6. Specifications required for HIST alternatives

Workshop participants agreed that a successful alternative to the HIST should be able to monitor all the functions (mechanisms) that are biologically relevant to the toxic activity of PTx (binding, enzymatic, and translocation). It should also possess a level of sensitivity at least equivalent to that of the HIST, be useful for the testing of final product, and be practical for quality control purposes.

Any alternative test proposed should be at least as sensitive as the HIST. Acceptance by a regulatory authority of an alternative assay is unlikely if such an alternative is unable to detect at least the same activity as the HIST of PTx per volume of final bulk aP vaccine. If the alternative test proposed is more sensitive, specifications may need to be adjusted on the basis of the consistency approach, and they may also need to be product specific. Different regulatory systems require different assay designs for residual PTx activity as an impurity (per the International Council on Harmonisation definitions, either as a quantitative test for its content or as a limit test for its control). The sensitivity of the alternative method (in terms of its lower limit of quantitation) can drive the setting of a specification for residual PTx activity if a limit test is used.

The sensitivity of an alternative method under certain ideal conditions may not be equivalent to the sensitivity of the same method in ‘real world conditions’, namely, in final vaccine samples. Therefore, the influence of the matrix (adjuvant and other excipients) on the assay should be adequately considered during method development.

4.7. Validation requirements for HIST alternatives

A vaccine that would pass the HIST should also pass the alternative test; likewise, a vaccine that would fail the HIST should also fail the alternative test. However, it is difficult to obtain ‘failing’ vaccines to perform the required demonstration to the regulatory authorities. In this regard, ‘spiking’ studies are useful, but they are limited in that they do not demonstrate what happens to PTx in vaccine upon storage. Unreasonable expectations for the performance of the alternative test should be avoided (e.g. 100 % agreement of results with HIST), and a plan should be established that would indicate what level of agreement of results will be acceptable and also take into account the inherent variability of the HIST. The alternative test should address the mechanisms that are relevant to the clinical effects of PTx, and should also be

able to monitor final bulk consistency without interference by the adjuvant and other excipients. In some regulatory systems, the alternative test should be shown to be similar or better to HIST; therefore, the outcome should be expressed as a consistent metric comparable to the one used to express HIST activity. Tests that explore individual steps relevant to PTx activity (e.g. enzymatic, binding activity) are valuable because they provide useful information about detoxification. However, their outcome is not equivalent to that of the HIST, because they are co-correlates (one of two or more factors that correlate with the activity being measured in alternative, additive, or synergistic ways); neither test by itself would be a correlate of intoxication [29] and thus would have a harder time being accepted by some regulatory systems as HIST replacements.

CHO cell clustering correlates with the ADP ribosylation of a Mr (relative molecular mass) = 41000 membrane protein by the toxin [30], and intoxication is mediated by the lectin-like binding of PTx to a 165-kilodalton glycoprotein [31]. In other regulatory systems the CHO cell assay is not accepted as an alternative to HIST at any stage of manufacturing, not even to monitor detoxification in process, because it is perceived that mice possess a mechanism favouring the dissociation of aggregated PTx molecules that fail to interact with their receptors in CHO cells, hindering the clustering response [32]. The studies in support of the contention of the superiority of HIST over the CHO cell clustering assay in detecting residual PTx in aP vaccines were limited to the use of one mechanism of PTx inactivation (formaldehyde) and one HIST of quantitative design (temperature drop outcome), and have not been reproduced due to the restricted practice of these conditions.

4.8. Regulatory challenges to acceptance of HIST alternatives

4.8.1. Methods to compare assays

Yuen *et al.* described a comparison between the HIST and a system combining two alternative assays to measure both binding and enzymatic activity [33]. However, the results of this study are not universally accepted. Two versions of the HIST are in worldwide use (see section 4.3), but they have not been strictly compared to each other. The version of HIST used in the Yuen *et al.* study (quantitative, temperature-drop endpoint) is only used by some regulatory systems. The alternative assays being compared to HIST (enzymatic and binding) are only 'partial' in nature (i.e. each one by itself does not completely address all the steps necessary for cell intoxication). The IU measurement for PTx activity was established using HIST and also reported using CHO cell clustering assays. Activity measured by either of the partial alternative tests is not directly comparable to the activity measurable by HIST or CHO cell assays; in fact, it has been shown that some vaccines pass HIST despite displaying substantial activity in the enzymatic assay [34]. To address this limitation, a mathematical strategy of using a combination of results was proposed. While this proposal is under further discussion, it may be complex to envision such a strategy as a routine quality control test.

4.8.2. Variations in reference material

There are two sources of PTx reference material available: one from the WHO and one from EDQM. Differences in the assignment of units of activity to the two PTx reference materials need to be resolved. IU were assigned to the 1st International Standard (WHO) on the basis of the lethal HIST, and the activity measured by CHO cell clustering was found to be not significantly different for this standard [35]. However, the IU assigned to the EDQM Biological Reference Preparation, also on the basis of the lethal HIST, were found to be significantly different than those obtained using the CHO cell clustering assay [28]. Calibration of any future standard will be based on the HIST, but it will also be tested in parallel by the CHO cell assay. The above discrepancy should be resolved if the CHO cell clustering method is shown successful as an alternative to HIST.

4.8.3. Differences in regulatory perspectives

Japanese regulators do not currently accept the CHO cell clustering assay at any stage of manufacturing because it is perceived that mice possess a mechanism that dissociates aggre-

gated PTx molecules that fail to interact with their receptors in CHO cells, thereby hindering the clustering response. The collaborative study that will address the transferability of the CHO cell assay will include Japanese and Korean participants; a positive outcome should help resolve this issue.

4.8.4. Confusion over the consistency approach

Another issue is the discordant interpretations of the consistency approach (safety vs. consistency). Manufacturing consistency should yield new lots that display consistency in testing outcomes. If the testing tool is changed, the new tool should have characteristics that make its performance consistent with those of the previous tool. Strategies other than direct comparison with HIST are needed to demonstrate consistency, since for both ethical and financial reasons it is not likely that an animal-intensive comparison will be done. Historical clinical lots cannot be compared with production lots using the biochemical alternatives without parallel testing, because no IU can be determined. Some consistency can be established between currently manufactured lots, but will require the establishment of new unitage and new specifications. Parallel testing in the HIST and alternative test would allow this comparison prospectively.

4.9. Achieving harmonisation and implementation of HIST alternatives

The existence of different regulatory requirements for HIST unquestionably increases the use of animals. Harmonisation of regulatory requirements will be critical to the success of identifying a HIST alternative method; setting fewer constraints on the replacement design will maximise the likelihood of successful harmonisation. While there are differences in regulatory requirements, some similarities do exist, and those similarities should be identified to achieve success in HIST replacement. The adoption of common basic principles is more likely than the embracing of detailed common protocols, due to variations in international regulations and manufacturing processes. Some activities that may promote the harmonised adoption of an alternative to HIST include:

- Adopting a uniform consistency approach.
- Agreeing on the principle that acceptable alternatives to HIST should be based on ADP ribosylation-mediated cell intoxication.
- Confirming a minimum assay sensitivity and defining its use.
- Continuation of multinational approaches involving national and international standardisation bodies.
- Organisation of suitable collaborative studies.

5. CONCLUSIONS

Regulators in attendance were strongly encouraged to take the information discussed in the workshop back to their respective agencies to ensure that all personnel would be informed of the agreements reached. While manufacturers have yet to begin submitting licence supplements/variations that include HIST alternatives, most regulators have indicated their willingness to consider them, as there is a strong desire to reach international consensus on a HIST replacement strategy to minimise animal use and make licence amendments and future testing strategies more efficient. Reviewers who will be examining applications that include a proposed alternative to HIST should be engaged ahead of time, so they have the opportunity to clarify any issues or concerns, and thereby be prepared to receive data from alternative methods once they are submitted.

The final discussion of the International Working Group on Alternatives to HIST was scheduled for March 2015 at the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) in London, UK. A primary objective of the meeting will be to review and discuss data generated in the Collaborative Study to determine if the CHO cell-based assay is a suitable alternative for replacement of HIST. If the CHO cell assay is deemed adequately valid based on the study results, pertussis vaccine manufacturers, national control

authorities, and regulatory agencies at the meeting will then work towards an implementation strategy for routine use of the CHO cell assay as an alternative to HIST.

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7. PARTICIPANTS

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8. PARTICIPANTS' AFFILIATION (BY ALPHABETICAL ORDER OF AFFILIATED BODIES)

ANSM:	Agence Nationale de Sécurité du Médicament et des produits de santé
ASCR:	Academy of Sciences of the Czech Republic
EDQM:	European Directorate for the Quality of Medicines & Healthcare
EURL-ECVAM:	European Union Reference Laboratory for alternatives to animal testing
ILS:	Integrated Laboratory Systems, Inc.
GSK:	GlaxoSmithKline
MFDS:	Ministry of Food and Drug Safety
NC3Rs:	National Centre for the Replacement Refinement & Reduction of animals in research
NIBSC:	National Institute for Biological Standards and Control
NICEATM:	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
NIEHS:	National Institute of Environmental Health Sciences
NIID:	National Institute of Infection Disease
PEI:	Paul Ehrlich Institute
UH:	University Hospital Hradec Kralove
US FDA:	United States Food and Drug Administration

9. ABBREVIATIONS

ADP: Adenosine Diphosphate; aP: acellular Pertussis; CHO: Chinese Hamster Ovary; DTaP: Diptheria, Tetanus and Pertussis; DTaP-IPV-HB-Hib: Diphteria Tetanus, Pertussis, Hepatitis B, Polio and Haemophilus influenzae type b; HIST: Histamine Sensitisation Test; HSD10:

Histamine Sensitising Dose, 10 %; HSD_{50}: Histamine Sensitising Dose, 50 %; HSU: Histamine-Sensitising Unitage; IU: International Units; LD_{50}: Lethal Dose, 50 %; Mr: Relative molecular mass; PTx: Pertussis Toxin; PTxd: Pertussis Toxin, deactivated; SHD: Single Human Dose; Tdap-IPV: Tetanus, Diphtheria, Pertussis, Polio; WHO: World Health Organization; wP: whole cell Pertussis.

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Table 1 – *HIST requirements specified in regulatory guidelines*

	USA	EU	Canada	China	Japan	WHO
Requirement according to	License dossier	Ph. Eur.	Ph. Eur.	Ph. Chin.	Ph. Ja.	TRS 979
Final lot (or bulk)/in process	Final lot (or bulk)	In process and/or final lot	Final lot	Bulk and final bulk	Final lot	Final bulk (one or more dilutions)
Injected vaccine volume	0.5 mL	2 HD	2 HD	0.5 mL	0.5 mL	1 or 2 HD
Vaccine sample storage	4°C	4°C and 37°C	4°C	4°C and 37°C	4°C and 37°C	1 or more dilutions, 4°C and or 37°C
Negative control	Diluent	Diluent	Diluent	n.s.	n.s.	Diluent or none
Positive control (and number of dilutions)	PTx (one)	n.s.	n.s.	PTx (several dilutions)	PTx (several dilutions)	PTx (1 or more dilutions)
Number of mice per group	20	5	5	10	10	10 or an appropriate number
Minimal number of groups	App. 3	3	2	App. 5	App. 6	App. 5
Histamine challenge	1 mg of histamine	2 mg of histamine	2 mg of histamine	2-4 mg of histamine	4 mg of histamine	defined dose of histamine (usually 1 or 2 mg)
Time interval between sample administration and challenge	5 days	5 days	5 days	4 days	4 days	4–5 days
Observation period	24 hours	24 hours	24 hours	30 minutes	30 minutes	30 minutes–24 hours
Minimum animal number 1 test	App. 60 animals	15 animals	10 animals	App. 50 animals	App. 60 animals	App. 50 animals
Readout parameter	Death	Death	Death	Temperature decrease	Temperature decrease	Temperature decrease or death

App.: approximately; HD: (human dose); n.s.: (not specified); Ph. Chin.: Chinese Pharmacopoeia; Ph. Eur.: European Pharmacopoeia; Ph. Jap.: Japanese Pharmacopoeia; PTx: pertussis toxin; TRS: Technical Report Series; WHO: World Health Organization.