



Alternative Safety Testing Strategies for Acellular Pertussis Vaccines

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Summary

The mouse histamine sensitization test (HIST) is the currently accepted regulatory method used to monitor 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, each of which monitors 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 most likely will 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.

Keywords: pertussis toxin, acellular pertussis vaccine, mouse histamine sensitization assay, *in vitro* assay

Infection by the Gram-negative bacterium *Bordetella pertussis* can lead to the development of pertussis, also known as whooping cough due to the characteristic sound infected infants make following a severe coughing fit. Infection by *B. pertussis* is fairly common, but it rarely causes serious complications in adults. However, the infection can be fatal in infants and young children if left untreated (CDC, 2011). Therefore, prevention of pertussis is a key health priority in most countries, and vaccination programs were initiated with the development of a killed whole-cell vaccine in the 1940's. Although the whole-cell pertussis vaccine is effective, the incidence of mild adverse events (e.g., swelling and pain at the sight of injection, fever, drowsiness) and difficulties in the manufacture of a product with consistent quality, led to the development of less reactogenic acellular pertussis vaccines (Berbers et al., 2009). These vaccines contain purified components of the *B. pertussis* bacteria, which

usually include bacterial hemagglutinin, pertactin, fimbriae 1 & 2, and inactivated pertussis toxin.

Due to its biological activity, the pertussis toxin (PTx) component of acellular pertussis vaccines must be inactivated prior to administration. This inactivation, or toxoiding, is most commonly done through the chemical modification of purified PTx to yield pertussis toxoid (PTd). To date, a number of chemical methods have been developed to inactivate PTx, including the use of formaldehyde and/or glutaraldehyde, as well as hydrogen peroxide (Corbel and Xing, 2004). A genetically inactivated PTx also has been developed (Peppoloni et al, 1995) but is not readily available.

The chemical detoxification process for PTx must be carefully balanced between detoxification and maintenance of effective antigenic epitopes (Fig. 1). The chemical detoxification process modifies PTx to render its biochemical functions inac-

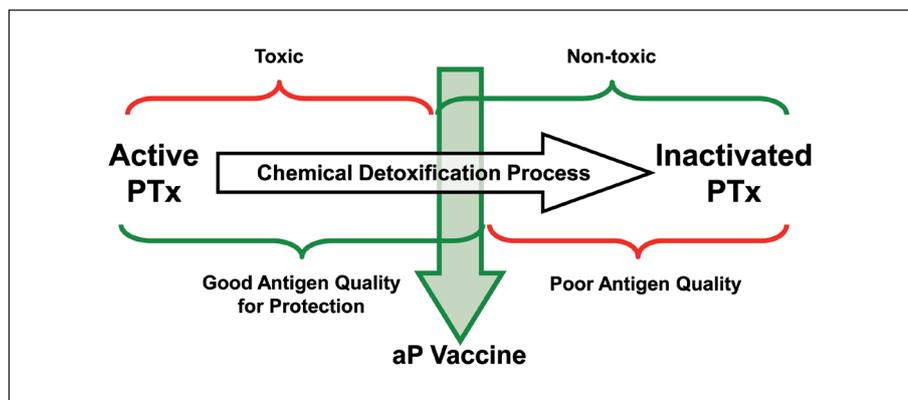


Fig. 1: The generation of a viable acellular pertussis (aP) vaccine requires careful control of the production process

The chemical detoxification of PTx must be balanced between sufficient inactivation to yield a non-toxic product, yet not overly modified toxin so as to maintain good antigen quality.



tive, but at the same time this reaction can potentially modify or destroy epitopes necessary to induce PTx-neutralizing antibodies. Therefore, each batch of PTD produced must be tested for both its immunogenicity and residual PTx activity. A highly sensitive cell-based assay exists that can be used to monitor purified PTD preparations for any remaining PTx activity. This assay is based on the observation that Chinese Hamster Ovary (CHO) cells will grow in cluster formations when incubated for 48 hours with PTx (Gillenius et al., 1985). Depending on the microscopy skills and abilities of the technician, this change in cell culture morphology is easily detected at concentrations of 0.1 ng PTx/ml or less, which is considered well below safe levels. It should be noted here that PTx has been administered to healthy adults in doses as high as 1 μ g/kg body weight without any reported adverse effects (Toyota et al., 1980). However, because pertussis vaccines are administered to infants, a large margin of safety is required for detectable levels of PTx that are consistent with safe and efficacious use of the product.

Due to the simplicity of the CHO cell assay, it is commonly used by vaccine manufacturers for in-process testing to monitor the detoxification of PTx during production. However, this assay cannot be performed with adjuvanted acellular pertussis vaccines due to the inherent cytotoxicity to cultured cells of the alum adjuvants contained in vaccines. Although in-process control testing ensures the absence of residual PTx activity in purified PTD bulk preparations, national regulatory authorities (NRAs) require the testing of the final acellular pertussis-containing vaccine to ensure the absence of residual PTx or reversion of the chemically-inactivated toxoid (WHO, 1996).

The commonly accepted assay to detect PTx activity in adjuvanted acellular pertussis vaccines is the mouse histamine sensitization test (HIST). This assay is based on the sensitization of mice to histamine by active pertussis toxin (Corbel and Xing, 2004). Mice are administered the test vaccine or controls and five days later are challenged with a histamine dose that would not normally be lethal to the animals. The mice are observed, and the number of deceased animals in the test group is compared to the number in the appropriate control group(s). Several variations of this assay exist, depending on specific NRA regulatory requirements for testing, and they include differences in the vaccine or histamine doses, mouse strain, number of mice per group, control groups, and whether the endpoint is the number of mice succumbing to the challenge within 24 hours or the change in their body temperature 30 minutes after the histamine administration. Using a body temperature method could be considered a refinement to the lethal endpoint protocol for the HIST; however, both methods require the same number of animals, and the majority of animals that die from the histamine do so in under 30 minutes. Therefore, in terms of animal usage and application of 3Rs, the advantages of a body temperature endpoint assay over a lethal endpoint test are minimal.

The HIST is the currently accepted method for testing acellular pertussis vaccines in all regulatory jurisdictions. However, as different HIST methods are required by differ-

ent NRAs, two or more HIST protocols may have to be used when vaccines from the same lot are distributed to different countries. Although acellular pertussis vaccines have been released using the HIST for decades, this method is less sensitive to PTx than the CHO cell clustering assay when used on pre-adjuvanted vaccines, and it is fraught with technical difficulties – most notably a high variability and high rate of false-positive outcomes. Combined, these problems lead to the use of a large number of mice due to the frequent retesting of acellular pertussis vaccine batches by manufacturers and/or NRA quality control labs.

During the past decade, there has been a growing effort to develop *in vitro* methods for monitoring PTx activity in adjuvanted acellular pertussis vaccines. The amount of research and development time invested in this project at the international level can be considered a reflection of the complexity of the PTx protein, as well as our limited understanding of its full biochemical and biological functions. For a detailed review of the PTx protein and its mechanism of action, the reader is referred to the article written by Mangmool and Kurose (2011). For the purposes of this summary, it is sufficient to note that the PTx protein is comprised of two binding regions, which recognize a variety of glycan structures on cell surface glycoproteins, and an enzymatic region, which ribosylates $G_{i/o}$ proteins within cells. The ribosylation of $G_{i/o}$ causes its inactivation and the removal of its inhibitory function on adenylyl cyclase, thereby resulting in the potential for an unregulated production of the signal transduction molecule cyclic AMP. The means by which PTx enters a cell to effect its enzymatic activity is still unknown, but based on the functions of other related bacterial toxins it is reasonable to assume that PTx has a translocation domain to control this function.

In vitro assays under development or evaluation as alternatives to HIST are based on either monitoring the individual biochemical functions – binding or enzymatic activities – or monitoring for a cellular response after a short-term exposure to the vaccine and before any cytotoxic effects of the adjuvant occur. The first of these assays to be developed was an enzyme-coupled HPLC (eHPLC) assay that uses an HPLC method to quantitate the amount of an ADP-ribosylated peptide substrate following incubation with PTx or the test sample (Cyr et al., 2001). As the enzymatic region of PTx is variably modified by the chemical methods used in the detoxification process, acellular pertussis vaccines tend to have a similarly variable range in their residual enzyme activity. Carbohydrate binding assays also have been developed (Gomez et al., 2006; Isbrucker et al., 2010) that use assay plates coated with the glycoprotein fetuin to capture PTx from the sample, followed by its detection using antibodies to PTx. Both the enzymatic and binding assays are very sensitive methods, but neither one alone should be used to monitor vaccines for PTx activity as each assay only provides an indication of a singular biochemical function of a multi-functional protein.

Also under development are cell-based assays that monitor cAMP accumulation (Hoonakker et al., 2010) or ATP reduction



in response to PTx exposure. These assays avoid the cytotoxicity problems associated with alum adjuvants by significantly reducing the incubation time to less than 6 hours instead of the 2 days required for the CHO cell agglutination test. Also, as these assays measure downstream cellular effects of PTx, the results reflect the levels of fully functional PTx rather than a specific biochemical activity that may remain viable within an inactive PTd. Despite the clear advantage of using a cell-based assay to test for residual PTx in vaccines, they currently require further development to reduce the variability and improve the sensitivity.

During the summer of 2011, two international workshops were held to discuss these *in vitro* assays and their use in monitoring residual PTx activity in acellular pertussis vaccines:

- “Workshop on Animal-Free Detection of PTx in Vaccines – Alternatives to HIST.” 9-10 June, 2011 at the Paul Ehrlich Institute (PEI) in Langen, Germany.
- “Alternative Safety Testing Strategies for Acellular Pertussis Vaccines.” 21 August, 2011 as a half-day satellite meeting to the 8th World Congress in Montreal, Canada.

The purpose of the June 2011 meeting at PEI was to (1) bring together those individuals/labs who were working on alternative methods to HIST, (2) exchange experiences with the HIST and alternative methods, and (3) define the criteria that alternative methods would have to meet for regulatory acceptance. In particular, the criteria to be discussed included assay sensitivity in relation to HIST, specificity, test precision and reproducibility, and assay validation criteria. The workshop opened with presentations and discussions of the different *in vitro* assays currently under evaluation or development, a weighing of their strengths and weaknesses, and discussions on measuring the limit of detection in the HIST. It was evident early in this workshop that results from different assays and/or labs are not readily comparable due to the different PTx preparations being used and that any future studies should use reference standards with values converted to International Units, rather than weight/volume.

Defining the criteria that any *in vitro* method should meet as an alternative to HIST proved difficult, as it is not possible to set a common level of sensitivity for these assays, due to the very nature of what they are actually measuring. Also, assay results can be vaccine-specific due to the different chemistries of their toxoiding process, other components of the vaccine, and the type of adjuvant used. The high variability of the HIST resulting from differences in mouse strain and protocols further complicates the discussion. A debate arose regarding whether the *in vitro* assay results should, or could, be correlated to HIST outcome, and whether the alternative methods need to be as sensitive as the animal test.

A possible solution to the problem was proposed: the adoption of a consistency of manufacture approach instead of attempting a direct correlation between HIST and any alternative method. As acellular pertussis vaccines have a history of safe use and are being released on HIST outcome, a set of new specifications could be established for each vaccine based on

in vitro assay outcomes. These specifications would then be considered indicators of a manufacturer’s consistent production of a vaccine with a history of safe use. Values for the specifications would be determined by the concurrent testing of the HIST and the alternative assay(s) for each vaccine batch until a sufficient number of outcomes are obtained to establish an accepted range. All subsequent vaccine batches then would be required to match the new specification(s) rather than being released on HIST outcome. Although this generally was considered a good approach to removing the HIST from vaccine quality control testing, questions remained as to which *in vitro* assay(s) should be selected, and whether they would be able to predict an outcome similar to the HIST. The need for validation and comparability studies of the HIST to the alternative methods was emphasized to ensure that the *in vitro* assays could differentiate between vaccine lots that passed or failed the animal test. However, as “failed” vaccine batches are not available for this purpose, the development of spiked vaccines would be necessary. Therefore, a working group was established to acquire acellular pertussis vaccine samples from manufacturers for distribution to research labs interested in the further development and evaluation of the alternative methods. The working group also would determine an appropriate method for spiking the vaccine with known quantities of PTx, and it would try to establish a framework for the assessment of the alternative methods.

The second international workshop was a satellite meeting to the 8th World Congress in Montreal and provided an opportunity to further discuss and clarify what NRAs would require to accept alternative methods to HIST and adopt a consistency of manufacture strategy, as well as to address the requirements for assay validation and comparability studies. As all NRAs do not accept the same HIST method, there was a difference in expectations regarding whether the *in vitro* methods should be a limit test or a quantitative assay. Other participants stated it would be inappropriate to validate any new alternative assays against the highly variable HIST. However, if a clear understanding of the intended purpose of the *in vitro* methods could be established, it would help define what would need to be validated. Although the new *in vitro* assays need to be at least as good as the HIST at detecting residual PTx in vaccines, concerns were raised that they may be far too sensitive and could lead to the incorrect failure of safe vaccine batches.

The participants were reminded during this satellite meeting that manufacturers use the *in vitro* CHO cell agglutination assay to monitor the inactivation of PTx prior to formulation and that the purpose of the HIST is to ensure the toxoid, PTd had not undergone reversion post-formulation. As this makes the HIST a form of stability test for PTd, it would seem appropriate that any alternative assay have this capability for monitoring the toxoid stability. Questions arose from this discussion as to why a vaccine must continue to undergo this stability testing when, historically, it has not been shown to undergo reversion, and whether it was appropriate to use an *in vivo* assay with a high variability to monitor this stability.



Consensus was attained among all of the participants that adopting a consistency of manufacture approach is an appropriate means for incorporating the accepted *in vitro* assays as an alternative to HIST. However, additional assay evaluation and development is required, particularly a better understanding on the effects of the adjuvant on assay outcome. Based on the discussions at this workshop, the participants agreed that a study using spiked vaccines to compare the sensitivities of the HIST and *in vitro* assays would be important. It also was agreed that the direct correlation between the *in vivo* and *in vitro* assays was not required and attempting to correlate the data could be detrimental to the study outcome. Finally, the participants felt that, although the biochemical methods that monitor the enzymatic and binding activities are much further ahead in their development, research into cell-based assays should continue as they could potentially provide a better indicator of whole PTx function and not just the individual activities of its components.

Combined, these two workshops brought together almost 50 participants representing 21 different organizations with interest in forwarding the implementation of alternative methods to the HIST. The success of these workshops is reflected in the on-going “spiked vaccine working group” and expansion in the list of individuals interested in participating in future studies. NICEATM-ICCVAM will be hosting the next workshop on Alternatives to HIST in November of 2012 in order for participants to present their findings on assays using the spiked vaccines as well as to discuss an international collaborative study to validate the *in vitro* assays.

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