HUMAN VACCINES & IMMUNOTHERAPEUTICS
2017, VOL. 13, NO. 11, 2522–2530
https://doi.org/10.1080/21645515.2017.1349585

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REVIEW

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Safety testing of acellular pertussis vaccines: Use of animals and 3Rs alternatives

Marieke Hoonakker[a], Juan Arciniega[b], and Coenraad Hendriksen[a]

[a]Institute for Translational Vaccinology (Intravacc), Bilthoven, The Netherlands; [b]United States Food and Drug Administration Center for Biologics Evaluation and Research, Silver Spring, MD, USA

ABSTRACT

The current test of acellular *Bordetella pertussis* (aP) vaccines for residual pertussis toxin (PTx) is the Histamine Sensitization test (HIST), based on the empirical finding that PTx sensitizes mice to histamine. Although HIST has ensured the safety of aP vaccines for years, it is criticized for the limited understanding of how it works, its technical difficulty, and for animal welfare reasons. To estimate the number of mice used worldwide for HIST, we surveyed major aP manufacturers and organizations performing, requiring, or recommending the test. The survey revealed marked regional differences in regulatory guidelines, including the number of animals used for a single test. Based on information provided by the parties surveyed, we estimated the worldwide number of mice used for testing to be 65,000 per year: ~48,000 by manufacturers and ~17,000 by national control laboratories, although the latter number is more affected by uncertainty, due to confidentiality policies. These animals covered the release of approximately 850 final lots and 250 in-process lots of aP vaccines yearly. Although there are several approaches for HIST refinement and reduction, we discuss why the efforts needed for validation and implementation of these interim alternatives may not be worthwhile, when there are several *in vitro* alternatives in various stages of development, some already fairly advanced. Upon implementation, one or more of these replacement alternatives can substantially reduce the number of animals currently used for the HIST, although careful evaluation of each alternative's mechanism and its suitable validation will be necessary in the path to implementation.

ARTICLE HISTORY
Received 4 April 2017
Revised 2 June 2017
Accepted 28 June 2017

KEYWORDS
3Rs alternatives; acellular pertussis vaccine; animal numbers; HIST; toxicity test

Introduction

Pertussis or whooping cough is a disease caused by a respiratory infection with the bacterium *Bordetella pertussis*. The disease is characterized by severe coughing, sometimes progressing to pneumonia, and can – on rare occasions and primarily in young infants – be fatal. The introduction of the first pertussis vaccines in the 1950s-60s led to a dramatic drop in pertussis incidence. These early vaccines were based on inactivated bacteria (wP vaccines). Downsides of these vaccines were their side effects and occasional but severe reactions temporally associated with their administration, which resulted in a search for safer vaccines. In the 1980s-90s, the acellular vaccines (aP) gradually replaced the wP vaccines in most industrialised countries (Table 1).[1,2] In non-industrialized countries, vaccination with wP vaccines has remained the preferred strategy, due to simpler manufacturing and consequent lower costs. aP vaccines consist of one or several purified proteins of the pathogen that are considered to contribute to protection.[3-5] Remarkably, the incidence of pertussis has increased since the 1980s, even in areas with high vaccination coverage.[6] Worldwide, the WHO estimated the number of pertussis cases to be about 16 million in 2008.[7,8] Because of the persistent circulation of *B. pertussis*, there will most likely be a continued need for aP and wP vaccines in the near future.

All aP vaccines include detoxified pertussis toxin (referred to as pertussis toxoid: PTd) and one or more of the following proteins: filamentous hemagglutinin, pertactin, fimbria type 2 and type 3. Testing for the presence of residual activity of pertussis toxin (PTx) and reversion of PTd to PTx is a regulatory requirement in all countries that use aP vaccines. For this purpose, the *in vivo* Histamine Sensitization test (HIST) is broadly applied, a procedure based on a discovery made in 1948 by Parfentjev and Goodline.[9] The test design consists of administering to groups of mice the vaccine lot under study, followed by a challenge with histamine 4–5 days post-vaccine injection. Originally the HIST was developed to test wP vaccines,[10] but nowadays it is only used for testing aP vaccines. In addition to its use for batch release testing, the HIST may also be performed in-process, and may be included in stability programs and optimization protocols of the vaccine production processes. Although widely used, the HIST is criticized for its poor reproducibility and the high variability in the sensitivity of the mouse strains.[11] Additionally, the test can inflict severe pain and distress, especially for mice receiving a reference PTx or mice that receive a vaccine containing residual PTx. For these reasons, manufacturers, as well as regulators, are highly motivated toward the successful development and implementation of a reproducible, validated *in vitro* method to replace the HIST.[12]

This review aims to estimate the impact of the HIST in terms of animal numbers used worldwide. In addition, it discusses the potential use of *in vitro* alternatives and the

CONTACT Marieke Hoonakker Marieke.Hoonakker@intravacc.nl Antonie van Leeuwenhoeklaan 9, 3721 MA Bilthoven, The Netherlands.

Table 1. Immunization programs.*

Acellular pertussis vaccines	Whole cell pertussis vaccines	Mixed
North America Europe	Africa South-East Asia	Eastern mediterranean Western Pacific

*[1][2]

information that may be required for their implementation. Before we discuss the alternatives that have been proposed or are under development we will provide some background information on the mechanism of PTx intoxication and the HIST. A preliminary version of the calculations summarized in this review were presented at the international workshop: "Alternatives to HIST for Acellular Pertussis Vaccines: Progress and Challenges" held on August 24, 2014 in Prague, Czech Republic.[13]

The mechanism of the HIST

PTx is an AB_5 toxin, composed of a B oligomer and an A subunit that are responsible for binding to the cell membrane and intracellular interference with the cAMP-PKA pathway, respectively (see Box 1 for a detailed description[12,14-26]). Since PTx by itself does not cause any noticeable acute effects in mice at the doses expected in aP vaccines, the HIST is based on the observation that PTx reduces the lethal dose of histamine about 30–300x.[10,27] Histamine is a compound involved in immunological responses, but can also act as a neurotransmitter.[28] It is typically released by mast cells upon activation by IgE antibodies and stimulates endothelial cells, increases blood vessel permeability and decreases blood pressure.[29,30] In the HIST, histamine administration causes a reduction in blood volume as a result of increased permeability of the blood vessels.[31] Bergman and Munoz have demonstrated that injection of compensatory volumes of saline into the bloodstream can prevent HIST-induced death.[27] Other studies have shown that PTx reduced the contractile properties of arteries, causing a hypersensitivity to histamine-induced vasodilation.[32,33] The histamine hypersensitivity is also observed in removal of adrenal glands and after blocking of β-adrenergic receptors of mice, suggesting that a common insensitivity to catecholamines is involved in both phenomena.[34] Taken together, these studies demonstrate that PTx affects essential properties of arteries and strongly suggest that these phenomena are responsible for PTx-induced sensitization to histamine.

HIST testing: regulatory requirements and test designs

aP vaccines were first manufactured and used in Japan in 1981.[35] Currently aP vaccines are also produced in Canada, Europe and China. These vaccines are widely used in the United States and Canada, the European region and the aforementioned Asian countries and are being gradually introduced in other parts of the world. Despite the widespread application of the HIST for aP vaccine testing, requirements for test performance are region-specific and vary with regard to mouse specifications (sex, age, etc.), inclusion of positive and/or negative control groups, test group sizes, time interval between vaccination and challenge, time interval between challenge and reading of the test outcome and histamine challenge dose. There are basically 3 versions of the test. The first version determines whether the level of PTx is at or below an acceptable threshold, by recording death of the mice after a histamine challenge. Similar to the first version, the second version is based on PTx-induced histamine sensitization; however, whether the level of PTx exceeds a specified threshold is determined by assessing a decrease in body temperature, rather than death of the mice.[36] The third version uses the same principle as the second version, but measures the actual level of PTx by comparing the associated decrease in body temperature caused by several doses of the vaccine on test, relative to a toxicity reference preparation. For the second and third versions, body temperature can be monitored by a rectal probe or by infrared detection of the dermal temperature.[37] Although changes in body temperature and death are both dose-dependent, there doesn't seem to be a direct link between both parameters,[37] and it is unclear whether both methods are based on the same mechanism.

Regional requirements and global guidelines (WHO) for the HIST, including relevant validity and acceptance criteria, are

Box 1. The cellular relevance of PTx in aP vaccines

The B oligomer of PTx consists of 5 subunits (S2, S3, two copies of S4, and S5).[14] and is of importance for binding the toxin to glycoconjugate cell receptors,[15] upon which the holotoxin enters the cell by endocytosis, followed by retrograde transport to the Golgi and the endoplasmic reticulum. Subsequently the S1 subunit is released into the cytosol.[16] Within the cytosol, the S1 subunit catalyzes the transfer of ADP-ribose from NAD^+ to the α-subunit of $G_{i/o}$ proteins,[17] thereby preventing interaction of these proteins with their cognate receptors. ADP ribosylation fixes the α-subunit of the G-proteins in their inactive (ADP-bound) form and the α-subunit is therefore unable to inhibit its target enzyme, adenylate cyclase. The consequent accumulation of the second messenger (cAMP) interferes with cellular signaling. PTx-induced changes in cell signaling can generally be detected within a couple of hours.[18] The B oligomer binds to several target proteins on the membrane of cells and thereby PTx influences several cellular processes. The B oligomer by itself causes proliferation of T cells,[19,20] results in glucose oxidation in adipocytes[19] and induces activation of TLR4.[21] These effects of the B oligomer appear 2-24 hours after exposure to PTx, while effects on intracellular cell signaling appear more rapidly.[22,23] Generally cellular effects of B oligomer require higher levels of PTx,[24] i.e. 250ng/mL-2μg/ml,[19-22] while ADP ribosylation by the S1 subunit is induced by 50ng/mL of PTx or lower concentrations.[25,26] The levels necessary for B oligomer effects are substantially higher than the levels accepted in aP vaccines (approximately 14.8ng PTx/mL[12]). The relevance of the direct effects of B oligomer for aP vaccines is therefore questionable, though B oligomer binding is essential for cell entry and therefore indirectly for S1 subunit functioning.

Table 2. Global requirements and recommendations for HIST.

		WHO	EU	US	Canada	China	Japan
		TRS 979	European Pharmacopeia	License dosier	License dossier	Chinese Pharmacopeia	Japanese Pharmacopeia
Vaccine	Testing stage	In-process (one or more dilutions)	Final lot	In-process or final lot*	In-process	In-process and final lot*	In-process and final lot*
	Residual or reversion to toxiciy**	Residual and or reversion	Residual and reversion	Residual***	Residual	Residual and reversion	Residual and reversion
	Volume	1 or 2 HD (one or more dilutions)	1–2 HD	500 μL	1 HD	500 μL	500 μL
Controls	Positive	PTx (one or more dilutions)	PTx (one dilution)	PTx (one dilution)	PTx (one dilution)	PTx (several dilutions)	PTx (several dilutions)
	Negative	Diluent or none	Diluent	Diluent	Diluent	N.S.	N.S.
Mice	Number of animals per group	10 or appropriate number	10	20	16	10	10
	Number of groups	App. 5	4	App. 3	3	App. 5	App. 6
	Min. animal number per test	App. 50	40	App. 60	48	App. 50	App. 60
Challenge and reading	Histamine dose	Defined dose (usually 1 or 2 mg)	2 mg	1 mg	0.7 mg	2–4 mg	4 mg
	Interval sample – challenge	4–5 days	5 days	5 days	5 days	4 days	4 days
	Period of observation	30 minutes-24 hours	24 hours	24 hours	24 hours	30 minutes	30 minutes
	Parameter	Decrease in temperature or death	Death	Death	Death	Decrease in temperature	Decrease in temperature

*In-process or final lot means that in-process products (antigen bulk or final bulk) or final lot are subjected to HIST, while in-process and final lot means both are subjected to HIST.
**Samples for residual toxicity are kept at 4°C, samples for reversion to toxicity are kept at 37°C for a specified period.
***Each manufacturer should put on stability testing at least one lot of each product per year, and it should be tested for HIST.
HD = Human dose.
N.S. = Not specified in the recommendation/guideline or regulation.

Table 3. Acceptance and validity criteria.

	WHO	EU	US	Canada	China	Japan
	TRS 979	European Pharmacopeia	License dossier	License dossier	Chinese Pharmacopeia	Japanese Pharmacopeia
Acceptance critaria	Residual activity of PTx or the number of animals that die is not higher than specified by the NRA. If a vaccine lot fails in a single test, it should pass 2 additional test for release.	The vaccine complies with the test if in the group that receives the vaccine stored at 2-8°C or 37°C, there are no deaths or no more deaths than in the group that receives the reference vaccine. If one mouse dies in one or both of the vaccine groups, repetition is allowed with the same number of mice or more. The vaccine is accepted when overall death rate is 5% or less.	One undiluted single human dose of 0.5 mL sensitizes no more than 10% of mice injected. If the vaccine fails to meet the criterion in a first test, it should pass 2 additional tests.	The vaccine complies with the test if in the group that receives the vaccine, there is no more than one death. If more than one mouse dies in the negative control group or the vaccine group, repetition is allowed with the same number of mice or more. The vaccine is accepted when overall death rate is 6.25% or less.	The histamine-sensitizing toxicity of both test samples at 4°C and 37°C shall be no higher than 0.8 HSU/mL in mice upon statistical analysis.	The histamine-sensitizing toxicity of both test samples at 4°C and 37°C shall be no higher than 0.4 HSU/mL in mice upon statistical analysis.
Validity criteria	1. Less than 5% deaths in the negative control group. 2. Demonstrated sensitivity of mice strain. 3. When linearity of log dose-response to PTx is demonstrated 1 positive control group suffice.	1. No mice die in the negative control group. 2. Sensitivity of the mice is demonstrated (e.g. 30% of the mice die in the positive control group). 3. A suitable mouse strain has a toxin LD50 between 6 IU and 50 IU.	1. PTx control group should show that mice used are sensitized by a dose of PTx below 100 ng, in terms of the HSD50. 2. No more than 10% of mice should die in the negative/diluent group.	1. There are at least 16 mice challenged per group. 2. No more than one mouse dies in the negative control group. 3. Sensitivity of the mice is demonstrated, i.e. at least 7 mice die in the positive control group (= 43.75%, mice injected with 400 ng of PTx).	N.S.	N.S.

NRA = National regulatory authority.
HSD = Histamine Sensitizing Dose.
HSU = Histamine Sensitization Units.
N.S. = Not specified in the recommendation/guideline or regulation.

provided in Tables 2 and 3. Both Japan and China apply the third version and assess the PTx content of a new batch of aP vaccine relative to a toxicity reference to which histamine sensitization units (HSU) have been assigned. The accepted levels of PTx are 0.4 and 0.8 HSU, for Japan and China, respectively. In Europe, the US and Canada, safety testing of aP vaccine batches is primarily based on the HIST with a lethal endpoint, although the second version of the HIST is also approved in Europe. Though the general outline of the lethal and temperature versions of the HIST are similar, there are notable regional differences (Tables 2 and 3). Independent of the test design, testing is performed in-process, on the final vaccine formulation or both. Apart from the regulatory requirements, the final lay-out of the test is influenced by the testing stage, that is, whether the test is the first one performed, a repeat of the first test or a retest. Repetition of a HIST is required when the validity criteria of the test are not met, and test validity is primarily determined by the responses of the positive and negative control groups. Generally, for repeated testing the same design and number of animals are used. Retests are performed when a vaccine lot does not meet the acceptance criteria. For retesting, in general, twice the number of animals is required for the lethal endpoint method, while the same number of animals is required when the temperature method is used. It appears, from this survey (data not shown), that repeated testing and retesting occurs more often for HIST protocols based on lethality as an endpoint (2.5%-7.5%), than for HIST protocols with the body temperature as a readout parameter (less than 1%), at least for the tests performed in Asian countries. The reason could be the inherent higher sensitivity of the temperature method and the relative evaluation of residual PTx to a PTx reference that enables compensation of assay variation.[37]

The HIST and laboratory animal use

To estimate the impact of the HIST in terms of animal numbers, a survey was conducted among manufacturers, national control laboratories (NCLs) and multinational organizations involved with vaccine quality. Sanofi Pasteur SA (France), Sanofi Pasteur Limited (Canada), GlaxoSmithKline Biologicals (Belgium) and Statens Serum Institut (Denmark) were asked to specify how many lots of vaccine containing an aP component were subjected to the HIST, either in the year 2012 or 2013 and to indicate the specifications of the HIST performed and procedural details, including the number of animals used. Information on test design was combined with literature on aP vaccine production in Japan and China.[38,39] Manufacturers provided their consent for publication of the data.

Following safety testing by the manufacturers, NCLs may also subject aP vaccine lots to the HIST. Eight NCLs/organizations (Pan American Health Organization (PAHO), US FDA, Health Canada (Canada), European Directorate for the Quality of Medicines & Health Care, Statens Serum Institut (Denmark), Indian Academy of Pediatrics (India), National Institute of Infectious Diseases (Japan), and National Institutes for Food and Drug Control (China)) were queried for their testing strategies, or the testing strategies of the NCLs in the region. The organisations were given random code numbers herein, not related to the order above. Organizations 1–4 provided complete information or as far as possible (for details see Table 4). Organizations 5 and 6 were unable to provide information, and therefore we assumed a testing regime in which 50% of the lots were tested. Precise information on testing by NCLs could not be provided by organization 7, while organization 8 indicated that aP vaccines were not produced in that region. Since exact numbers were only partly available, some of the data were estimated as shown in Table 4. Although our calculations attempt to provide an estimation of the number of mice required for the HIST on a global scale (Table 5), in reality they cover only the manufacturers and NCLs in the regions in which aP vaccines are mainly produced and used (Table 1). For example, one organization reported the purchase of aP-containing vaccines for 14 countries from 3 of the manufacturers surveyed, but data on additional HIST testing by NCLs at the local level, if any, were not available. The data are presented masked.

To calculate animal numbers used for the HIST, 2 assumptions were made, in consultation with the manufacturers and NCLs. Firstly, the percentages of repeated testing and retesting were known or estimated to occur between 0% and 16% and 0% and 7.5% of the cases (as indicated), respectively. Secondly, when information on testing by a NCL was not available, we assumed that 50% of the lots being released by these NCLs were tested, and that repeated testing and retesting frequencies were similar to those of the manufacturers. Evaluation on the HIST performance revealed substantial regional differences in the number of animals used for a single test, as well as group size (10 to 20 animals per group), and inclusion of positive and negative control groups (data not shown). Based on the evaluation we estimated that worldwide approximately 65,000 mice are used for the HIST each year, ~48,000 by the manufacturers and ~17,000 by the NCLs (Table 5). These tests covered the release of approximately 850 final lots and 250 in-process lots of aP vaccines each year, of which 152 were destined for the Canadian and international markets, 251 for the United States, 474 for Europe, and 193 for China and Japan. 27 additional lots/batches were produced for non-commercial purposes. These vaccines were deployed mostly for the protection of the population of industrialised countries, and a few emergent economies, against pertussis.

The HIST and 3R alternatives: perspectives

The scientific and animal welfare concerns associated with the HIST provide a strong incentive for the development and application of 3R methods. In general, replacement of an animal method by an animal-free method takes time during which there may be opportunities for refinement and reduction before replacement. In this context, we evaluated whether there were any possibilities to reduce animal numbers for the HIST. Surprisingly, there were marked differences in group sizes used, primarily for the lethal version of the HIST, ranging from 10 to 22 animals per group (data not shown). These differences in numbers are mainly associated with local regulatory practices although they may be justified by assay variability, as a consequence of the use of different mouse strains[11] or different vaccination-challenge schedules. There is no evident scientific justification for applying various group sizes, since the various

Table 4. Origin of information on animal numbers.

Manufacturer/ Organization code	Year	Number of in-process lots	Number of final lots	Average number of final lots/ in-process lots per test*	Average number of animals/ test	Total animal number	Percentage of animals used for first test	Percentage of animals used for repeated tests	Percentage of animals used for retesting
M1	2013	N.T.	Known	Estimated	Calculated	Known	Estimated	Estimated	Estimated
M2	2012	Known	N.T.	Known	Known	Calculated	Estimated	Estimated	Estimated
M3	2013	N.T.	Calculated	Estimated	Calculated	Known	Estimated	Estimated	Estimated
M4	2012	N.T.	Known	Known	Known	Known	Known	Known	Known
Or1	—	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
Or2	—	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
Or3	2012	N.T.	Known	Known	Known	Calculated	Known	Known	Known
Or4	2012	N.T.	Known	Known	Known	Calculated	Known	Known	Known
Or5	—	N.T.	Estimated	Estimated	Estimated	Estimated	Estimated	Estimated	Estimated
Or6	—	N.T.	Known	Estimated	Estimated	Estimated	Estimated	Estimated	Estimated
Or7	—	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
Or8	—	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.

N.T. = Not tested, testing is not done or required by indicated organization.
M = Manufacturer.
Or = Control, Regulatory or Guiding Organization.
Known: Numbers were provided.
Estimated: Numbers could not be provided and were therefore estimated in consulation with the organization/manufacturer.
Calculated: Exact numbers were unavailable, but could be calculated based on the information indicated as 'known'.

performances of the HIST are based upon the same principle, and since each group size is accepted by one or more NCLs. Therefore, a possible approach to reduce animal numbers for the HIST would be using the smallest group size (i.e. 10 animals per group). Recalculations revealed that universal application of this group size would reduce total animal numbers by 10% (data not shown). Another option to diminish the animal use, might be the adoption of testing plans by NCLs with reduced testing frequency. It should be noted, however, that the number of mice used by NCLs may be lower than the number reported. As the actual frequency is often confidential, we had been forced to estimate it. In any event, application of either of these approaches requires international consensus, which has proven to be a challenging process. In addition to reducing animal numbers, another 3R option is to minimize the distress inflicted to the animals. As mentioned before, the presence of PTx can be assessed by changes in body temperature, a method applied in some Asian countries and by SSI in Denmark.[36] A drop in rectal or dermal temperature as a consequence of PTx is assessed within 30 minutes of histamine challenge.[37] Application of this method might therefore reduce the duration of potential suffering.[36] However, the majority of the deaths occur within the 30 minutes following the histamine injection,[37] and these fatalities most likely happen among the animals that experience the highest level of suffering. Using body temperature as a readout parameter may only reduce suffering of animals that receive a borderline lethal level of PTx. In a study of the transferability of the non-lethal test for PTx in aP vaccines,[40] the maximum decrease in temperature post-histamine challenge was achieved with a dose as low as 1.75 IU of PTx per mouse, a dose which already caused ≥ 50% death in groups of 10 mice, in 4/10 experiments. This represents a high level of lethality in a non-lethal alternative, if we consider that a collectively agreed suitable baseline HIST sensitivity is 2.0 IU of PTx per mL of vaccine.[12,13] Taken together, possibilities to reduce and refine the HIST are available, but it is questionable whether the gain in animal welfare justifies the efforts needed for validation and implementation, considering the number of animals needed for validation. In addition, refinement and reduction alternatives still encompass the variation inherent to the principle of the HIST, and several promising *in vitro* methods are in the final stage of development. Thus, given the lack of clear benefits of the refinement and reduction alternatives, some National Regulatory Authorities (NRA), NCLs and manufacturers may opt not to pursue them.

In addition to refinement and reduction, several research groups have attempted to develop animal-free methods for replacement of the HIST. Generally, the acceptance of an alternative method would be easier to justify if it is based on a principle which has similarity with pharmacologically relevant mechanisms of PTx.[41-43] As discussed before, the mechanisms primarily involved in the HIST have been elucidated. However, the relationship between the mechanisms of the HIST and the pharmacological effects of PTx in humans is not totally clear. As mentioned in Box 1, theoretically B oligomer

Table 5. Estimated annual number of animals used for HIST.

		Total
Manufacturer	Number of final lots or in-process lots tested	1100
	Number of tests	640
	Number of animals per group	10–22
	Average number of animals per test*	40–132
	Total animal number	**47700**
	*Of which used for first test***	43800
	*Of which used for repeats****	1100
	Of which used for retesting§*	2800
Con Or	**Total animal number**	**17100**
	Of which used for first test§§*	15600
	Of which used for repeats§§§*	400
	Of which used for retesting§*	1100

Con Or = Control organization.
*Numbers depend on group size, inclusion of positive and negative controls and the number of final lots/in-process products tested.
**Percentage of animals used for this purpose varied between 79–100%.
***Percentage of animals used for this purpose varied between 0–16%.
§Percentage of animals used for this purpose varied between 0–7.5%.
§§Percentage of animals used for this purpose varied between 90–100%.
§§§Percentage of animals used for this purpose varied between 0–2.5%.

and S1 may have direct and independent effects on cell functioning. Nevertheless, the concentration of PTx that has raised concern for vaccine safety is one at which only S1 is able to induce cellular effects, while the direct activity of the B oligomer at these concentrations (Box 1) has not been described. Noteworthy is that a functional B oligomer is necessary for cell binding and internalisation and therefore essential for S1 intracellular effects.

There has been debate on what the acceptable and clinically relevant level of PTx in aP vaccine is to guarantee vaccine safety.[12,44] Only indirect evidence is available to approach this question, since – for ethical reasons – it is not possible to carry out clinical studies to quantify toxic PTx effects in the clinic. Nonetheless, a study of the therapeutic effects of PTx for diabetes revealed that the intravenous injection of 1 μg/kg of the protein did not cause any pronounced adverse effects in healthy volunteers.[45] Additionally, clinical evidence for a relevant level of PTx is provided by studies showing that the PTx content of whole cell vaccines is approximately 0.3 μg/mL,[11,46] while these vaccines are generally considered to be safe and efficacious. Also, the HIST itself provides information by means of its sensitivity and acceptance criteria. According to the guidelines of the European Pharmacopeia (Ph. Eur.), a mouse strain is generally considered suitable for the HIST if the LD_{50} for Ph. Eur.'s Biological Reference Preparation (BRP) for PTx is between 6 IU and 50 IU.[47] A vaccine is accepted if not more than 5% of the immunized mice die when challenged with histamine. Based on this information and data from a collaborative study organized by the EDQM,[48] the LD_5 for the BRP1 PTx standard was estimated to be approximately 2.22 IU/ml [12] (corresponding to 14.8 ng/mL for BRP PTx batch 1).

There has been a range of initiatives aimed at replacing the HIST by suitable animal-free methods. One advanced alternative entails a combination of 2 analytical methods. The first of the 2 methods assesses the binding of PTx to a fetuin coated ELISA plate,[49,50] and mimics B oligomer's binding to glycoproteins on the cell surface.[15] The second method is based on the HPLC measurement of the enzymatic activity of the A subunit, using a fluorescent peptide.[51-53] The combination of these assays reflects properties essential for PTx toxicity, although not simultaneously, and has proven to be a useful tool to study the inactivation procedures.[54] However, the assays do not cover the internalisation and translocation of the toxin from the cell membrane to the cytosol, nor is intracellular ADP-ribosylation or its effects measured. These latter aspects are an inherent part of the pharmacological effects of the toxin, and are addressed by the 3 *in vitro* cell culture alternatives. The first of these methods is the Chinese Hamster Ovary cell (CHO) test, based on a PTx-induced clustered growth pattern.[55,56] A study in which genetically modified CHO cells constitutively expressed the S1 subunit demonstrated that this moiety was ultimately responsible of the cell clustering.[57] Nevertheless, under physiological conditions, B oligomer binding and internalisation of the toxin will be necessary for S1 to execute its effect in the cytosol. This CHO cell clustering test is only suitable for evaluation of pre-formulated and non-adjuvated products, due to inherent toxicity of the adjuvants for CHO cells,[44] though recent studies have demonstrated that these cytotoxic effects can be overcome by dilution or the use of transwell inserts.[58] Another hurdle is the visual reading of the cell clustering. Despite attempts of several research groups, automatic analysis reading is not yet possible. Nevertheless, the CHO-cell assay was deemed by the International Working Group on Alternatives to HIST at a recent workshop in London as deserving further development/validation.[59] The other 2 cell-based methods assess the intracellular effects of PTx on adenylate cyclase regulation, by measuring changes in hormone-stimulated cAMP or ATP levels.[25,26,44] Since toxin binding, internalisation and disturbance of the regulation of adenylate cyclase are intrinsic elements of the adverse outcome pathways, the methods cover all cellular aspects relevant for the safety of aP vaccines. Proof-of-principle for the cAMP-PTx assay has been positive and further development (pre-validation) of this quantitative method has been supported.[44] In conclusion, there are various animal-free alternatives in different stages of development, which reflect several distinct properties of PTx. A strategic, stepwise plan was proposed at the London meeting of the International Working Group on Alternatives to HIST,[59] in which an alternative test would be adopted for release purposes first, and then, once sufficient confidence in its suitable performance has been gained, its use would be extended to stability testing. On the other hand, representatives of regulatory organizations present agreed, in principle, that the approval of the elimination of the HIST testing requirement from the stability program might be entertained if manufacturers would be able to provide unequivocal evidence that the chemical treatment used to detoxify PTx yields irreversible toxoid. Such a removal would obviously have a major impact on the number of animals used for aP vaccine safety testing, while toxin inactivation by chemical procedures will continue, being demonstrated in-process by the CHO-cell assay on every bulk of PTd before its adsorption to adjuvant.

Concluding remarks

A survey of vaccine manufacturers and regulatory bodies has allowed us to estimate the use of mice for safeguarding the safety of recipients of aP vaccines worldwide at about 65,000. Although this figure constituted a useful baseline for discussing a plan for the replacement of HIST,[59] its accuracy should not be overestimated, and the challenges associated to its estimation (summarized in Table 4) should not be ignored. For example, while manufacturers test 100% of the lots introduced into the market, the proportion of lots tested by some NCLs had to be estimated, due to their confidentiality policies. Though the HIST has ensured the release of safe aP vaccines for many years, there is now reasonable scientific and animal welfare justification to change this testing regime. There are means to refine the test and reduce the animal number for the existing testing strategy. However, it is questionable whether there would be a meaningful gain in animal welfare, given that there is often a lethal outcome in the ostensibly non-lethal test. Thus, given the lack of a clear benefit, some NCLs and manufacturers may not consider worthwhile the efforts needed for validation and implementation of these interim alternatives in the path to replacement, especially since various *in vitro* alternatives are under development. However, if an interim reduction strategy is sought, NCLs may consider a reduction scheme (if one is not already in place), where only a limited number of batches of

each product is tested per year. This paper discusses several *in vitro* alternatives that have scientifically sound mechanistic substantiation, a more permanent solution. After appropriate validation, these alternatives have the potential to replace the HIST all together, although a careful comparative evaluation of their mechanisms, as well as the sensitivity of each test, will be part of the selection process. With regard to replacement, a reference should also be made to one of the conclusions of the International Working Group on Alternatives to HIST's London meeting, suggesting a stepwise adoption of one or a combination of these *in vitro* methods. An eventual elimination of the HIST testing requirement might be considered if there is unequivocal evidence that reversion to toxicity cannot occur. Any of these approaches will significantly reduce the number of animals that are currently used for the HIST.

Disclosure of potential conflicts of interest

The authors declare that there is no conflict of interest.

Acknowledgments

We are grateful to all the manufacturers, national control laboratories (NCLs) and multinational organizations for their input and for providing the information essential for this survey.

Funding

This study was part of a project funded by the Dutch Ministry of Health, Welfare and Sports and the Dutch Ministry of Economic Affairs that aims to develop alternatives to animal use.

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