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ORIGINAL ARTICLE

Differences in epitope-specific antibodies to pertussis toxin after infection and acellular vaccinations

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Abstract

Objectives. Pertussis toxin (PT) is a component of all acellular pertussis vaccines. PT must be detoxified to be included in acellular vaccines, which results in conformational changes in the functional epitopes of PTs. Therefore, induced epitope-specific antibodies to PT may vary after vaccinations or natural infections, and this information could reveal biomarkers implicated for protection and successful immunisation. **Methods.** Pertussis toxin epitope-specific antibodies in sera from 152 vaccinated children and 72 serologically confirmed patients were tested with a blocking ELISA, based on monoclonal antibodies that target protective PT epitopes. **Results.** All study groups induced considerable antibody titres to subunit 1 (S1). Of interest, S3 7E10-specific antibodies were present in patients, but not after vaccinations ($P < 0.001$). The impact of glutaraldehyde treatment of PT was visible on epitope 1D7 (S1), whereas epitopes 1B7 (S1) and 10D (S1) were more preserved. Antibodies to these epitopes were higher after three primary vaccine doses than after a single booster dose. **Conclusion.** The high amount of 7E10-specific antibodies in patients suggests this epitope might be functionally relevant in protection. The overall characteristics of epitope-specific antibodies are influenced by infection or vaccination background, by the used detoxification method of PT and by the amount of the toxin used in immunisation.

Keywords: ELISA, epitope, monoclonal antibody, pertussis, pertussis toxin

INTRODUCTION

Since the introduction of acellular pertussis vaccines (aP) for primary and booster immunisations, the epidemiology of pertussis has changed substantially all around the world. The noted resurgence of the disease seems to be caused by multiple factors, including the adaptation of circulating *Bordetella pertussis* strains, improved diagnostic methods, better

surveillance and waning of vaccination-induced immunity.[1–8] Although aPs have been in use for more than two decades, a challenge for their further development has been the lack of well-established immune correlates for protection (in humans) to evaluate the protective efficacy of vaccines.[9] Further, general assumptions towards protective properties of aPs are not straightforward, as aPs from various manufacturers vary in the quantity of antigens, in methods of purification and inactivation of vaccine antigens.

The effectiveness and duration of immunity after a pertussis vaccination are related to both cellular and humoral immune responses. Several studies have demonstrated qualitative differences in T-cell responses, as T-cell responses after recent infection and whole-cell pertussis vaccination tend towards priming of Th1/Th17 cells, whereas aPs induce a more dominant Th2/Th17 response.[10] When considering the humoral immunity, antibodies may function by neutralising bacterial antigens, preventing bacteria from binding to epithelial cells or enabling the uptake and destruction of bacteria by phagocytes. Even though antibody responses decline rapidly after immunisation with most aPs, cell-mediated immunity is maintained for several years.[11–13] Thus far, studies with aPs illustrate that serological correlates against pertussis toxin (PT), pertactin and fimbrial antigens could contribute to the protection against pertussis.[14]

Pertussis toxin is one of the main virulence factors of *B. pertussis*, and it is included in every aP.[15] The toxin consists of five subunits: an enzymatically active catalytic ADP-ribosyltransferase subunit 1 (S1) (also referred as the A-oligomer) and subunits 2–5 (S2–S5), which form a heteromultimeric complex (the B-oligomer), which has multiple roles, including target cell receptor binding.[16–20] Because of the toxic properties of PTs, native PT (PTx) must be detoxified (PTd) for it to be used in vaccines either chemically with formaldehyde, glutaraldehyde and hydrogen peroxide, or through genetic modifications. The detoxification procedures inactivate the mitogenic, hemagglutinating and ADP-ribosyltransferase activities of PT and stabilise the molecule, but the process also modifies the protein structure and surface epitopes. More specifically, formaldehyde treatment of PT inactivates the enzymatic activity of S1 more effectively than glutaraldehyde, and conversely less in S2–S5.[21–24] Structural changes following the inactivation of PT with hydrogen peroxide have been reported to impair the epitope binding activities of mAbs towards the molecule. However, fewer epitopes appeared to be affected in comparison with other chemical treatments. [21] These modifications cause vaccine-induced antibody responses to preferentially recognise PTd over PTx, and could, therefore, affect the protective outcome through the quality of antibodies.[21,25,26]

Multiple studies have described PT-specific (m)Abs, antigenic determinants and antibody recognition sites of PT from either peptide fragments, recombinant truncated S1 molecules, synthetic peptides or plasmablast clones.[27,28] Furthermore, the functional protective characteristics of these (m)Abs have been evaluated in numerous ways, with many of these (m)Abs successfully neutralising either the A- or B-oligomer functions of PT. In addition, their exact sequence specificity, as well as the connection between their antibody-mediated neutralisation capabilities to specific PT epitopes, has been addressed in the field. Despite this knowledge, the binding sites of human-induced antibodies and their effect on protection have not been studied thoroughly, except for mAbs targeting two epitopes 1B7 and 11E6.[26–28]

Sato & Sato developed a total of 32 mAbs to different PT subunits and compared their protective characteristics regarding ADP-ribosylation inhibition, CHO cell clustering, leucocytosis-promoting activity and intracerebral and aerosol challenge in a mouse model.[29–31] Many of these mAbs effectively neutralise activity of PTs *in vivo*, and mAbs 1B7 and 11E6 have also been proven to be protective against the disease in baboons.[32] Hence, measurement of vaccine-induced antibodies to similar epitope determinants may aid in the development of future vaccines as more precise correlates of protection that could be applied more broadly and ideally even to all pertussis vaccines. We aimed to study the presence and magnitude of specific antibodies against three subunits with five distinct PT binding mAbs in pertussis patients as well as vaccine recipients. Further, the effect of different detoxifications was compared with serum samples collected from Finland, where glutaraldehyde- and formaldehyde-treated PT is used in the vaccines, and from Denmark, where a vaccine containing hydrogen peroxide-treated PT

Table 1. Pertussis vaccination programmes in Denmark and Finland at the time of the study

Country	Acellular pertussis vaccine since	Primary vaccination	Booster vaccinations	Antigens	Approximate coverage (%) (primary)
Denmark	2004	3, 5, 12 months (DTaP-IPV/HIB)	5 years, Tdap-IPV	Solely hydrogen peroxide-treated pertussis toxin (PT) (40μg)	> 95
Finland	2005	3, 5, 12 months (DTaP-IPV-Hib)	4 years, dTaP-IPV, 14–15 years dtap	Glutaraldehyde-/formaldehyde-detoxified PT (25 μg in primary, 8 μg in booster), pertactin, filamentous hemagglutinin	> 95

has been used until recently (Table 1). Further, in this paper, we refer to the name of these mAb binding sites as names of corresponding epitopes. Our findings highlight a deficit of vaccination-induced antibodies especially towards subunit 3 epitope 7E10, and to an extent towards epitopes 11E6 (S23) and 1D7 (S1). Such differences in PT epitope-specific antibody responses between vaccination and natural infection could be considered as promising serological correlates of improved protection in humans, or even as biomarkers for separating infection- and vaccination-induced anti-PT antibodies.

RESULTS

Epitope-specific antibodies after infection and vaccinations

Since the anti-PTx IgG concentrations were in a similar range in each group, the results between vaccinated subjects and patients were comparable. Moreover, within countries, the anti-PTx IgG concentrations did not differ significantly between the study groups. The inter-assay coefficient of variation (CV) was calculated from the values of negative sample control in epitope measurements, that included all study samples, resulting in a 13.7% variation in epitope 1B7 and 15.0% in 7E10. The intra-assay CV was counted from 5 parallel wells of 1% BSA-PBS, negative and positive control samples resulting in 1.54%, 2.42% and 3.5% variation, respectively.

The initial screening of 27 PT-specific mAbs with sera after infection revealed a weak response to certain epitopes, mainly including those to S4 and S5 targeting epitopes. Further, mAbs 1B7, 1D7, 10D6, 11E6 and 7E10 were tested with the Finnish study groups. The specific inhibition readout for each group and tested epitope is presented as mean values in Table 2 and as box plots in Figure 1. Altogether, there was significantly more antibody binding to 1D7, 11E6 and 7E10 after infection in comparison with vaccinations ($P < 0.001$). In further comparisons between the individual Finnish study groups, significantly fewer antibodies were quantified against 1D7 from both Finnish single-dose booster study groups in comparison with patients and primary vaccinated babies ($P < 0.001$). The primary vaccinated group had also significantly more bound antibodies to the 10D6 epitope in comparison with other Finnish study groups ($P < 0.05$).

Table 2. Mean values of specific inhibition to 1B7, 1D7, 10D6, 11E6 and 7E10 PTx/PTdg binding epitopes by the study groups

	Anti-PTx IgG (median, range)	Mean[a] (95% CI) of epitope-specific inhibition (%)								
		1B7 (S1) (PTx)	1D7 (S1) (PTx)	10D6 (S1) (PTx)	11E6 (S23) (PTx)	7E10 (S3) (PTx)	1B7 (PTdg)	1D7 (PTdg)	10D6 (PTdg)	7E10 (PTdg)
Finnish patients $N = 51$	118 (55–255)	16.9 (14.9–19.0)	12.8 (10.6–15.1) $N^b = 37$	13.4 (11.2–15.6) $N = 37$	7.8 (5.4–10.2)	22.6 (18.8–26.3)	–	–	–	–
Finnish 13-m primary vaccine $N = 50$	101 (63–238)	19.1 (16.2–22.1)	11.9 (9.4–14.4) $N = 31$	22.4 (19.2–25.5) $N = 31$	3.4 (2.0–4.7)	6.8 (5.1–8.6)	–	–	–	–
Finnish 4- to 5-year booster vaccine $N = 30$	104 (60–233)	14.1 (10.6–17.7)	4.4 (2.3–6.6) $N = 26$	14.4 (11.9–17.0) $N = 26$	2.2 (0.5–4.8)	7.1 (3.6–10.5)	42.5 (36.7–48.3)	40.4 (34.6–46.2) $N = 26$	41.4 (35.8–47.0) $N = 26$	14.1 (10.8–17.5)
Finnish 11- to 13-year booster vaccine $N = 50$	94 (50–232)	13.8 (11.4–15.9)	5.3 (3.3–8.3) $N = 31$	8.0 (6.7–10.0) $N = 31$	4.6 (3.0–6.2)	5.2 (3.1–7.3)	–	–	–	–
Danish patients $N = 21$	149 (62–266)	22.0 (18.8–25.3)	13.3 (9.2–17.2)	–	–	19.3 (15.5–23.2)	39.5 (35.4–43.6)	37.6 (33.6–41.5)	–	18.7 (15.3–22.1)
Danish 5- to 6-year booster vaccine $N = 22$	134 (52–261)	15.1 (12.1–18.1)	9.8 (5.7–14.0)	–	–	5.2 (1.6–7.5)	30.9 (24.3–37.5)	26.4 (19.3–33.5)	–	11.9 (8.3–15.6)

[a]Arithmetic mean of the sample group's specific inhibition.
[b]Sample size is specified separately for epitopes, which did not include all samples in the group.

A significant difference in the level of antibody binding to epitope 7E10 of S3 was observed between Finnish patients and Finnish vaccinated study groups ($P < 0.001$). In this epitope, specific inhibition by antibodies after infection averaged 22.5%, which correlated with overall anti-PTx IgG titres [Pearson's $r = 0.35$, 0.079–0.617 95% confidence interval (CI), $P = 0.012$]. In comparison, the inhibition by antibodies after vaccination averaged 6.3% without correlation to overall anti-PTx IgG titres (Pearson's $r = 0.05$). Moreover, when sera from vaccinees ($N = 12$) with a very high concentration (> 300 IU mL^{-1}) of anti-PTx IgG were tested, only a portion of samples had measurable 7E10 antibodies (Supplementary figure 1). We further examined three other S3 epitopes 2E12, 6F8 and 10B8 with sera from Finnish patients ($N = 21$) and acellular vaccinations at the 13-month and 4- to 5-year age groups ($N = 21$ and 21); however, no such difference as in 7E10 was observed (data not shown).[29]

Based on these findings, we further tested Danish sera, collected from serologically confirmed patients and from individuals who had received acellular booster vaccinations, against epitopes 1B7, 1D7 and 7E10. Both groups expressed high antibody titres towards the S1 epitopes (Table 2, Figure 2). Similar to the Finnish vaccination groups, the Danish vaccine group did not express high titres against 7E10 either (Pearson's r = 0.266, −0.184–0.715 95% CI). In contrast, the Danish patients possessed significantly higher 7E10-specific antibodies ($P = 0.003$), which correlated scarcely with overall anti-PTx IgG titres (Pearson's $r = 0.37$, 0.07–0.82 95% CI, $P = 0.096$). Danish patients had also a significantly higher level of antibodies to 1B7 in comparison with vaccination ($P = 0.009$), but the groups were similar towards 1D7 ($P = 0.246$).

Specific antibodies to S1 cannot be differentiated following PTd-induced immunity

Epitope-specific antibodies to glutaraldehyde-detoxified PT (PTdg) were tested for epitopes 1B7, 1D7 and 7E10 with Danish study samples as well as with those from the Finnish 4- to 5-year-olds' booster group. With the PTdg capture surface, the measured specific inhibition increased up to two- or threefold in comparison with PTx (Table 2). However, the level of inhibition to 7E10 remained the same in the Danish patient sera, but the

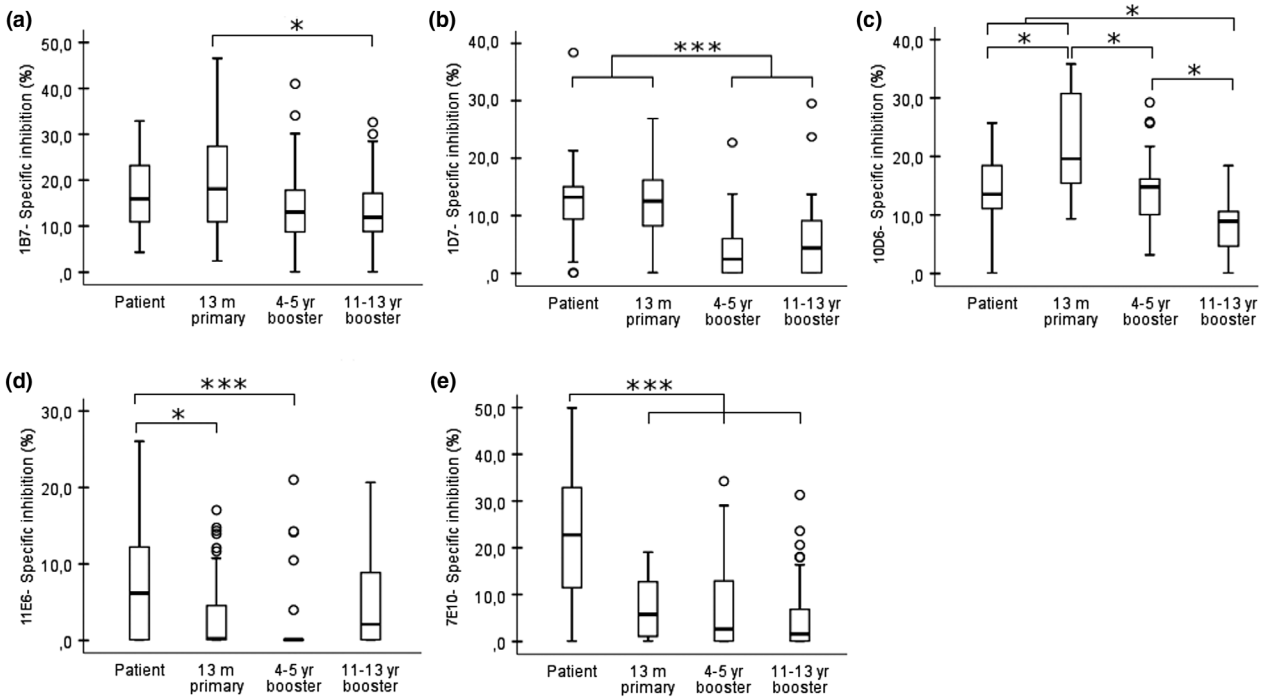

Figure 1. Epitope-specific antibody responses to native pertussis toxin from Finnish sera after pertussis infection and acellular vaccinations. Specific inhibition to epitopes **(a)** 1B7 (S1), **(b)** 1D7 (S1), **(c)** 10D6 (S1), **(d)** 11E6 (S23) and **(e)** 7E10 (S3), which demonstrates the relative amount of antibodies, was measured by blocking ELISA and was counted from background reduced signals as 1 − absorbance (sample)/absorbance (BSA-PBS control). The box plots demonstrate the median, quartile range and 1.5 times the quartile range of inhibition of the study groups. Significance by the two-tailed Mann–Whitney *U*-test for independent samples comparing each study population is indicated as * ($P < 0.05$) and *** ($P < 0.001$). The number of samples varied between the tested epitopes (see Table 2). The experiment was performed in duplicate for epitopes 1B7, 1D7, 7E10 and once for 10D6 and 11E6. O = values exceeding 1.5 times the interquartile range.

correlation between the PTdg/PTx responses turned out to be nonexistent (Pearson's $r = -0.004$) (Table 3 & Figure 3). Further, 1B7 and 10D6 were still well-presented in PTdg based on the correlations of PTdg/PTx responses, whereas there was no correlation of 1D7-specific antibody responses between PTdg and PTx in the Danish patient and the Finnish vaccine group. Remarkably, the overall correlation between epitope-specific responses of 1B7 and 1D7 increased significantly in comparison with PTx (Figure 4). In particular, the correlation increased from 0.62 (0.25–0.98 95% CI) to 0.98 (0.90–1.0 95% CI) with PTdg after Danish vaccination, and from 0.62 (0.29–0.95 95% CI) to 0.99 (0.927–1.0 95% CI), respectively, after Finnish vaccination.

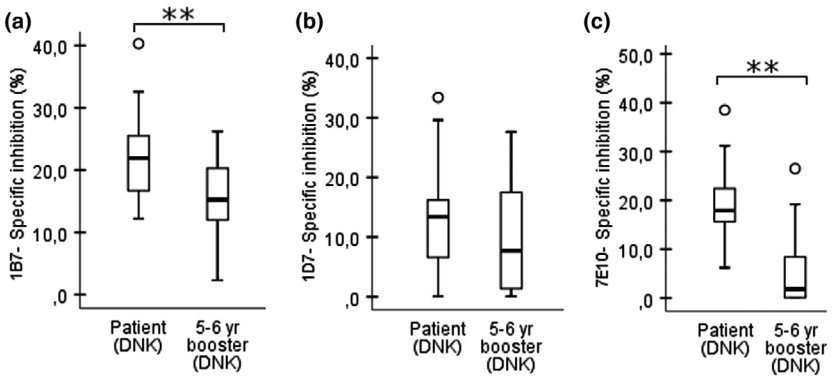

Figure 2. PTx epitope-specific antibody responses from Danish sera to **(a)** 1B7, **(b)** 1D7 and **(c)** 7E10 after pertussis infection and acellular booster vaccination. Significance between the groups is indicated as ** ($P < 0.01$). The experiment was performed once.

Table 3. Pearson correlations of epitope-specific responses between glutaraldehyde-detoxified PT and native PT capture surface

	Danish patients	Danish 5- to 6-year booster vaccine	Finnish 4- to 5-year booster vaccine
1B7	0.705**	0.526*	0.443*
1D7	0.036	0.639**	−0.011
10D6	–	–	0.534*
7E10	−0.004	0.241	0.154

*Correlation is significant $P < 0.05$.
**Correlation is significant $P < 0.01$.

However, within Danish patients, the correlation between 1B7 and 1D7 decreased non-significantly from 0.55 ($P = 0.01$) (PTx) to 0.44 ($P = 0.046$) (PTdg), mostly because of two strongly deviating outliers. If these were removed, the trend was similar to vaccinations: correlation increased from 0.70 (0.30–1.0 95% CI) to 0.94 (0.83–1.0 95% CI) with the PTdg antigen. Finally, the phenomena of S1 epitopes persisted also in the 10D6 epitope-specific antibodies, which were only measured with the Finnish booster group, as the correlation of 10D6 response to 1B7 and 1D7 increased from an average of 0.63–0.92.

DISCUSSION

To characterise differences in PT-induced antibody responses in humans, we developed a blocking ELISA based on PT-specific murine monoclonal antibodies, which compete for binding with antibodies in samples of human sera. The PT epitope-specific human antibody responses were compared between serologically confirmed pertussis patients and children who had received primary or booster doses of aPs. Even though the individual study groups had similar IgG antibody titres against PTx, the PT epitope-specific antibody responses varied between different exposure backgrounds. It stands out that especially single doses of booster aPs induced a notably lower quantity (or affinity) of epitope-specific antibodies to all tested epitopes. Multiple encounters with PTdg, as indicated by the primary vaccination group, seem to enforce the antibody maturation towards epitopes 1D7 and especially 10D6, as both the specific inhibition and its correlation with overall anti-PTx IgG titres were increased in these epitopes. Nevertheless, all tested acellular vaccine groups seem to induce considerable antibody titres to most of the protective epitopes in the immunodominant region of S1. This was somewhat expected as these chemical treatments have been reported to alter the function of B-oligomer more than as opposed to the A-oligomer.[21,23,24,33]

There was a marked difference in antibodies induced after infection compared to those after vaccination in the epitope 7E10 of PT S3, which likely has a role in carbohydrate-binding that facilitates the binding of PT to cell membrane receptors.[34,35] After infection, these antibodies appeared in substantial amounts and correlated with the overall anti-PTx IgG titres, whereas children who received aPs had much lower and non-correlative levels of 7E10-specific antibodies regardless of dose amounts or detoxification treatment. In other tested S3 epitopes, there was no such significant difference between the groups, suggesting 7E10 to be distinct from any other S3 epitope. Desired alterations of the toxin structure should only allow for the inactivation of the toxin without the destruction of the epitopes important to produce effective antibodies. However, both glutaraldehyde/formaldehyde and

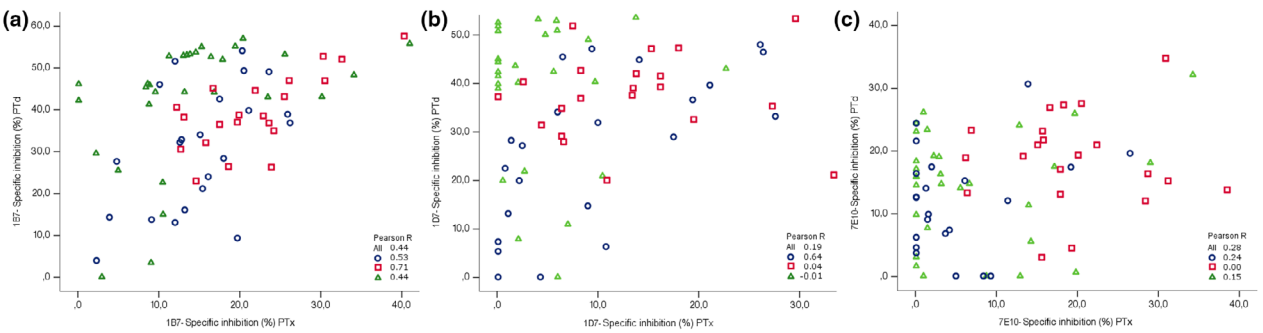

Figure 3. Specific inhibition between native PT and glutaraldehyde-detoxified PT capture surface is here illustrated with mAbs **(a)** 1B7, **(b)** 1D7 and **(c)** 7E10. The samples are labelled as blue O = booster vaccination in 5- to 6-year-old Danish children ($N = 22$); red = Danish patients ($N = 21$); green Δ = booster vaccination in 4- to 5-year-old Finnish children ($N = 26$). The experiment was performed once.

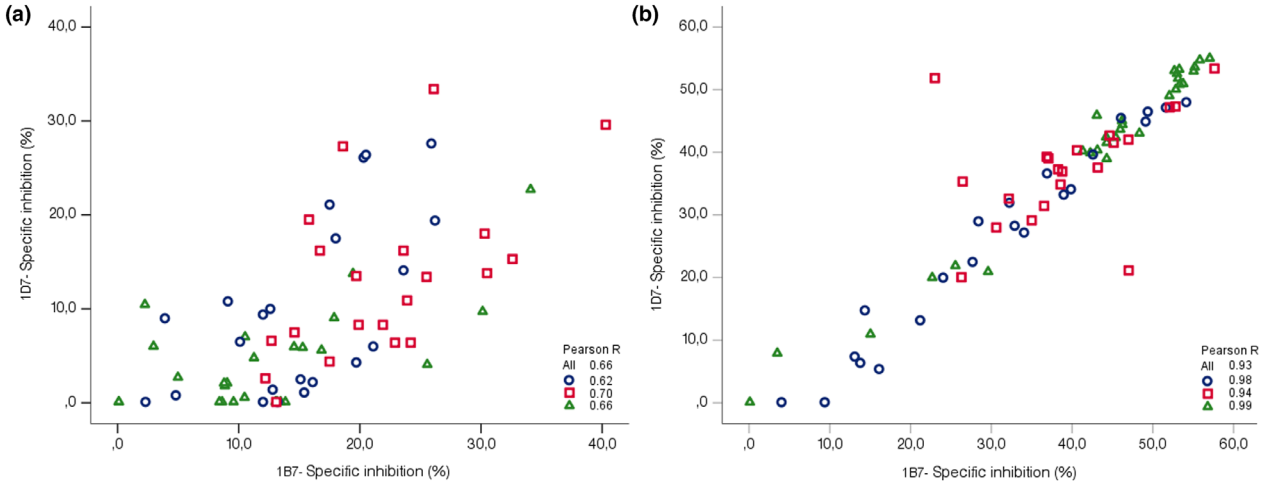

Figure 4. Correlation of epitope-specific inhibition between S1 epitopes 1B7 and 1D7 of native **(a)** and glutaraldehyde-detoxified PT **(b)**. The samples for tested PTd epitopes are labelled as blue O = booster vaccination in 5- to 6-year-old Danish children (*N* = 22); red = Danish patients (*N* = 21); green Δ = booster vaccination in 4- to 5-year-old Finnish children (*N* = 26). The overall Pearson correlation coefficient of the two epitope-specific antibody responses was increased from the use of PTx capture surface to PTd from 0.662 (0.468–0.857 95% CI) to 0.928 (0.84–1.0 95% CI), respectively.

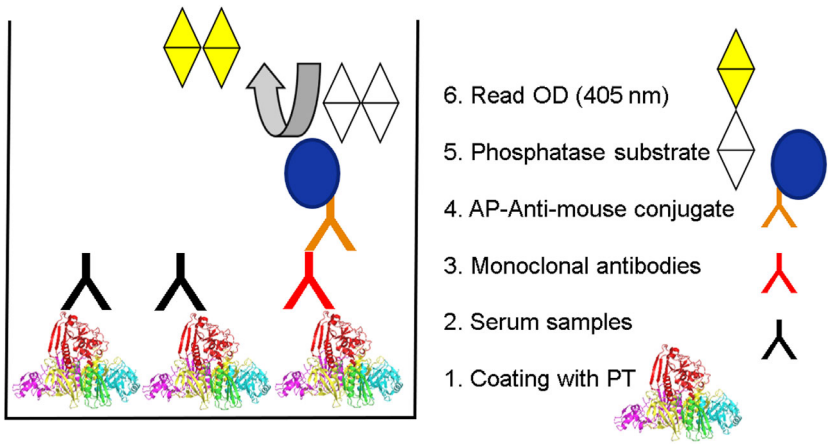

Figure 5. Schematic of the blocking assay. The wells were first coated with native or glutaraldehyde-detoxified pertussis toxin and blocked with BSA; then, serum samples, monoclonal antibodies, an alkaline phosphatase conjugate and a phosphatase substrate were added in their respective assay steps, with washing between the steps. Absorbance was measured at 405 nm from the wells until the blank sample reached an absorbance value of 1.5. The measured absorbance decreases as antibodies from serum block the binding epitopes of the monoclonal antibodies.

hydrogen peroxide treatment of PT manifest in drastically less epitope specificity towards 7E10, which presumably induces functional antibodies as, otherwise, these would be present as ineffective molecules in patients.

Sutherland *et al.*[26] made a similar observation between infection and acellular booster vaccination in adults with the S23 targeting mAb 11E6. However, our assay provided relatively weak inhibition to this epitope regardless of the sample background. Nevertheless, the difference between the Finnish patients and all Finnish vaccinees combined was statistically significant for this epitope. As the murine mAb 11E6, in particular, has a very strong affinity to PTx in comparison with the other tested mAbs, it is probable that the human antibodies in the blocking ELISA cannot compete with the murine 11E6 mAb's affinity despite high antibody titres (Supplementary table 1).[29] The variation in mAb affinities also affects how reliable comparisons between separate epitopes are, making it challenging to claim whether a group expresses say more 7E10- than 11E6-specific antibodies. This

Table 4. Average age, gender, time from the latest vaccination and median amounts of anti-PTx IgG of the study subjects

Sample cohort	*N*	Median of age Years (range)	Female/male	Median (range) concentration of anti-PTx IgG antibodies (IU mL^{-1})	Time from the latest pertussis vaccination
Finnish patients	51	14.3 (3–70)	27/24	118 (55–255)	–
Primary vaccination	50	13 months	18/32	101 (63–238)	1 month
Booster vaccination	30	4.3 (4–5)	17/13	104 (60–233)	2 weeks–1 year
Booster vaccination	50	11.6 (11–12)	24/26	94 (50–232)	1 month
Danish patients	21	13 (11–17)	11/10	149 (62–266)	5–16 years
Booster vaccination (DNK)	22	5 (5–6)	10/12	134 (52–261)	10–580 days

emphasises our analysis to focus on comparisons of groups within a tested epitope. Moreover, in a more general notion, it is important to consider the epitope-like character of the measured antibodies, which limits our conclusions to consider that these antibodies are simply targeting protective epitopes without certainty of the protective abilities against PTx. Further confirmation of the function to inhibit PT activity for each of the human-induced epitope-specific antibody profiles would be necessary to enable the use of these biomarkers to predict successful protection. Unfortunately, because of the limited amount of sera available for this study, we were not capable of performing a PT neutralisation assay for these samples.

In addition to native PT, we also studied the epitope-specific antibody responses to glutaraldehyde-detoxified PT. Interestingly, the correlation between the S1 epitope-specific responses increased remarkably, and as a result, 1B7, 1D7 and 10D6 responses could not be distinguished from each other any longer, suggesting a loss of epitopes in PTdg and a close relation of these binding sites. This could be further confirmed as a loss of 1D7 in PTdg as there was no correlation between the PTx- and PTdg-specific antibody responses in either Danish patients or the Finnish vaccine recipients. Based on specific inhibition levels, there were vaccine-induced antibodies in the Finnish vaccination group to glutaraldehyde-treated '1D7-like' epitope, but these are not binding to PTx. On the contrary, patients had high 1D7 titres to PTx but the binding of these antibodies to the PTdg '1D7-like' epitope increased to a nearly constant level in all samples, likely because of other S1 binding antibodies overtaking closely related and better-presented epitopes (Figures 3 and 4). However, PTx 1D7-binding antibodies were induced after a primary vaccination of three doses of glutaraldehyde-/formaldehyde-treated PT, which would suggest this epitope's capability of inducing antibodies to be based strongly on the quantity of PT antigen during immunisation. This was further supported by the observation that the hydrogen peroxide-treated PT vaccine also induced these antibodies, which has a much higher PT dose in the vaccine. It is also possible that hydrogen peroxide promotes a slightly differently altered, but closely related epitope to 1D7, which is equally present (or not present) in PTx and PTdg. As for S3 7E10 epitope-specific antibodies in PTdg, the signal levels were near equal between patients and vaccine recipients. However, the level of inhibition remained unchanged in patients but the correlation between PTx and PTdg 7E10 responses was nonexistent, which further demonstrated 7E10 to be unique to PTx. Altogether, these observations indicate changes in the presentation of PT epitopes after glutaraldehyde detoxification treatments, and even though it remains to be confirmed in a similar test setting, based on PTx readout, the hydrogen peroxide treatment also affects the 7E10 epitope.

There are certain limitations to this study, the first being that the number of subjects was limited in certain study groups. Also, the exact vaccination history of Finnish patients was unavailable, which left the effect of the whole-cell vaccine and aP priming to the patient's specific antibody responses unreliable to analyse. Similarly, it is uncertain whether patients or vaccinated individuals have had pertussis infections in the past. It certainly varies to an extent how much the small number of subjects, the apparent shortcomings of the aPs and the individual characteristics of the donors truthfully reflect the immunogenic qualities of PTx and PTd.[36] The vaccination studies conducted for 13-month-old babies and 11- to 13-year-old booster vaccination groups happened 10 and 19 years ago, respectively, and the long storage could possibly lead to a degradation of epitopes of antibodies: however, the anti-PTx IgG levels of

these samples were re-measured for this study. Only marginal decreases in concentrations of anti-PTx IgG antibodies were found. Additionally, we have no certainty what changes happen in the 3D structure of the toxin/toxoid because of the adsorption to the plate as compared to the native structure. This further highlights a need for individual PT subunit-specific assays to a wide variety of assays to study these phenomena without interference from other PT subunits.

This study compared antibodies after infection and acellular pertussis vaccinations, and with the developed blocking ELISA, it is possible to differentiate serum epitope-specific anti-PTx or anti-PTd antibodies. The different expression of antibodies between patients and vaccine recipients in epitopes 7E10, 11E6 and 1D7 emphasises the importance of structural preservation of PT to prevent the antigenic alterations of these regions in the current detoxification procedures. Additionally, it would be important to determine, in the future, whether genetically modified PT-induced epitope-specific antibody responses would resemble the immunological properties of PTx better than PTd does, as it retains a more native-like structure in comparison with PTd.

METHODS

Study design

The study included sera from 72 pertussis patients and 152 children who received aP doses at different ages and with vaccines containing differently detoxified PT from Finland and Denmark. The samples chosen for this study were selected within a similar range of anti-PTx IgG antibodies between 50 and 266 international units per mL (IU mL^{-1}). The lower cut-off of > 50 IU mL^{-1} was used to define a recent pertussis infection during the last 12 months.[37] The study samples (Table 4) included (1) Finnish patients with serologically diagnosed pertussis (2015–2016). The diagnosis of these patients was based on IgA and IgM antibody levels measured by ELISA using sonicated *B. pertussis* bacteria as a coating antigen[38]; (2) Finnish infants who had received three primary doses of two- or three-component aPs [Tetravac, Sanofi Pasteur, Lyon, France, or Infanrix, GlaxoSmithKline (GSK), Rixensart, Belgium] at 3, 5 and 12 months of age in 2008–2010[39]; (3) Finnish children who had received a booster dose of a two-component aP (Tetravac vaccine, Sanofi Pasteur) at the age of four years. The samples were collected from routine diagnostic samples without relation to respiratory infections, during 2014–2017; (4) Finnish adolescents who received a booster dose of a three-component dTaP vaccine (Boostrix, GSK) in 1997, and their serum sample was collected one month after vaccination. They had all received four doses of whole-cell pertussis vaccine in earlier childhood[40]; (5) Danish children who were recently vaccinated with the Danish monocomponent booster vaccine (Statens Serum Institut, Copenhagen, Denmark); (6) Danish adolescents with confirmed pertussis by serology, who were vaccinated in childhood with the Danish aP (3 months + 5 months + 12 months + 5 years); however, four subjects had only received the infant series and not the booster. The diagnosis of these patients was based on anti-PTx IgG ELISA.[41] All sera in this study were stored either at −20 or at −70°C, and their anti-PTx IgG antibodies were measured with standardised ELISA at the Finnish National Reference Laboratory for Pertussis as previously described.[25,42]

Study approval

Prior to the inclusion into the study, all subject data, except for age and gender, were anonymised. The Finnish sera of patients and 4- to 5-year-old children had been sent for diagnostic purposes of pertussis and Lyme borreliosis, to the diagnostic laboratory of the Department of Medical Microbiology and Immunology, at the University of Turku, Finland. The use of these clinical samples was approved by the Ethics Committee of the Hospital District of Southwest Finland by the chief of the operative group of Turku University Hospital (Decision 14/17 MBG). The regional research ethics committee in Denmark did not require discrete approval for the use of Danish sera for this study.

Epitope mapping with blocking ELISA

A total of 27 murine mAbs were obtained from the National Institute for Biological Standards and Control (NIBSC, Potters Bar, UK). They targeted the five subunits of PT (10 to S1, 2 to S2, 5 to S23, 6 to S3, 3 to S4 and 1 to S5) and were first screened against PTx (Supplementary figure 2), and with a set of sera (N = 11, anti-PTx IgG range 0–387 IU mL^{-1}) from Finnish patients. Based on the amount of binding to PTx, and the biological protection in mice (Supplementary table 1), two mAbs 1B7 and 7E10 targeting subunits 1 and 3 were further tested with all study subjects (N = 224).[30] Sera were also tested with mAbs 1D7 (S1), 10D6 (S1) and 11E6 (S23), but with a fewer number of samples (N = 168, 125 and 181, respectively) because of sample restrictions. Based on PTx readout, mAbs 1B7, 1D7, 10D6 and 7E10 were further studied with glutaraldehyde-detoxified PT as the capture antigen with the Danish sera and sera from the Finnish 4- to 5-year booster group.

The blocking of mAb binding to PTx and PTdg by serum antibodies was determined by ELISA (Figure 5)[43]: first, 96-well plates (Art. No. 655061; Greiner Microlon, Frickenhausen, Germany) were coated with 250 ng of PTx (GSK) or PTdg (Sanofi Pasteur) in 100 μL of PBS (pH 7.4). The wells were washed with PBS and blocked with 150 μL of 1% BSA-PBS (cat no 810033; MP Biomedicals, Solon, Ohio) for 1 h at 37°C. Three washes between all the following assay steps were done with 0.9% NaCl-0.05% Tween (Sigma P-1379, St. Louis, USA) buffer. Next, the serum samples, diluted 1:20 in 100 μL of 1% BSA-PBS, were incubated for 2 h at 37°C. All samples, including a blank sample (1% BSA-PBS) and anti-PTx IgG-negative and IgG-positive controls from in-house serum sample pools with each test plate, were tested in duplicate wells. After

washing the plates, 100 μL of mAb in PBS was added according to NIBSC recommended dilutions and incubated for 1 h at 37°C. Goat anti-mouse IgG conjugate (AP124; Merck, Espoo, Finland) was then diluted 1:2000 in 100 μL of BSA-PBS and incubated for 1 h at 37°C. Last, 100 μL of p-nitrophenylphosphatase substrate (cat no S0942; Sigma, Helsinki, Finland) in diethanolamine-$MgCl_2$-buffer (cat no 170057; Reagena, Toivala, Finland) was incubated and covered from light until the blank sample reached an absorbance value of ~ 1.5. Absorbance was measured at 405 nm with Multiskan EX device (Thermo Scientific, Vantaa, Finland). The reduction/blocking of the maximum signal of mAb binding caused by the similar epitope-like antibodies from study sera, referred to as 'specific inhibition', was counted from background reduced signals as 1 − absorbance (sample)/absorbance (blank).

Statistics

Specific inhibition values were analysed using IBM SPSS statistics 25.0 software for Windows. All values of specific inhibition equal to 0% or less were converted to 0.1% for data analysis. Since only 1B7 responses followed normal distributions across all groups, the differences in means between the groups were tested with Mann–Whitney *U*-tests with Bonferroni corrections, and two-sided *P*-values < 0.05 were considered as statistically significant. Correlation of epitope-specific antibody responses to the overall anti-PTx IgG IU mL^{-1} titres and between each other was calculated with the Pearson correlation coefficient.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Aapo Knuutila: Data curation; Investigation; Methodology; Writing-original draft. **Tine Dalby:** Investigation; Methodology; Writing-review & editing. **Alex-Mikael Barkoff:** Data curation; Formal analysis; Methodology; Writing-review & editing. **Charlotte Sværke Jørgensen:** Data curation; Investigation; Writing-review & editing. **Kurt Fuursted:** Data curation; Investigation; Writing-review & editing. **Jussi Mertsola:** Data curation; Investigation; Resources; Writing-review & editing. **Kevin Markey:** Investigation; Methodology; Resources; Writing-review & editing. **Qiushui He:** Investigation; Methodology; Funding acquisition; Resources; Data curation; Writing-original draft; Writing-review & editing.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.