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Stimulation of secretory antibodies against *Bordetella pertussis* antigens in the lungs of mice after oral or intranasal administration of liposome-incorporated cell-surface antigens

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Lipopolysaccharide (LPS) and outer membrane protein (OMP) preparations of *Bordetella pertussis* were incorporated into multilamellar liposomes composed of soya bean-derived phospholipids which were then used for oral and intranasal immunization of mice. Specific antibody responses of animals immunized by either route were measured in lung washes. A specific IgA response to LPS was detected after immunization with the OMP-containing liposomes but not with the LPS-containing liposomes, indicating adjuvant activity of the proteins. The OMP-containing liposomes were significantly more effective in inducing immune responses than the OMP preparation alone. Responses were highest when mice were given a booster 30 days after primary immunization. Maximum responses occurred 20 days after the booster but specific antibody was still detected 75 days after the secondary immunization. These results suggest that this liposome antigen delivery system has potential in stimulating secretory antibody responses which may be helpful in protecting against infection from *B. pertussis*.

Key words: *Bordetella pertussis*; liposomes; vaccine; outer-membrane proteins; lipopolysaccharide.

Introduction

The incidence of pertussis in the developed countries has been largely reduced through large scale parenteral vaccination with heat-killed whole-cell preparations of *Bordetella pertussis*. Pertussis is, however, still a worldwide problem owing to (i) the paucity of vaccination programs in underdeveloped countries where 95% of the incidence of

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pertussis occurs; (ii) a reduced acceptance rate of current vaccines as a result of the side effects associated with vaccination, and (iii) variability of vaccine efficacy.

One possible reason for the lack of reproducible efficacy of vaccines is that parenteral administration of *B. pertussis* antigens may induce poor and variable mucosal immune responses which are required as first line defence against infection. Epidemiological evidence suggests that current vaccines protect more against the disease than against infection.[1] Oral or intranasal (i.n.) administration of antigens may not only give better protection against infection but may also circumvent the side reactions associated with parenteral vaccination. However, orally administered antigens often induce short-lived immune responses and require adjuvant containing boosters to maintain these responses.

Phospholipid bilayer vesicles (liposomes) have been used as delivery systems for a wide variety of biologically active substances to specific tissues, and more recently have been used as immunological adjuvants to enhance the immune response to several bacterial and viral antigens.[2] Here we report the potential of liposomes as a delivery system to stimulate immunological responses to *B. pertussis* surface antigens in the lungs.

Results

Analysis of outer membrane protein (OMP)-coated vesicles

The OMP-coated vesicles and the OMP preparation were analysed by SDS–PAGE (Fig. 1) and found to contain essentially the same proteins (Fig. 1, lanes 1 and 2), indicating that the incorporation technique did not preferentially select specific proteins. No proteins were directed in the uncoated vesicle preparation (Fig. 1, lane

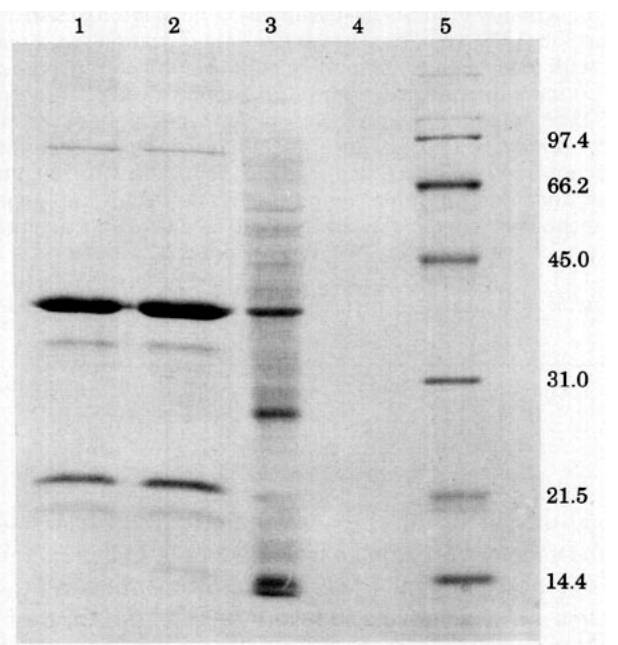

Fig. 1. SDS–PAGE of vesicles. Samples were analysed with a 11% separating gel. Lanes: 1, OMP preparation of *B. pertussis*; 2, OMP-coated vesicles; 3, whole cells of *B. pertussis*; 4, uncoated vesicles; 5, molecular weight markers in kDa. Reproduced here at 90%.

4). Silver staining of the OMP-coated vesicles also indicated the presence of lipopolysaccharide (LPS) (result not shown).

Electron microscopy of the uncoated and OMP-coated vesicles revealed that both vesicles were multilamellar and varied in size from 0.2–2 μm (Fig. 2A–D). However, the OMP-coated vesicles were different in morphological appearance when compared with the uncoated vesicles (compare Figs 2A and B with C and D, respectively); following incorporation of OMP, the vesicles exhibited blebs (Fig. 2D). Incubation of such vesicles with anti-OMP antibodies resulted in an intense labelling of the vesicles (Figs 2E and F) demonstrating the location of the OMP material on the outer surface of the vesicles. In control experiments no labelling was observed (Fig. 2G and H).

Immunization with OMP-coated vesicles

To investigate the adjuvanticity of the coated vesicles, groups of mice were vaccinated i.n. with OMP-coated vesicles or the OMP preparation used to coat the vesicles, at the same protein concentration. For mice immunized with OMP-coated vesicles, the IgA and IgG titres in the lung washes, in ELISA units, were 4.9 ± 0.9 and 6.7 ± 0.8, respectively. The corresponding values in mice immunized with the OMP preparation were 1.2 ± 0.4 and 1.6 ± 0.3, respectively. The OMP-coated vesicles therefore gave an approximately four-fold higher IgA and IgG response than for mice immunized with the OMP preparation. The serum IgG titre for mice immunized with OMP-coated vesicles was about twice that for mice immunized by the OMP preparation. Mice immunized with uncoated vesicles showed no immune response to OMPs (results not shown).

Although the existence of a common mucosal immune system is well established, it was of interest to compare the effectiveness of immunization by the i.n. and oral routes. Titres in the lung washes were similar whether mice were vaccinated i.n. or orally (Table 1). A primary response was detected 10 days after the second immunization at day 4 but titres increased substantially after a booster was given at day 30. It is not known when the peak primary response occurred but it clearly occurred after 10 days as titres were higher for mice killed at day 40 without having received a booster. The IgG titre in the serum paralleled that of the lung response (results not shown).

The dose required to give the maximum response in the lungs was 6–12 μg protein (total dose from two primary immunizations plus one booster) (Fig. 3). Mice immunized with 120 μg protein gave a weaker response indicating an inhibitory effect at higher concentrations. However, with doses as low as 1.25 μg, specific antibodies could still be detected in the lung washes. To determine the duration of the immune responses,

Table 1 Antibody titres (ELISA units $\pm$ SD) to OMP of *B. pertussis* in mouse lung washes after oral and intranasal administration of OMP-coated vesicles

Route of immunization	Days after immunization					
	10		40			
			Without booster[a]		With booster	
	IgA	IgG	IgA	IgG	IgA	IgG
Oral	0.10 ± 0.06	0.11 ± 0.05	ND	ND	4.6 ± 2.0	6.5 ± 1.1
Intranasal	0.11 ± 0.03	0.35 ± 0.17	0.40 ± 0.18	0.48 ± 0.35	5.1 ± 1.5	6.6 ± 1.5

[a] Booster was given 30 days after the first immunization.
ND = not determined.

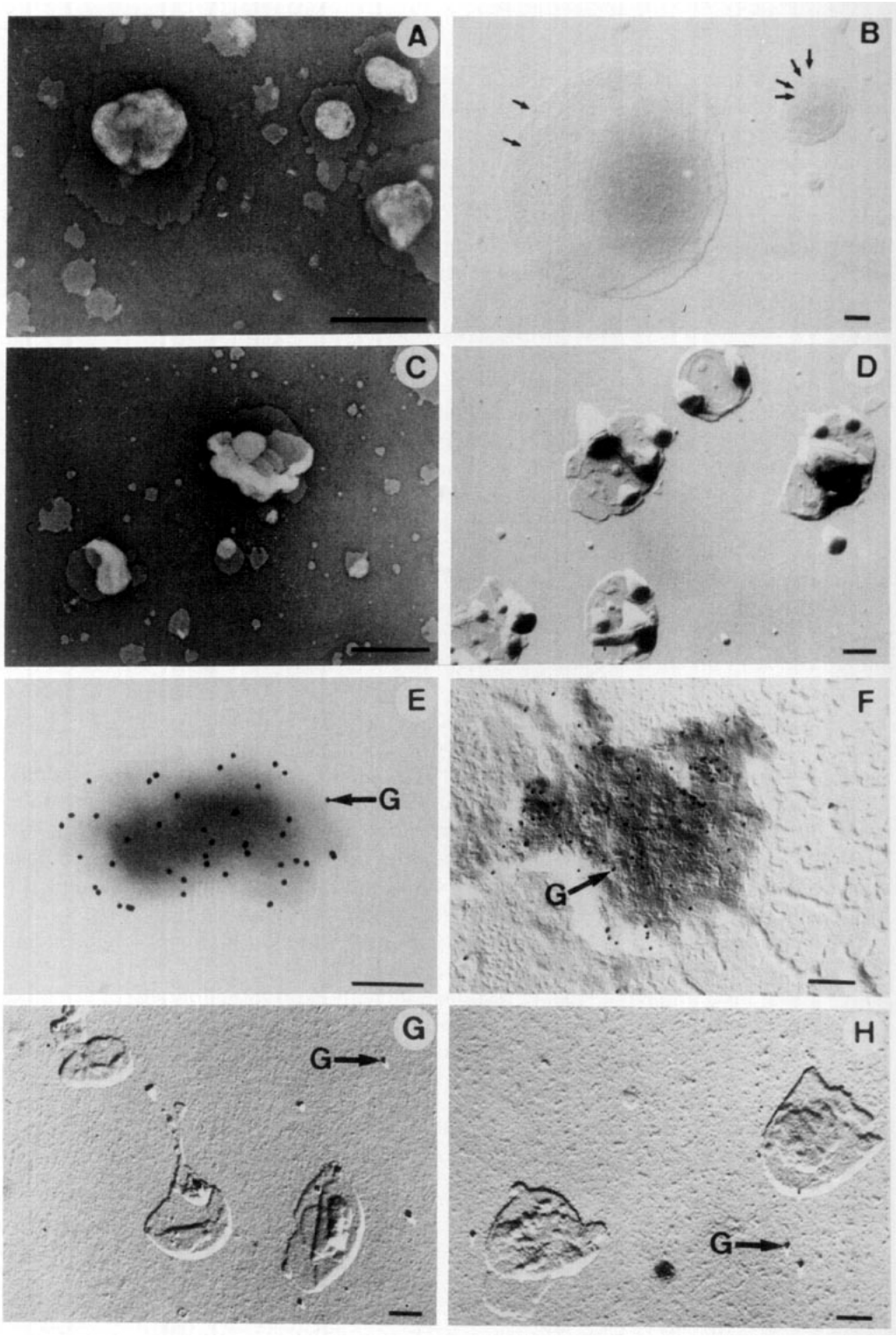

Fig. 2. Electron microscopic examination of vesicles. Uncoated vesicles (A) and OMP-coated vesicles (C) were negatively stained with 4% uranyl acetate. Uncoated vesicles (B) and OMP-coated vesicles (D) show vesicles after unidirectional metal-shadowing. OMP-coated vesicles were incubated with anti-OMP antibodies followed by Protein A-gold complexes (E, unstained vesicles; F, after metal shadowing); the gold particles (indicated by arrows) indicate the OMP material located at the outer surface of the OMP vesicles. In control experiments, OMP-coated vesicles were incubated with preimmune immunoglobulin followed by protein A-gold complexes (G) or with the protein A-gold complexes alone (H); no labelling of the OMP-coated vesicles was detectable. Bars represent 0.2 μm.

mice were sacrificed at various time intervals after the 30 day booster (Fig. 4). The maximum IgA and IgG responses both occurred 20 days after the booster but specific immunoglobulin could still be detected in the lungs after 75 days.

Western blot analysis of the immune response

Lung washes and serum samples from mice immunized orally and intranasally were pooled together and analysed by Western blotting (Fig. 5). Lung washes from mice immunized with OMP-coated vesicles reacted with several OMP bands although the signals were notably stronger for anti-mouse IgG than for anti-IgA. This may be due to differences in avidity or sensitivity between the two detection systems or alternatively because IgG was the more prominent immunoglobulin in the lower respiratory tract.[3] No signals against OMP bands were detected from lung washes obtained from mice immunized with uncoated vesicles (Fig. 5, lanes 3 and 4). Pooled serum from mice immunized with OMP-coated vesicles also reacted with several OMP bands (Fig. 5, lane 5). Both serum and lung washes reacted with the major 32 kDa OMP, but the serum response showed a preference for lower molecular weight bands whereas the secretory response showed a preference for higher molecular weight bands.

Immunization with LPS-coated vesicles

Lipopolysaccharide isolated from *B. pertussis* and LPS-coated vesicles were analysed by SDS–PAGE and silver staining. Both showed the same migration pattern (Fig. 6) and was of the 'ab' phenotype as described by Peppler,[4] wild-type *B. pertussis* possesses two distinct rough LPSs revealed as two bands, designated 'a' and 'b', on SDS–PAGE. After oral and i.n. immunization of mice with vesicles coated with LPS, no specific IgA could be detected in the lung washes (Table 2) although a specific IgG response was detected. However, a specific IgA response to LPS was detected in mice immunized with the OMP-coated vesicles. This suggests that proteins present in the OMP-coated vesicles are required for adequate presentation of the LPS antigens. The IgG response was also higher in mice immunized with OMP-coated vesicles than in mice immunized with the LPS-coated vesicles. Immunization by the i.n. route appeared to be more effective in inducing an immune response than oral immunization. Serum anti-LPS specific IgG titres were similar for OMP vesicle and LPS vesicle immunized mice.

Discussion and conclusions

Allison and Gregoriadis[5] were the first to report that a protein antigen entrapped within liposomes stimulated higher antibody responses than in its free form. Since then, liposome technology for the use of targeted vaccine and drug delivery has advanced considerably and various bacterial, viral and parasite antigens have been incorporated

Table 2 Antibody titres (ELISA units $\pm$ SD) to LPS of *B. pertussis* in mouse lung washes after oral and intranasal administration with LPS- and OMP-coated vesicles

	Oral		Intranasal	
	IgA	IgG	IgA	IgG
LPS-vesicles	0 ± 0.05	0.37 ± 0.12	0 ± 0.05	0.46 ± 0.21
OMP-vesicles	0.66 ± 0.51	1.1 ± 0.34	1.6 ± 0.23	1.6 ± 0.14

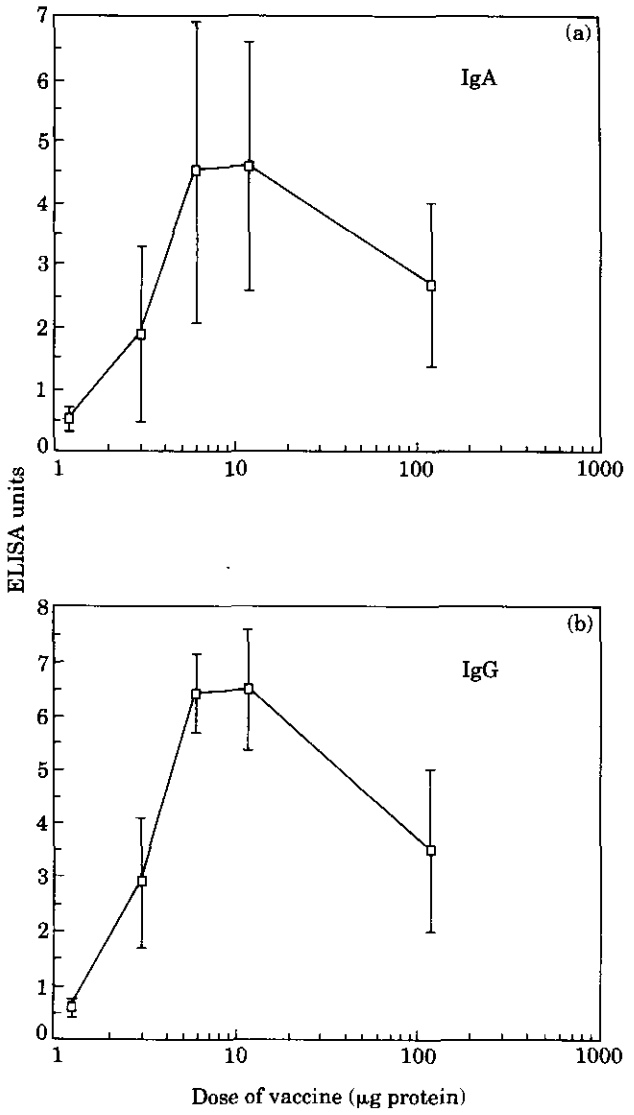

Fig. 3. The effect of dose of vaccine on antibody titres (anti-OMP) in lung washes of mice after intranasal vaccination with OMP-coated vesicles. Bars indicate SD.

and tested for vaccine potential. However, while the chemical composition of liposomes becomes more and more complex with the use of sophisticated chemical derivatives, so does the ultimate cost of the vaccine preparation. A critical parameter in vaccine development is cost-effectiveness while achieving high levels of protection with minimal side effects.

The pathogenesis of pertussis is complex and it is still unclear which antigens should be included in acellular vaccine. Parenteral vaccines based on pertussis toxin (PT) and filamentous haemagglutinin (FHA) gave some protection in Swedish field trials,[6] although it is clear that these vaccines were contaminated with other antigens which may have contributed to protection.[7] Data from this study demonstrated lower than expected efficacy and a lack of serological marker for protection, which raised serious

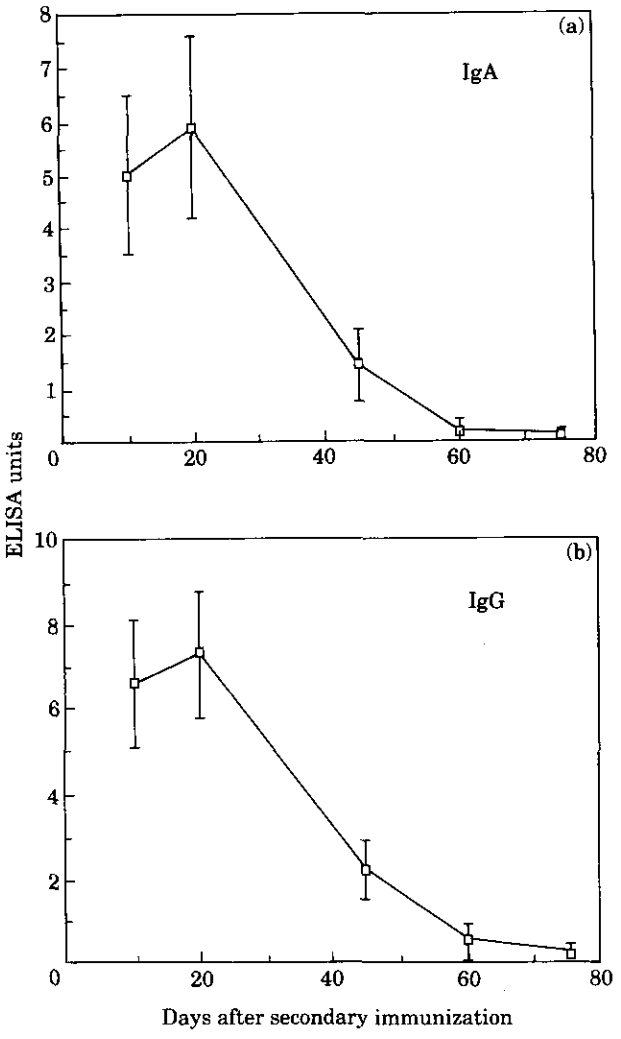

Fig. 4. Duration of antibody response after intranasal immunization of mice with OMP-coated vesicles. Mice were vaccinated and killed at the days indicated and the anti-OMP titres from lung washes were determined. Bars indicate SD.

questions about the adequacy of PT (alone or combined with FHA) as an acellular vaccine and suggest that consideration should be given to inclusion of other antigens.[8]

Here we report the use of soya bean-derived phospholipids (a cheap source) to prepare liposomes carrying OMPs and LPS from *B. pertussis* for oral delivery to stimulate a generalized mucosal immune response. Immunoelectron microscopy analysis of antigen-incorporated and control liposomes provided interesting data (Fig. 2) showing that, although the general assumption is that antigens are entrapped within the multilamellar structure of liposomes, we find a significant number of antigenic regions are actually exposed on the surface of the vesicles. This observation is of interest since the antigen-incorporated vesicles may be capable of direct B-cell stimulation as well as T-helper cell-mediated B-cell proliferation.

The adjuvant activity of the liposomes was confirmed by the observation that mice immunized with OMP vesicles gave secretory antibody titres about four-fold higher

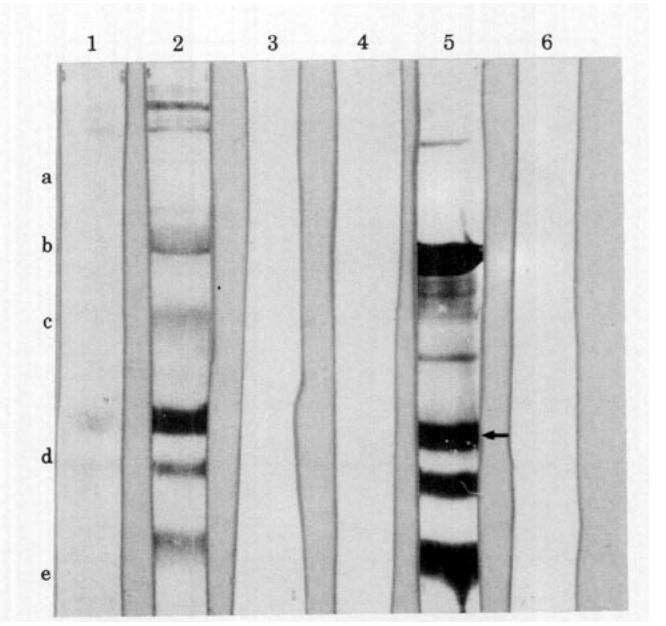

Fig. 5. Westerm blot analysis of lung washes and serum. OMPs of *B. pertussis* were separated by SDS–PAGE with a 11% separating gel and blotted. Strips: 1 and 2 were incubated with pooled lung washes from mice immunized with OMP-coated vesicles; 3 and 4 with pooled lung washes from mice immunized with uncoated vesicles; 5 with pooled serum from mice immunized with OMP-coated vesicles; and 6 with pooled serum from mice immunized with uncoated vesicles. Strips 1 and 3 were incubated with peroxidase conjugated anti-mouse IgA, as second antibody, and strips 2, 4, 5 and 6 with anti-mouse IgG. Arrow indicates the 32 kDa protein. Molecular weight markers are indicated on the right and in kDa they represent: a, 97.4; b, 66.2; c, 45.0; d, 31.0; e, 21.5.

Fig. 6. Silver staining of LPS-coated vesicles. Lanes: 1, LPS extracted from *B. pertussis*; 2, uncoated vesicles; 3, LPS-coated vesicles. Arrows show the 'a' and 'b' LPS bands. Reproduced here at 80%.

than mice immunized with the OMP preparation itself. The titres to OMP were similar in mice vaccinated orally or i.n., although mice vaccinated i.n. appeared to give a higher titre to LPS. Further, the OMP preparation itself may have adjuvant activity in stimulating an IgA response to LPS, since we observed that secretory IgA responses against LPS could only be detected after immunization with the OMP-coated vesicles (containing residual quantities of LPS) and not the LPS-coated vesicles.

Although the role of anti-LPS antibodies in protection is not clear, it has been suggested that anti-LPS antibodies in humans may play a role in preventing colonization.[9] The inclusion of LPS in the liposome-based vaccine may also contribute to the OMP-specific immune response, since it is now known that the immunogenic potency of liposomes can be increased by the inclusion of immunostimulators such as bacterial LPS, lipid A or muramyl dipeptide.[10–12]

Maximum antibody responses observed occurred 20 days after the 30 day booster in mice immunized i.n. with OMP-coated vesicles, although, secretory antibody could still be detected 75 days after immunization. This is of particular interest in view of the observation of Goodman *et al.*,[3] that anti-pertussis IgA can be detected in nasopharyngeal secretions 3 months after natural infection in humans, but not after parenteral vaccination. Furthermore, it is generally accepted that natural infection of pertussis results in better and longer lasting immunity than parenteral vaccination.

The oral route of administration may indeed circumvent many of the toxic side effects associated with parenteral administration of crude vaccines, especially those associated with LPS. Side effects associated with pertussis toxoid preparations may also be reduced. It is of interest to note that in a study of 15 000 infants, a whole-cell vaccine given orally was as effective as vaccine given parenterally but the reactogenicity was essentially eliminated with oral immunization.[14]

The OMP-coated liposome preparation used in this study to elicit a secretory response is undoubtedly a crude vaccine. However, the liposome delivery system, which enhanced antibody stimulation to the OM antigens, could equally be used to incorporate purified secreted antigens such as FHA, detoxified PT (or protective peptides), the 69 kDa OMP associated with virulence, which have been shown to be protective in mouse models,[15] and possibly others such as the adenylate cyclase toxin-haemolysin and dermonecrotic (heat-labile) toxin. The soya bean lipid source used to manufacture these liposomes is relatively cheap and therefore attractive for large scale vaccine production.

Materials and methods

Bacterial strains and cultivation. B. pertussis D300421 (serotype 1.2.3)[16] was grown on Bordet Gengou base (Difco) with 1% (v/v) glycerol and 15% (v/v) defibrinated horse blood. For large scale production, *B. pertussis* was grown in Modified Hornibrook medium.[17]

Preparation of lipopolysaccharide. Two litres of a 48 h *B. pertussis* culture were harvested by centrifugation and washed once with phosphate-buffered saline (PBS; NaCl 8 g/l, KCl 0.2 g/l, KH_2PO_4 0.2 g/l, $Na_2HPO_4.2H_20$ 2.9 g/l, pH 7.4). The cells were resuspended in distilled water to a final volume of 75 ml and incubated at 65°C for 30 min. The suspension was then mixed with an equal volume of 90% (w/v) phenol and incubated at 65°C for 45 min with constant mixing. The suspension was allowed to cool on ice and centrifuged at 10 000×*g* for 10 min. The top aqueous layer was removed and centrifuged at 10 000×*g* for 10 min to remove debris. The LPS was then precipitated by the addition of two volumes of ice cold acetone and incubation overnight. The precipitate was collected by centrifugation at 10 000×*g* for 10 min at 4°C and washed with 70% acetone. The dried pellet was resuspended in 20 ml distilled water and ultracentrifuged at 100 000×*g* for 2 h. Pellets were washed twice more with distilled water and lyophilized.

Preparation of OMPs. Two litres of a 48 h *B. pertussis* culture were harvested by centrifugation and cells were washed once in PBS. Cells were resuspended in 50 ml PBS and heat-killed (56°C for 30 min). Phenyl-methane-sulphonylfluoride (PMSF) was added to a final concentration of 0.1 mM and the cells disrupted by sonication (ten 1 min pulses on ice with a 1 min pause between pulses). Unbroken cells were removed by centrifugation at 5000×*g* for 10 min and the envelopes collected by centrifugation at 100000×*g* for 1 h. The envelopes were then resuspended in 20 ml 2% Triton X-100, 7.5 mM $MgCl_2$, 50 mM Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) pH 7.4, allowed to stand for 1 h on ice, and collected by centrifugation at 100000×*g* for 1 h. They were then washed once with the same buffer and twice with distilled water. Protein concentrations were determined using a modification of the Lowry method.[18]

Preparation of vesicles. Lyphazome™ a proprietary form of small multilamelar phospholipid vesicles, containing *B. pertussis* LPS and OMP preparations were prepared using the Solvent Dilution Microcarrier technique developed by Fountain Pharmaceuticals Inc.,[19] using purified soya bean phosphatides supplied by American Lecithin Company, New York. The composition of the purified lipid mixture was phosphatidylcholine, phosphatidylethanolamine, phosphatidic acid and neutral lipids in an approximate ratio of 8:1:0.7:0.3. The amount of OMP which was incorporated into the liposomes was estimated using the techniques described previously,[20] and the estimated concentration of encapsulated protein was 80 μg/ml while the amount of unencapsulated protein was less than 2 μg/ml. This material, which was subjected to protein analysis, had already been passed over a Sephadex G-25 column. The antigen encapsulated liposomes were excluded from the gel bed and ran in the excluded volume while the non-encapsulated material was retarded and maintained in the included volume. The presence of liposomes with incorporated antigen was measured by turbidometric measurement on half-ml collected fractions as described previously.[21] The encapsulated liposomes were stored at 4°C and without any further separation or washing were used for immunization within 3 weeks.

Immunization of mice. Four to 5-week-old female BALB/c mice were immunized in groups of five. Mice immunized intranasally were anaesthetized by ether and 50 μl of vaccine dilution in PBS was deposited on the external nares and allowed to be inhaled. For mice immunized orally, vaccine was diluted in PBS and an equal volume of 3% sodium bicarbonate in PBS (pH 8.0) was added just prior to immunization to neutralize gastric acidity. Mice deprived of water for 6–8 h, were gently fed with 50 μl. Unless otherwise stated, mice were immunized on days 1 and 4 and given a booster on day 30. One dose consisted of 4 μg protein for OMP-coated vesicles, and 15 μg dry weight for LPS-coated vesicles and uncoated vesicles. Unless otherwise stated, mice were killed by cervical dislocation 10 days after booster and bled from the brachial artery. Lung washes were obtained by cannulating the trachea with a syringe and filling and emptying the lungs four times with 0.7 ml ice-cold PBS containing 0.1 mM PMSF. About 0.5 ml of lung wash was recovered from each mouse, centrifuged at 4°C at 10000×*g* for 10 min to remove debris and stored at −20°C.

Antiserum to OMP. OMP preparation was emulsified with Freunds incomplete adjuvant at a ratio of 1:1 in a final volume of 1 ml and a 3 month Chinchilla rabbit was injected subcutaneously and intramuscularly on day 1 (200 μg), day 30 (100 μg) and day 40 (100 μg). The rabbit was killed on day 50, serum obtained and immunoglobulin purified by protein A-sepharose CL4B chromatography.

SDS–PAGE and Western blot analysis. Proteins and LPS were electrophoretically separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) as previously described.[22] Proteins were stained by Coomassie Blue and LPS by silver staining.[23] Western blotting was carried out as previously described.[24] Briefly, proteins were transferred to nitrocellulose using a semi-dry transfer cell with 25 mM Tris. 192 mM glycine, 20% methanol pH 8.3 as transfer buffer and 5% bovine serum albumin (BSA) in PBS as a blocking agent. Blocked membranes were incubated for 1 h at 37°C with either lung washes, diluted 1 in 4 in PBS, or serum sample diluted 1 in 10 in PBS. After washing three times with PBS, the membranes were incubated with peroxidase conjugated goat anti-mouse IgA (Southern Biotechnology Associates Inc.) or anti-mouse IgG (Jackson Immunoresearch Laboratories) for 1 h at 37°C. Membranes were washed three times in PBS and developed using 4-chloro-1-naphthol as a substrate.

ELISA. Microtitre plates (Nunc Maxisorp) were coated overnight at 4°C with either OMP in 0.1 M $NaHCO_3$ pH 9.6 (5 μg in 50 μl per well) or LPS in 50 mM Tris-HCl pH 9.6, 20 mM $MgCl_2$ (1 μg in 50 μl per well). The plates were washed and blocked with 10% foetal calf serum (FCS) in PBS (100 μl per well) for 1 h at 37°C. After washing, plates were incubated with various dilutions of serum or lung washes in 10% FCS in PBS (50 μl per well) for 1 h at 37°C. Plates were washed and 50 μl (per well) of peroxidase conjugated goat anti-mouse IgA or IgG diluted 1:1000 in 10% FCS in PBS was added. Plates were incubated as before and after washing, plates were developed by addition of 50 μl (per well) of substrate (0.25 M citric acid, 0.25 M Na_2HPO_4, 0.0015% H_2O_2, 0.3 mg ml^{-1} O-phenyl-diamine dihydrochloride). After 30 min at room temperature, the reaction was stopped by the addition of 50 μl of 0.25 M H_2SO_4 and the A_{490} was determined using a Biorad model 3550 microplate reader. The results refer to the average values obtained from samples of five mice and ELISA units were calculated by taking the mean values of the optical densities at A_{490} for each immunization group and multiplying it by the respective serum or intestinal fluid dilution factor. Standard deviations represent variations between individual mouse samples in each group. The A_{490} values were always taken from the linear part of the titration curve. Undiluted lung washes and serum samples (diluted 1:50) from control mice gave essentially null readings.

Electron microscopy. Uncoated and OMP-coated vesicles were negatively stained with 4% aqueous uranyl acetate, pH 4.5, according to Valentine *et al.*[25] For metal-shadowing the two vesicle samples were absorbed onto freshly prepared formvar covered nickel grids, washed with distilled water, air-dried and unidirectionally metal-shadowed with platinum (20° angle).

Immunoelectron microscopy. Uncoated and OMP-coated vesicles were absorbed onto freshly prepared collodium covered nickel grids and carefully washed with distilled water. After air drying at room temperature the grids were treated with purified antibodies (100 μg IgG protein ml^{-1}) for 30 min at room temperature. Unbound antibodies were removed with a mild spray of PBS (0.1 M potassium phosphate, 0.15 M NaCl, pH 6.9) from a plastic bottle. The bound antibodies were made visible for electron microscopy by incubating the grids on drops of protein A-gold complexes (10 nm gold particle size and A_{520} of 0.03) for 10 min at room temperature. Grids were subsequently rinsed with PBS containing 0.01% Tween 20 followed by distilled water. After air drying the grids were unidirectionally metal-shadowed with platinum (20° angle). In control experiments, samples were treated with purified preimmune immunoglobulin or with protein A-gold complexes alone. Samples were examined with a Zeiss electron microscope CEM 902 or 10B at an acceleration voltage of 80 kV and at calibrated magnifications.

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