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Effect of different detoxification procedures on the residual pertussis toxin activities in vaccines

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ABSTRACT

Pertussis toxin (PTx) is a major virulence factor produced by *Bordetella pertussis* and its detoxified form is one of the major protective antigens in vaccines against whooping cough. Ideally, PTx in the vaccine should be completely detoxified while still preserving immunogenicity. However, this may not always be the case. Due to multilevel reaction mechanisms of chemical detoxification that act on different molecular sites and with different production processes, it is difficult to define a molecular characteristic of a pertussis toxoid. PTx has two functional distinctive domains: the ADP-ribosyltransferase enzymatic subunit S1 (A-protomer) and the host cell binding carbohydrate-binding subunits S2–5 (B-oligomer); and in this study, we investigated the effect of different detoxification processes on these two functional activities of the residual PTx in toxoids and vaccines currently marketed worldwide using a recently developed *in vitro* biochemical assay system. The patho-physiological activities in these samples were also estimated using the *in vivo* official histamine sensitisation tests. Different types of vaccines, detoxified by formaldehyde, glutaraldehyde or by both, have different residual functional and individual baseline activities. Of the vaccines tested, PT toxoid detoxified by formaldehyde had the lowest residual PTx ADP-ribosyltransferase activity. The carbohydrate binding results detected by anti-PTx polyclonal (pAb) and anti-PTx subunits monoclonal antibodies (mAb) showed specific binding profiles for toxoids and vaccines produced from different detoxification methods. In addition, we also demonstrated that using pAb or mAb S2/3 as detection antibodies would give a better differential difference between these vaccine lots than using mAbs S1 or S4. In summary, we showed for the first time that by measuring the activities of the two functional domains of PTx, we could characterise pertussis toxoids prepared from different chemical detoxification methods and this study also highlights the potential use of this *in vitro* biochemical assay system for in-process control.

1. Introduction

Pertussis toxin (PTx) is a major virulence factor produced by *Bordetella pertussis* and is one of the major protective antigens in vaccines against whooping cough. Ideally, PTx in the vaccine should be completely detoxified while still preserving immunogenicity. However, this may not always be the case and a safety test for residual PTx activities present in these vaccines are required by authorities before they are released to the public. PTx has two functional distinctive domains: the enzymatic subunit S1 (A-protomer), which is an ADP-ribosyltransferase and the subunits S2–5 (B-oligomer), which is a carbohydrate binding molecule that facilitates host cell binding and entry of PTx into the cells [1]. The current detoxification processes include chemical treatments of the toxin with formaldehyde and/or glutaraldehyde, hydrogen peroxide and genetic detoxification [2]. Formaldehyde reacts with side chains of lysine, arginine, cysteine, histidine and N-terminal amino acids and formaldehyde-glycine Schiff-base adduct could also react with the primary N-terminal amino group, arginine, tyrosine as well as asparagine, glutamine and tryptophan [3]. Glutaraldehyde is a bifunctional protein cross-linking reagent and reacts similarly

Abbreviations: AsF, asialofetuin; BSA, bovine serum albumin; D, diphtheria toxoid; EDTA, ethylene diamine tetra-acetic acid; Fet, fetuin; FHA, filamentous haemagglutinin; Fims2/3, fimbriae type 2 and type 3; F, formaldehyde; g-PTd, genetically inactivated PTx toxoid; G, glutaraldehyde; Hib, Haemophilus influenzae type b; HPLC, high performance liquid chromatography; HIST, histamine sensitisation test; HRP, horseradish peroxidase; H_2O_2, hydrogen peroxide; IPV, inactivated poliovirus vaccine; i.p., intra-peritoneal; mAb, monoclonal antibody; OD, optical density; PRN, pertactin; PTx, pertussis toxin; PTd, pertussis toxoid; PBS, phosphate buffered saline; PBSG, PBS gelatine; pAb, polyclonal antibody; rcf, relative centrifugation force; SHD, single human dose; T, tetanus toxoid.

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to formaldehyde to form more complex high molecular weight protein species [4–7]. In PTx toxoid prepared by oxidation of hydrogen peroxide, nearly all methionine and cysteine were oxidised and tryptophan, histidine, tyrosine and asparagine were also modified [8]. Genetic detoxification of PTx could be achieved by substitution of two amino acids (9K/129G) at the enzymatic active site in subunit S1 and the resultant recombinant inactive PTx molecule could then be stabilised by low dose of formaldehyde [9].

Due to multilevel reaction mechanisms of chemical detoxification, characterisation of sites of modification of the resultant PTx toxoid (PTd) is often difficult. Varying degrees of alternation either an enhancement or a decrease in the PTx antibody binding or neutralising epitope of the PTd had been reported [2]. Presence of heterogeneous high molecular weight species had also been shown in PTds detoxified with increasing formaldehyde concentration [10]. In a recent study of chemically detoxified PTx using a patented procedure [11] with formaldehyde, glutaraldehyde and both, Oh et al. [12] found that different aldehydes could affect differently on the PTx functional domains. They showed that formaldehyde inactivated the enzymatic subunit S1 better than glutaraldehyde while the opposite was true for the carbohydrate binding subunits S2–5. The authors could also demonstrate that under their detoxification conditions, the stability or reversion of the toxoids were different between the chemical reagents.

In one of our earlier studies on carbohydrate binding activity of PTx and PTd, we demonstrated that a glutaraldehyde-detoxified PTd lost almost all of its ability to bind trisialylated tri-antennary *N*-glycans but the binding capacity to neutral tri-antennary *N*-glycans was less affected [13]. The degrees of carbohydrate binding activities had also been shown to relate to local reactogenicity due to changes to vaccine detoxification and production methods in Japan [14]. This clearly illustrates that different production methods of PTd would result in vaccines with different biochemical and biological residual PTx activities. Here we reported our further investigation into the functional changes of PTx in different PTds and pertussis vaccines, of which some are currently marketed worldwide, using the *in vitro* biochemical and *in vivo* biological assay methods.

2. Materials and methods

2.1. Reference pertussis toxin

B. pertussis toxin (PTx, NIBSC Code: 90/518) was prepared in-house at National institute for Biological Standards and Control (NIBSC). This preparation has a nominal protein concentration of 20 μg/ampoule and an assigned unitage of 2,100 IU/ampoule calibrated against the 1st IS, JNIH-5 by histamine sensitisation test (HIST) [15].

2.2. Vaccines and toxoids

Acellular pertussis vaccines used in this study was all licensed, commercially available products (Table 1). The pertussis antigens PTd, filamentous haemagglutinin (FHA), pertactin (PRN) and fimbriae type 2 and type 3 (Fims2/3) were either co-purified/or individually purified, detoxified and were in combination with diphtheria toxoid (D), tetanus toxoid (T) or other antigens such as Haemophilus influenzae type b (Hib) capsular polysaccharide conjugated to T or inactivated poliovirus vaccine (IPV). Information on pertussis antigen composition in these vaccines is presented in Table 1. Genetically inactivated pertussis toxoid (g-PTd), hydrogen peroxide (H_2O_2)-detoxified toxoids, glutaraldehyde (G)-, formaldehyde (F)- and glutaraldehyde + formaldehyde (G + F)-detoxified pertussis vaccines were kindly provided by different vaccine manufacturers. Anti-PTx sheep polyclonal (pAb, NIBSC Code: 97/572) and anti-PTx subunits monoclonal antibody (mAb) S1 (NIBSC Code 99/506), S2&3 (NIBSC Code 99/534), S4 (NIBSC Code 99/554) were prepared at NIBSC. Fetuin (Fet, F3004) and asialofetuin (AsF, A4781); 2, 2′azinobis (3-ethylbenzothiazoline-6-sulfonic acid) di-ammonium salt (A9941); hydrogen peroxide (H3410); horseradish peroxidase (HRP)-labelled anti-sheep IgG (A3415) and HRP-labelled anti-mouse IgG (A0168) were from Sigma–Aldrich (Poole, UK).

2.3. ADP-ribosyltransferase activity assay

The determination of PTx ADP-ribosyltransferase activity in PTds and vaccines was performed using a synthetic fluorescein-labelled peptide substrate, F-$G_{\alpha i3}$C20 (Cat # HCAM-2; AnaSpec Inc., San Jose, CA, USA) and the separation and quantitation of the ADP-ribosylated product was by reverse-phase HPLC as described in details previously [16,17]. Prior to enzymatic determination, appropriate dilutions, range between neat to 1:20, of PTds and vaccine samples were made in ovalbumin solution (2 mg/ml) so that the resultant ADP-ribosyltransferase activity would be within the PTx (90/518) standard curve range (0–400 ng/ml). Total enzymatic activity is presented as E-units (EU or 1000 mEU) where 1 EU equals to fluorescence produced by 1 μg of PTx (90/518) under identical assay and analysis conditions. Unless otherwise stated, all assays were performed in duplicates and met the in-house assay validity criteria. The results are presented as means ± standard deviation (SD) or percentage coefficient of variation (%CV).

2.4. Carbohydrate-binding assay

The PTx carbohydrate-binding activity in vaccines using either Fet (containing trisialylated triantennary *N*-glycans) or AsF (containing non-sialylated triantennary *N*-glycans) as carbohydrate ligands was performed as described by Gomez et al. [13]. Vaccines and PTx were treated 1:1 (v/v) with freshly prepared desorption reagent (0.15 M EDTA disodium salt dissolved in 0.52 M di-sodium hydrogen orthophosphate) overnight at 37 °C to release the antigen from the aluminium adjuvant. After the desorption process, vaccines were centrifuged at 2000 RPM (400 rcf) for 10 min and the supernatant, usually used as neat solution and up to 3 serial twofold dilutions in diluent (3% BSA-PBS-0.05% TWEEN 20) were used in the assay. To construct standard binding curves, one ampoule of PTx

Table 1
Acellular pertussis vaccine information. Vaccines (V1–3) were chemically detoxified by either glutaraldehyde (G), formaldehyde (F) or glutaraldehyde + formaldehyde (G + F); absorbed onto either alum phosphate (PO_4) or hydroxide (OH) adjuvants; vaccine formulation and types were as indicated: PTd, detoxified PT; FHA, filamentous haemagglutinin; PRN, pertactin; Fims, fimbriae type 2 and type 3; DTaP, diphtheria tetanus and acellular pertussis; IPV, inactivated poliovirus vaccine; Hib, Haemophilus influenzae type b; n/a, not containing.

Vaccine code	# of batches	Detoxification	Adjuvant-type	Pertussis antigen concentration (μg/SHD)				Vaccine type
				PTd	FHA	PRN	Fims	
V1	49	G	PO_4	20	20	3	5	DTaP + IPV + Hib
V2	19	F	PO_4	23.4	23.4	n/a	n/a	DTaP
V3	23	G + F	OH	25	25	8	n/a	DTaP ± IPV

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