

HOST RESPONSE TO INFECTION

A nasal whole-cell pertussis vaccine induces specific systemic and cross-reactive mucosal antibody responses in human volunteers

A. K. H. BERSTAD, J. HOLST, L. O. FRØHOLM*, I. L. HAUGEN, E. WEDEGE, F. OFTUNG and B. HANEBERG†

Departments of Vaccinology and *Bacteriology, National Institute of Public Health, N-0403 Oslo and †Department of Microbiology, Institute of Pharmacy, University of Oslo, Norway

A whole-cell pertussis vaccine, each dose consisting of 250 µg of protein, was given intranasally four times at weekly intervals to six adult volunteers. All vaccinees responded with increases in nasal fluid IgA antibodies to *Bordetella pertussis* whole-cell antigen. Three vaccinees with high nasal antibody responses also developed increased serum IgA and IgG antibodies to this antigen. Salivary antibody responses to the whole-cell antigen, as well as antibodies in serum and secretions to pertussis toxin (PT) and filamentous haemagglutinin (FHA) were negligible, except for a moderate increase in nasal fluid antibodies to FHA. Unexpectedly, the same vaccinees developed significant rises in nasal and salivary IgA antibodies to meningococcal outer-membrane antigens, whereas corresponding serum IgA and IgG antibodies were unchanged. Thus it appears that mucosal immunisation may induce secretory antibodies with broader specificities than can be found in serum.

Introduction

The predominating immunoglobulin in mucosal secretions, IgA, is actively secreted by the co-ordinated function of mucosal epithelial cells and polymeric IgA-producing local plasma cells [1]. Specific IgA can block attachment of micro-organisms or neutralise their secreted toxins at the mucosal surface by so-called immune exclusion [2]. The appearance of IgA in mucosal secretions has been shown to correlate with protection against infection with bacteria and viruses that reside primarily in mucosal tissues [3–6]. Such antibodies are usually elicited by natural infection or vaccines administered on to mucosal surfaces.

The pathogen that causes whooping cough in man, *Bordetella pertussis*, also remains in mucosal tissues during the disease, i.e., it does not invade the interior of the body [7]. Secreted toxins may affect other parts of the body, and serum antibodies induced by parenterally administered pertussis vaccines can thus protect against disease symptoms after exposure to *B.*

pertussis [8]. However, the difficulties in establishing a serological correlate of protection after pertussis vaccination may indicate a role of mucosal antibodies in protecting against colonisation and transmission in which serum antibodies are far less effective [9]. In animal models of respiratory tract infection with *B. pertussis*, protection correlated with local mucosal antibodies induced by nasal immunisation, rather than with serum antibodies [10, 11]. Antibodies specific to *B. pertussis* in airway secretions are induced after natural infection or mucosal immunisation, but not by current parenteral pertussis vaccines [12–15].

Induction of mucosal immunity against *B. pertussis* through mucosal delivery of pertussis vaccines has been tried in two previous studies in man. After oral administration of five doses of whole-cell vaccine to 15 000 newborn babies, salivary agglutinating antibodies as well as cellular immune responses were observed [16]. In another study comprising eight adult volunteers, one dose of whole-cell vaccine delivered by inhalation induced specific antibodies in nasal secretions [17]. Significant serum antibodies were not reported in these experiments. However, even after natural infection serum antibody changes are highly variable and sometimes very weak [18–20]. Therefore, it is possible that serum antibodies induced by infection or mucosal immunisation act differently to those

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Corresponding author: Dr A. K. H. Berstad (e.mail: aud.herland.berstad@folkehelsa.no).

induced by injectable vaccines in protection against pertussis.

Mucosal delivery of non-proliferating vaccine antigens usually requires mucosal adjuvants to elicit immune responses, and cholera toxin (CT) is considered to be the most effective [15]. However, two recent studies in mice [21, 22] showed that CT did not enhance systemic or secretory antibody responses to a whole-cell pertussis vaccine given nasally, or secretory antibodies to a nasal group B meningococcal outer-membrane vesicle (OMV) vaccine. In a study in human subjects, the OMV vaccine without adjuvant, given as a nasal vaccine, was sufficiently immunogenic to induce local mucosal antibodies, as well as serum antibodies with strong bactericidal activity [23]. Mucosal immunogenicity of non-proliferating antigens may increase when formulated as particles, through sampling and transport by M cells in nasopharyngeal epithelium to immune-inductive lymphoid tissues [24]. The aim of the present study was to investigate the immunogenicity in human volunteers of intranasally administered whole-cell pertussis vaccine, another particulate vaccine containing no adjuvant.

Materials and methods

Subjects

Six healthy adults were recruited for the study (two men and four women, 25–46 years of age). They had all been immunised with whole-cell pertussis vaccine in childhood. When screening pre-vaccination serum samples for antibodies to *B. pertussis*, one vaccinee had high titres in all antibody assays. She described a history of long-lasting paroxysmal coughing, probably pertussis, c. 1 year earlier. She was still given the nasal vaccine to see if any booster immune response could be elicited. Each participant gave their informed written consent before the start of the study. The study was approved by the Regional Committee of Medical Research Ethics and the Norwegian Medicines Control Authority.

Samples of serum, saliva and nasal secretions from the 12 participants in the previous nasal immunisation study with the meningococcal OMV vaccine [23] were tested for antibodies to *B. pertussis* before and after immunisations. They had also received pertussis vaccine in childhood. These volunteers (nine women and three men, 25–61 years of age) were given OMV in the form of drops or spray four times at weekly intervals. Each nasal dose consisted of 250 µg of OMV protein, equivalent to 10 times the intramuscular dose.

Vaccine and immunisations

The whole-cell pertussis vaccine consisted of formalin-inactivated *B. pertussis*, 7×10^{10} cells/ml, from vaccine strains 3803, 3825, 3843 and 3860 (Statens

Seruminstitut (SSI), Copenhagen, Denmark). This concentrated bulk, intended for SSI's licensed parenteral formulation, was kindly provided by Karen Gibsholm-Madsen, SSI. The vaccine was preserved with merthiolate 0.01%, was not adsorbed to aluminium adjuvant, and was suspended in phosphate-buffered saline (PBS). The protein content was 2.3 mg/ml (Lowry). Each vaccine dose was adjusted to 250 µg of protein in a 0.5-ml volume, half of which was sprayed into each nostril with a Minigrip metered spray device (Apodan, Copenhagen, Denmark) with the vaccinee seated. The vaccine was given four times at weekly intervals.

Monitoring of side-effects

After each vaccine dose, participants were asked to fill in case report forms for 7 days, registering any local side-effects such as irritation, stuffiness or sneezing, as well as systemic symptoms like fever or any other discomfort. Albumin in nasal fluid was chosen as a marker of inflammation [25] and was measured by ELISA after each vaccination. Mucosal total IgE, a more sensitive marker for nasal reactions than serum IgE [26], was measured in nasal fluid at the start of the study and after each dose. At the same time, a white blood cell count was determined as a marker of systemic absorption of any active pertussis toxin [27].

Collection of samples

Blood samples, saliva and nasal fluids were collected at the start of the study, 1 week after each vaccine dose and at 2, 4 and 6 weeks after the last dose. Nasal fluid was obtained after spraying c. 0.4 ml of luke-warm PBS, pH 7.2, into each nostril and collecting the fluid flowing out of the nostril by four absorbent cylindrical wicks measuring 2×25 mm (Polyfiltronics Group, Rockland, MA, USA). Saliva was collected after salivation had been induced by chewing gum, by placing two wicks between the lower gum and buccal mucosa at each side for 1 min. The wicks were then placed into pre-weighed 1.5-ml microcentrifuge tubes, and the net weights of captured fluids were calculated. All samples were frozen immediately and stored at -20°C before extraction and assay.

Nasopharyngeal and tonsillar swab samples were obtained for cultivation of *B. pertussis* and *N. meningitidis*, respectively, at the start of the study and at the second and fourth immunisations [20].

Extraction of immunoglobulins from wicks

Proteins were extracted by addition of 500 µl of PBS with the following protease inhibitors: 0.2 mM 4-(2-aminoethyl)-benzenesulphonyl fluoride (AEBSF) (Boehringer Mannheim GmbH, Mannheim, Germany), aprotinin (Sigma) 1 µg/ml, 10 µM leupeptin (Sigma) and 3.25 µM bestatin (Sigma). After vortex mixing for

1 min, a small hole was punched into the bottom of each tube, which was placed into another tube measuring 1.2×8 cm. The extracts were collected into the second tube by centrifugation at c. 2000 g for 5 min at room temperature, assayed immediately or stored at -20°C .

Measurement of antibody responses

IgG and IgA antibodies to the whole-cell vaccine antigen, pertussis toxin (PT) and filamentous haemagglutinin (FHA) were quantified by ELISA as described previously [18, 21]. PT and FHA were kindly donated by Dr George Siber, Massachusetts Public Health Biologic Laboratories, USA. Antigens (pertussis vaccine containing 1×10^5 cells/ml, PT $3 \mu\text{g/ml}$, FHA $3 \mu\text{g/ml}$, all in PBS, OMV $4 \mu\text{g/ml}$ in Tris. HCl, pH 7.2) were adsorbed on to microtitration plates (Nunc, Roskilde, Denmark) and incubated at 4°C overnight. The plates were washed five times with PBS containing Tween 20 0.05% v/v, and blocked with non-fat dry milk 5% w/v in PBS. Serum from one vaccinee with high pre-vaccination titres of pertussis antibodies and a recent history of pertussis-like disease was used as standard for all pertussis-specific assays. A pool of sera from different donors with high titres of IgG and IgA antibodies to OMV served as standard for OMV antibodies in serum, and a sample of saliva from a donor with a high titre of IgA antibodies was used as standard for antibodies to OMV in secretions.

Concentrations of IgA and IgG antibodies to B. pertussis were expressed as arbitrary units (U) based on the standard serum and read from the linear part of the standard curve. The intra-assay coefficient of variation (CV) for serum IgA and IgG was $\leq 15\%$, for nasal IgA $\leq 20\%$ and for saliva IgA $\leq 25\%$. A vaccine response based on a titre rise of $\geq 50\%$ thus corresponded to at least two times the intra-assay variation. The inter-assay CV was calculated to be $\leq 25\%$.

Concentrations of total IgA in secretions were determined by ELISA in the same way with purified human IgA (DAKO A/S, Glostrup, Denmark) as standard. Concentrations of specific antibodies in secretions were expressed as the ratio of IgA antibody:total IgA concentrations to correct for dilution during nasal fluid collection and for variations in salivary flow.

Immunoblot analysis of serum and nasal fluids

IgA and IgG antibodies to OMV antigens were analysed on immunoblots loaded with OMV as described previously [28]. Sera were diluted 1 in 200 and nasal fluid 1 in 10 in bovine serum albumin (BSA) 3% in PBS. Antibody binding was detected with 1 in 500 dilutions of peroxidase-conjugated goat anti-human IgA (Sigma) or peroxidase-conjugated rabbit anti-

human IgG (DAKO). Antibody binding to different OMV antigens was determined visually.

Statistical analysis

Rises of 1.5-fold or higher from baseline in pertussis antibodies were considered as a vaccine response, a definition used in a previous study [20], and entered in the analyses of differences of significance by Wilcoxon signed rank test or Mann-Whitney U test for paired and unpaired samples, respectively. Coefficients of correlation were calculated and simple linear regression curves were plotted with GraphPad Prism for Windows.

Results

Clinical observations

One of the six vaccinees reported nose bleeding lasting a few minutes and on four occasions within 1 week of the first vaccine dose, but received the three ensuing doses without any bleeding. She had experienced episodes of nose bleeding before vaccination. Otherwise, no local or systemic symptoms were recorded during the observation period. One vaccinee was lost to follow-up 3 weeks after the last dose due to intercurrent viral infection. There were no notable changes in blood leucocyte counts or nasal albumin concentrations in any of the vaccinees. IgE in nasal fluids was not detectable, except in one male vaccinee with known pollen allergy and seasonal symptoms of rhinoconjunctivitis. His total nasal IgE rose to 1.4 ng/ml after the last dose, but was undetectable on follow-up a few weeks later. Cultures from throat and nasopharynx did not show growth of meningococci or B. pertussis on any occasion.

Antibody responses to the whole-cell antigen

Concentrations of antibodies to the B. pertussis whole-cell antigen in serum, nasal fluid and saliva were followed for up to 6 weeks after the last vaccine dose (Fig. 1). Nasal fluid IgA antibodies were elevated from baseline 3 weeks after the first vaccine dose, with the mean level reaching a maximum of a seven-fold increase above baseline at week 7. At the end of the study (week 9), mean nasal antibody levels were still three-fold elevated compared with the baseline. Defining a minimum increase of at least 1.5-fold as a vaccine response in this study, all vaccinees responded to the nasal immunisations with increases in nasal fluid antibody concentrations (range 1.5–20-fold, $p < 0.05$). In contrast, salivary IgA antibody increases were weak and their kinetics thus less distinct (data not shown). Therefore, saliva was not useful for measuring vaccine-induced responses in this study.

The mean level of serum IgA antibodies for the whole group increased four-fold from the start of the study to week 3, rising further to a maximum of six-fold at

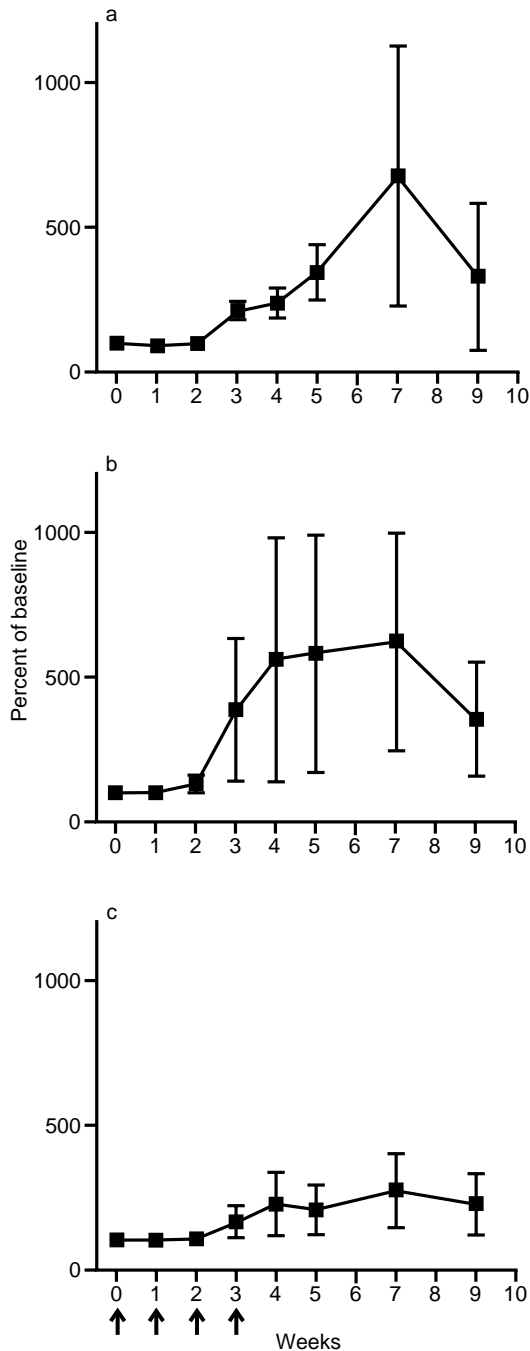


Fig. 1. Nasal fluid IgA (a), serum IgA (b) and IgG (c) antibodies to the whole-cell *B. pertussis* antigen in six vaccinees, after four intranasal immunisations (\uparrow) with a whole-cell pertussis vaccine. The concentrations of IgA antibodies in nasal fluid were measured in ELISA arbitrary units and expressed as ratios of specific IgA: total IgA. Results are shown as the mean percent of baseline (100%) concentration at each time point. Bars represent SEM.

week 7, and was three-fold above baseline at week 9 (Fig. 1). The kinetics as well as the magnitude of IgA antibody responses in serum and nasal fluid were, therefore, quite similar. Corresponding serum IgG responses were lower, but exhibited similar kinetics to IgA responses in nasal fluid and serum (Fig. 1). However, according to the definition of a 1.5-fold increase or more as a vaccine response, elevations of

serum IgA and IgG antibodies did not reach statistical significance.

Antibodies to the *B. pertussis* whole-cell antigen in nasal fluid and serum were also measured in 12 persons who received a nasal meningococcal OMV vaccine following the same schedule as pertussis vaccinees [23]. Changes in these antibody concentrations from baseline to 2 weeks after the last nasal dose were compared between the two vaccine groups. Compared with OMV vaccinees, the pertussis vaccine group had significant antibody increases in nasal fluid IgA ($p = 0.009$), serum IgA ($p = 0.008$) and serum IgG ($p = 0.008$) (data not shown). Thus, it may be concluded that significant responses to *B. pertussis* whole-cell antigen were induced both in nasal fluid and in serum by this nasal pertussis vaccine.

Antibody responses to FHA and PT

The FHA and PT antigens are the most commonly used antigens for diagnosis of infection with *B. pertussis*, as well as for inclusion in acellular pertussis vaccines. The whole-cell vaccine used in the present study induced only a modest rise in nasal IgA antibodies to FHA in five individuals, range 1.5–2-fold (data not shown). Thus, the magnitude of these responses was lower than the corresponding whole-cell antibody increases, and nasal IgA responses to PT were not detected. Serum IgG antibodies to PT and FHA were not observed, except in one vaccinee who had an eight-fold rise in PT antibodies, but with no known exposure to *B. pertussis*.

Antibody responses to meningococcal antigens

All participants in the pertussis vaccine group showed nasal and salivary IgA antibody rises to the OMV antigens (range 1.6–7-fold, $p = 0.0005$) (Fig. 2). The peak response in nasal fluid IgA for the whole group occurred 1 week after the third vaccine dose, and these antibodies were still raised, compared with the pre-vaccination level, in all vaccinees 5 weeks from the start of the study. Thus, these mucosal responses were strikingly similar among individuals, in magnitude as well as kinetics. However, none of them showed anti-OMV antibody increases in serum. This was confirmed by immunoblots demonstrating individual binding patterns to OMV antigens that did not change after immunisation. Corresponding blots with nasal fluid gave patterns different from those in serum, but bands were generally weak and, due to insufficient sample volumes, no further analyses could be performed.

The concentrations of antibodies to meningococcal OMV in nasal secretions among pertussis vaccinees at the start and at weeks 2, 3 and 5 were compared with nasal anti-*B. pertussis* whole-cell antibody concentrations at the same time points. These antibody responses correlated moderately but significantly (Spearman's

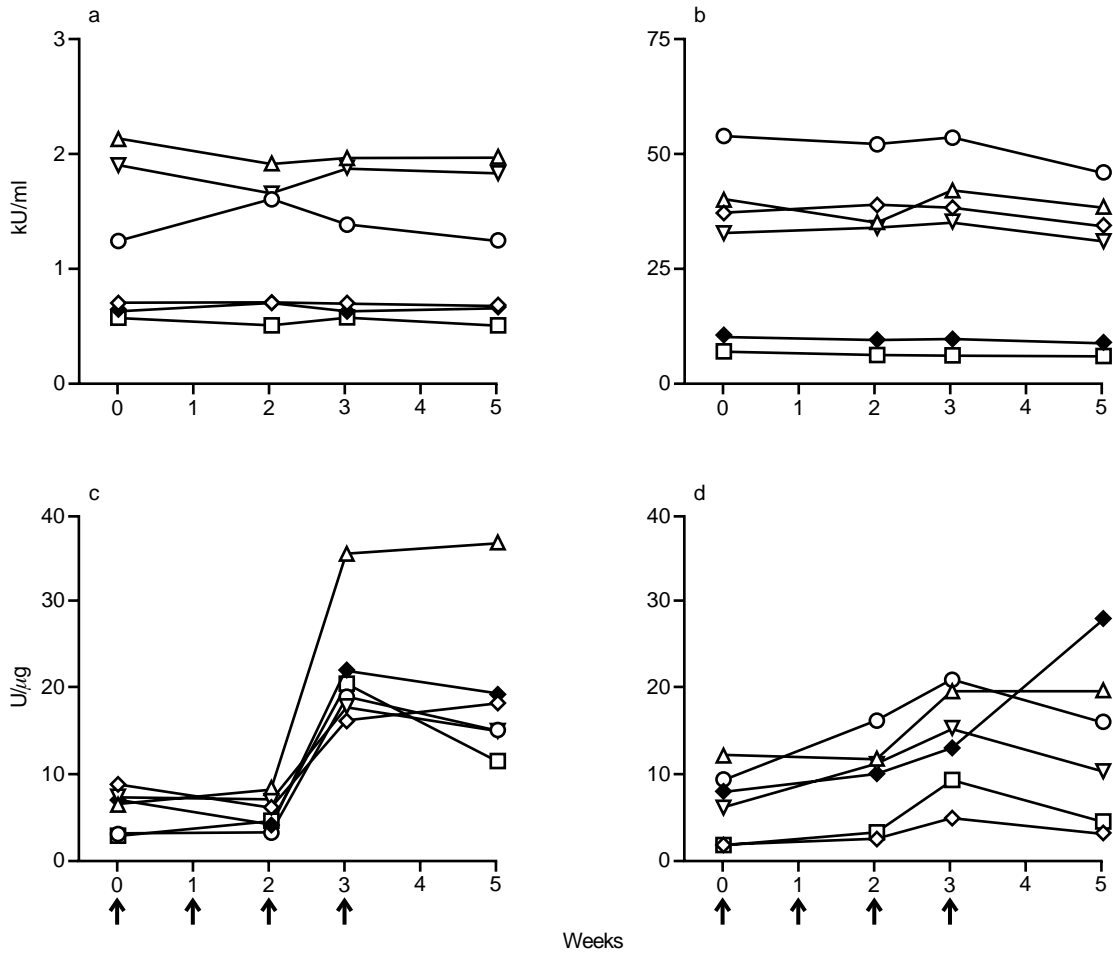


Fig. 2. Individual concentrations of antibodies (by ELISA) to meningococcal OMV antigens in serum (a, IgA; b, IgG), nasal fluid (c, IgA) and saliva (d, IgA), at the start of the study and after nasal immunisations (↑) with whole-cell pertussis vaccine. The concentrations of IgA antibodies in secretions, measured in arbitrary units (U), are expressed as the ratios of specific IgA: total IgA (U/μg).

coefficient of correlation, $r = 0.55$, $p = 0.005$). Therefore, it seems likely that the OMV antibody increases were caused by the nasal pertussis vaccine.

Discussion

The normal immune responses in mucosal tissues discriminate between harmless antigens and pathogenic micro-organisms in dietary and airborne particles as well as in the indigenous flora [29]. These mucosal immune defences are adapted to protect against invasion by pathogens without disrupting the normal function or integrity of the epithelial lining [1, 30]. Therefore, in the present study of nasal immunisation in human volunteers, subjective as well as objective parameters of local or systemic inflammation were monitored carefully. From these results, there was no reason to suspect that the nasal immunisations caused any systemic or mucosal adverse effects.

As vaccine antigens delivered on to mucosal surfaces generally are less immunogenic than when administered parenterally, formulation of the mucosal vaccine is crucial. A vaccine consisting of bacteria-derived

particles takes advantage of the preferential uptake and non-degraded delivery by epithelial M cells to local immune inductive tissues [31]. Therefore the whole-cell pertussis vaccine, intended for parenteral administration, was administered intranasally in this study, and antibody responses in serum as well as in airway secretions were monitored by recently established methods of sampling and assaying [23].

This study focused primarily on measuring antibody responses to the whole-cell antigen. The intranasally delivered pertussis vaccine without adjuvant induced significant increases in antibodies to this antigen in nasal secretions, and in some participants the levels remained elevated throughout the study. Antibodies to surface structures of the pathogen, contained in the whole-cell vaccine, probably play an important role at the mucosal surface to accomplish protection by immune exclusion [2, 7, 32].

In saliva, increases of IgA antibodies to the whole-cell antigen were only $\leq 50\%$ of those in nasal secretions. Even after natural infection, immune responses against *B. pertussis* in saliva are difficult to detect, in contrast to the regular appearance of antibodies in nasal

secretions [12–14, 33]. As *B. pertussis* primarily adheres to the ciliated cells in the nasopharynx, this may be consistent with the concept of compartmentalisation within the common mucosal immune system, i.e., the maximum response is seen at the site of immune induction [1].

Although *B. pertussis* itself does not invade beyond mucosal tissues, systemic antibodies may protect against systemic effects of bacterial products [7, 18]. Serum antibodies may also constitute an important defence line for the lower airways, which normally lack organised mucosa-associated lymphoid tissue [1]. Nasal delivery of pertussis whole-cell vaccine in this study induced significant serum IgA and IgG responses to the vaccine, which represents a multitude of different soluble and cell-associated antigens [32]. However, it cannot be excluded that childhood parenteral vaccinations or previous natural infection could have primed the vaccinees for responses to the mucosal vaccine. Nevertheless, secretory and serum antibodies were induced in the same individuals, with the potential of protection against colonisation as well as the systemic effects of *B. pertussis*.

Protective immunity can be predicted by measures other than ELISA antibody concentrations alone. For instance, human subjects given a nasal meningococcal OMV vaccine showed serum bactericidal activity at the same level as that induced by parenteral immunisation, despite only low concentrations of antibodies as measured by ELISA [23]. Whooping cough is a result of *B. pertussis* attached to and remaining at ciliated nasopharyngeal epithelium, and, therefore, immune responses induced by a nasal pertussis vaccine might be better evaluated by measuring inhibition of bacterial adherence to human ciliated airway cells [34]. As parenteral vaccines depend on systemic immune responses as the principal conveyor of protection, serological correlates of protection established for such vaccines may not be appropriate for mucosal vaccines, which offer additional protection at the mucosal surface.

Whole-cell vaccines administered parenterally protect against pertussis disease, in spite of frequently low contents of the PT and FHA components [8]. The absence of mucosal as well as serum antibody responses to PT in this study might be due to the reported negligible amount of this antigen in the whole-cell vaccine preparation used [32]. Nevertheless, modest antibody increases in nasal fluid to FHA were induced in five of the six vaccinees. As this is an important antigen for the adherence of *B. pertussis* to ciliated cells, antibodies against FHA and to other antigens in the whole-cell preparation may act synergically against colonisation [7].

Unexpectedly, significant rises in nasal and salivary antibodies to OMV antigens from *N. meningitidis* were

found after immunisation with pertussis vaccine, whereas serum IgA and IgG antibody concentrations to OMV were largely unchanged. In contrast, the nasal meningococcal OMV vaccine did not induce mucosal antibodies to *B. pertussis*. This is in line with the results of a recent study in mice [35]. The correlation in the present study of nasal antibodies to *B. pertussis* with nasal antibodies to OMV indicates that the pertussis immunisation caused rises of mucosal IgA antibodies which are cross-reactive with meningococcal antigens.

The functional significance of the meningococcal mucosal antibodies induced by the nasal pertussis vaccine is unknown. However, it has been reported that antigen-specific secretory IgA antibodies may have broader reactivities compared with their counterpart in serum through binding of unrelated epitopes [36]. Secretory IgA against surface antigens of one pathogen induced by mucosal immunisation may thus protect against other pathogens at the mucosal surface [37]. This may represent a beneficial effect of mucosal immunisation that should be explored in further studies.

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