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Antibody Responses in the Serum and Respiratory Tract of Mice following Oral Vaccination with Liposomes Coated with Filamentous Hemagglutinin and Pertussis Toxoid

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Mice were orally vaccinated with liposomes coated with filamentous hemagglutinin (FHA) and detoxified pertussis toxin (PT) of *Bordetella pertussis*. FHA- and PT-specific immunoglobulin G (IgG) was detected in serum, and both IgG and IgA were detected in lung washes following the immunization. Antibody responses in mice immunized with liposomes coated with FHA and PT were significantly higher than those in mice immunized with free FHA and PT, which demonstrated the adjuvanticity of the liposome carrier. The results indicate the potential usefulness of this approach for eliciting immune responses against FHA and PT (and perhaps other pertussis antigens) in humans and its possible utility in large-scale vaccination to protect against both *B. pertussis* infection and disease.

Bordetella pertussis is the etiological agent of whooping cough, an infection of the human respiratory tract. The disease is particularly severe in young children and may lead to neurological disorders and death (48). The impact of pertussis morbidity and mortality on health is usually underestimated because of underreporting; however, 60,000,000 cases and more than 500,000 deaths per year are estimated to have a pertussis etiology or to be pertussis related (30). The prevalence of pertussis in countries where vaccination is not mandatory is very high, with 95% of the sera of 17- to 19-year-old individuals containing antibodies against pertussis (16).

In developed countries, the incidence of whooping cough has been reduced largely by mass immunization with a heat-killed whole-cell vaccine (10). The benefits associated with this vaccine clearly outweigh the risks of rare but severe adverse effects, but nevertheless, public concern about safety and immunogenicity has resulted in decreased vaccination rates (22, 29). Moreover, the efficacy of the whole-cell vaccine has been questioned in a few countries such as the United States because, although immunization rates are high, between 30,000 and 125,000 cases of pertussis have occurred annually (43). Therefore, a new generation of nontoxic and highly immunogenic vaccines, consisting of well-defined components, is urgently needed. Several virulence factors of *B. pertussis* have been considered for inclusion in defined acellular vaccines, and detoxified pertussis toxin (PT) and filamentous hemagglutinin (FHA) are prime candidates (27, 34). Conventional vaccines administered by the parenteral route seem to primarily elicit humoral immunity, and the secretory antibody responses are poor (17, 41, 44). *B. pertussis* bacteria, however, infect the body through the respiratory tract mucosal membrane, and specific immunoglobulin A (IgA) is elicited after natural infection (44). Such antibodies may well protect the host from both colonization and disease. It would, thus, seem worthwhile to explore means of stimulating mucosal immunity of airways and to evaluate its utility in protecting from infection and disease.

Liposomes have been successfully used as delivery systems for drugs, antigens, hormones, and genetic material (18). Promising results have also been obtained following immunization with liposome-associated antigens; the oral (3, 19, 35, 37, 47) and parenteral (4, 8, 13, 24, 25, 38, 39, 42) routes have been used for antigens of parasites (4, 24, 25, 37, 39), viruses (13, 32), and bacteria (3, 8, 19, 35, 38, 42, 47). Their potential as adjuvants has been demonstrated in several studies, in which the use of liposome-associated antigens resulted in protective immunity (4, 8, 19, 24, 25, 37, 39, 42) or at least cell-mediated (8) and humoral responses (3, 13, 38). The adjuvanticity of liposomes seems to depend on several factors including vesicle size and structure, lipid constitution, surface charge, antigen localization, the animal species immunized, route of immunization, and the distribution and number of lamellae. The association of orally administered antigens with liposomes enhances their absorption, targeting them to processing cells, and favors their presentation to T cells and uptake into regional lymph nodes. This process improves the induction of humoral, secretory, and cell-mediated immune responses (1, 2, 18, 45).

In the present report, we describe the ability of FHA- and PT-coated liposomes to induce specific systemic or secretory immune responses following oral immunization of mice.

MATERIALS AND METHODS

Preparation of antigen-coated liposomes. FHA was purified from *B. pertussis* Tohama as previously described by Sato et al. (40), and PT was kindly supplied by S. Cryz (Swiss Serum and Vaccine Institute, Berne, Switzerland) and detoxified as described by Munoz et al. (31). Lyphazome liposomes, a

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commercial form of small multilamellar phospholipid vesicles, containing *B. pertussis* FHA and PT, were prepared by the solvent dilution microcarrier technique developed by Fountain Pharmaceuticals, Inc. (11), using purified soybean phosphatides supplied by American Lecithin Company, New York, N.Y. The composition of the purified lipid mixture was phosphatidylcholine, phosphatidylethanolamine, phosphatidic acid, and neutral lipids at an approximate ratio of 8:1:0.7:0.3. The unincorporated antigen was removed by size exclusion chromatography. The final-preparation vaccine was passed over a column whose gel matrix was Sephadex G-25 medium. The coated liposomes were excluded from the gel bed and ran in the excluded volume, while the nonincorporated antigen was retarded and was retained in the included volume. The presence of liposomes and antigen was measured by turbulometric measurements on collected fractions as previously described (12).

Size determinations of liposomes. Size analysis studies of the FHA-coated and PT-coated liposomes were performed immediately after synthesis by using a Coulter submicron particle analyzer N4MD (Coulter Electronics, Inc., Hialeah, Fla.).

Western blot analysis. Monoclonal antibody P12H3 against FHA (14) and a cocktail of monoclonal antibodies reactive against PT subunits S1 (E19), S4-S5 doublet (E205), and S2 and S3 (E251) (51) were used in Western blotting (immunoblotting) experiments (5). Free antigens and FHA-coated and PT-coated liposomes were mixed with loading buffer at a ratio of 1:1, and protein was electrophoresed according to the procedure of Laemmli (28) using a 3.85% acrylamide stacking gel and a 10% acrylamide separating gel. Proteins were transferred to a nitrocellulose membrane (Bio-Rad Laboratories SRL, MI, Segrate, Italy) with a semidry transfer cell (Bio-Rad) by using 25 mM Tris–192 mM glycine–20% methanol (pH 8.3) as the transfer buffer and a 10% solution of low-fat (0.3%) milk in phosphate-buffered saline (PBS) (137 mM NaCl, 27 mM KCl, 14 mM $Na_2HPO_4 \cdot 2H_2O$, 15 mM KH_2PO_4), pH 7.4, as the blocking reagent. The blocked membrane was incubated for 2 h with the first antibody, either P12H3 or a cocktail of E19, E205, and E251 hybridoma supernatant fluids diluted 1:20 in PBS (pH 7.4). After three washes with PBS, the membranes were incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse IgG diluted 1:500 in PBS (Southern Biotechnology Associates, Inc., Birmingham, Ala.). Membranes were washed and developed by using 4-chloro-1-naphthol as a substrate. Prestained molecular weight markers were purchased from Bio-Rad.

Electron microscopy. Uncoated, FHA-coated, and PT-coated liposomes were negatively stained with 4% aqueous uranyl acetate, pH 4.5, according to the method of Valentine et al. (46). For metal shadowing, the liposome samples were absorbed onto freshly prepared collodium-covered 300-mesh nickel grids, washed with distilled water, air dried, and unidirectionally metal shadowed with platinum at an angle of 15°.

For immunoelectron microscopy, uncoated or antigen-coated liposomes were absorbed onto freshly prepared collodium-covered 300-mesh nickel grids and carefully washed with distilled water. After being air dried at room temperature, the grids were treated with protein A-purified anti-FHA or anti-PT polyclonal (21, 50) or monoclonal (14, 51) antibodies (125 μg of IgG protein ml^{-1}) for 60 min at room temperature. Unbound antibodies were removed by a mild spray of PBS (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 (gold particle size = 10 nm, A_{520} = 0.01) for 15 min at room temperature. Subsequently, the grids were rinsed with PBS containing 0.01% Tween 20 and then with distilled water. After being air dried, the grids were unidirectionally metal shadowed with platinum (angle, 15°) or examined without metal shadowing. In control experiments, the samples were either treated with purified preimmune serum or with protein A-gold complexes alone. For postembedding labeling, the PT-coated liposomes were embedded according to the method of progressive lowering of temperature by using Lowicryl K4M resin and applying the labeling protocol as described elsewhere (50). Samples were examined with a Zeiss electron microscope EM 10B at an acceleration voltage of 80 kV and at calibrated magnifications.

Mouse immunization. Five- to six-week-old female BALB/c mice (Charles River) were orally immunized in groups of five as follows: group a, FHA-coated liposomes; group b, PT-coated liposomes; group c, FHA-coated and PT-coated liposomes; group d, free FHA; group e, free PT; group f, free FHA and PT; control group, uncoated liposomes. The animals were caged separately. The vaccination regimen was one dose of protein (4 μg) on days 0 and 4, followed by a booster of an identical dose on day 30. Mice that had been deprived of water for 6 to 8 h were orally given 50 μl of vaccine diluted in PBS and an equal volume of 3% sodium bicarbonate in PBS, pH 8.0, which was added immediately before in order to neutralize gastric acidity. The animals were anesthetized with ether and sacrificed by cervical dislocation 10 days after the booster. The blood samples were collected by cutting the brachial artery, and the serum was separated and stored at −20°C. Lung wash samples were collected by pertracheal cannulation and gentle washing with 0.7 ml of ice-cold PBS containing 2 mM phenylmethylsulfonyl fluoride as a protease inhibitor. About 0.5 ml of lung wash was recovered from each mouse, centrifuged at 4°C at 10,000 × *g* for 5 min to remove debris, and stored at −20°C.

Determination of specific antibodies against FHA and PT. For the determination of class-specific antibodies against FHA and PT present in serum and lung washes, enzyme-linked immunosorbent assays (ELISA) were performed as follows. Nunc Maxisorp Immunomodule 96-well plates were coated with FHA or PT diluted in 0.1 M $NaHCO_3$ (pH 9.6) at 60 ng in 50 μl per well and incubated at 4°C overnight. The wells were blocked with 100 μl of 10% fetal calf serum (ICN-Flow Biomedicals Societá per Azioni MI, Cassina de' Pecchi, Italy) in PBS for 2 h at 37°C. Plates were subsequently washed three times with PBS, and a 100-μl serum sample diluted 1:50 or lung wash sample diluted 1:10 in 10% fetal calf serum in PBS was added to each well. After 60 min at 37°C, the plates were again washed, 100 μl of alkaline phosphatase-conjugated goat anti-mouse antibodies for IgG, IgM, or IgA heavy chains (Southern Biotechnology Associates, Inc.) diluted 1:500 in 10% fetal calf serum in PBS was added to each well, and the mixtures were incubated for 2 h at 37°C. The plates were again washed and then developed by the addition of 100 μl of the substrate solution (10 mg of *p*-nitrophenylphosphate disodium salt per ml in diethanolamine buffer, pH 9.8) per well. After 30 min at room temperature, the reaction was stopped by the addition of 50 μl of 3.0 M NaOH, and the A_{405} was determined with a Titretek Multiskan MCC microplate reader (ICN-Flow Biomedicals Societá per Azioni). All samples were processed simultaneously on the same day, each serum or lung wash sample was individually assayed in duplicate, and uncoated-lipo-

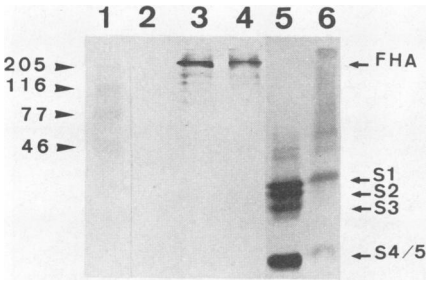

FIG. 1. Incorporation of FHA and PT into liposomes. Lane 1, molecular mass standards; lane 2, uncoated liposomes; lane 3, free FHA; lane 4, FHA-coated liposomes; lane 5, free PT; lane 6, PT-coated liposomes. FHA and PT subunits are indicated on the right, and the molecular masses of the standards in kilodaltons are indicated on the left.

some-immunized mouse serum or lung wash samples were used as blanks for the ELISA readings (nonspecific activity, 0.025). Results are expressed as mean values for each immunization group; standard deviations represent variations between individual mouse samples in each group. Titration assays of representative samples demonstrated that the A_{405} values obtained for the single point dilutions chosen fall on the linear part of a standard curve.

Statistical calculations. Antibody responses of mice immunized with FHA- or PT-coated liposomes and those immunized with free FHA or PT were analyzed for significance by analysis of variance and Student's t test. Differences were considered significant at $P \leq 0.05$.

RESULTS

Size determinations of liposomes containing FHA and PT. The mean diameters (with 95% confidence intervals in parentheses) of uncoated and FHA- and PT-coated liposomes were 227 (211 to 243) nm, 236 (219 to 253) nm, and 244

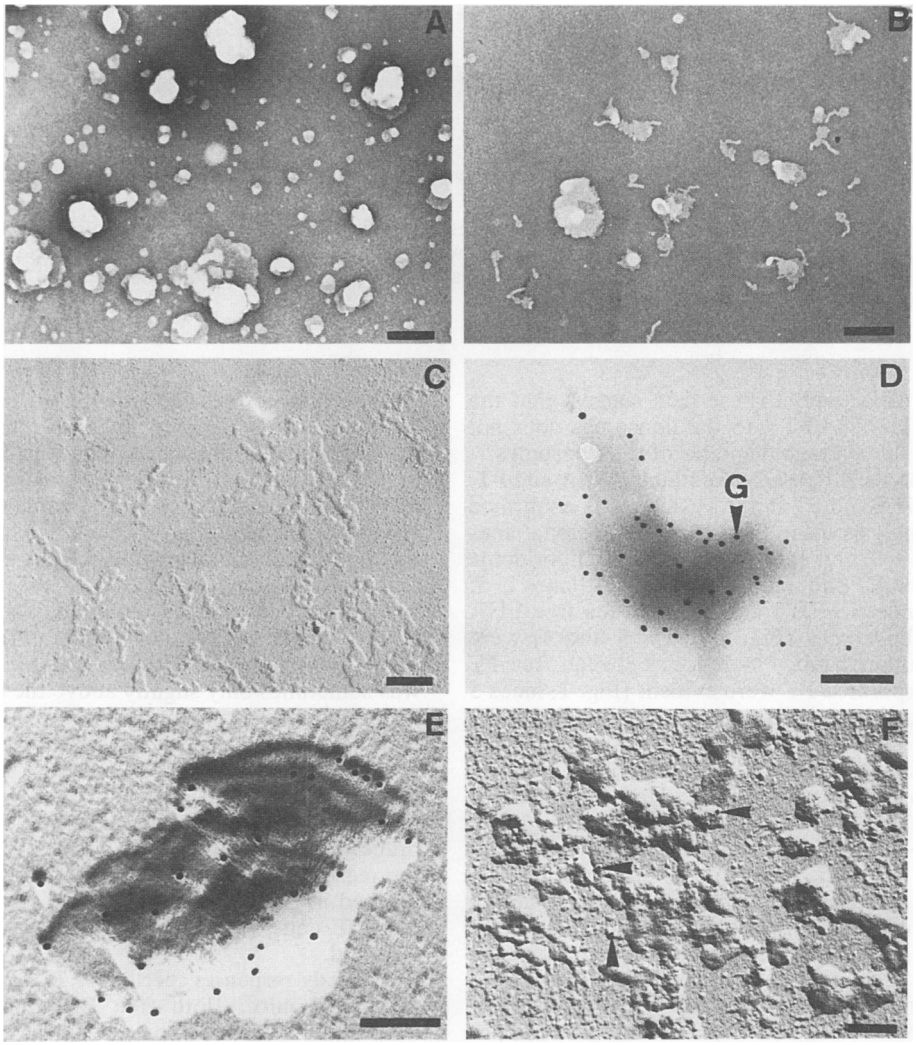

FIG. 2. Electron-microscopic examination of FHA-coated liposomes that were negatively stained (B), unidirectionally metal shadowed (C), incubated with polyclonal anti-FHA antibodies and then with protein A-gold complexes without (D) or with (E) metal shadow, or incubated with preimmune serum and protein A-gold complexes (F). Negatively stained uncoated liposomes are shown in panel A. G, gold particle. Bars, 0.2 μm.

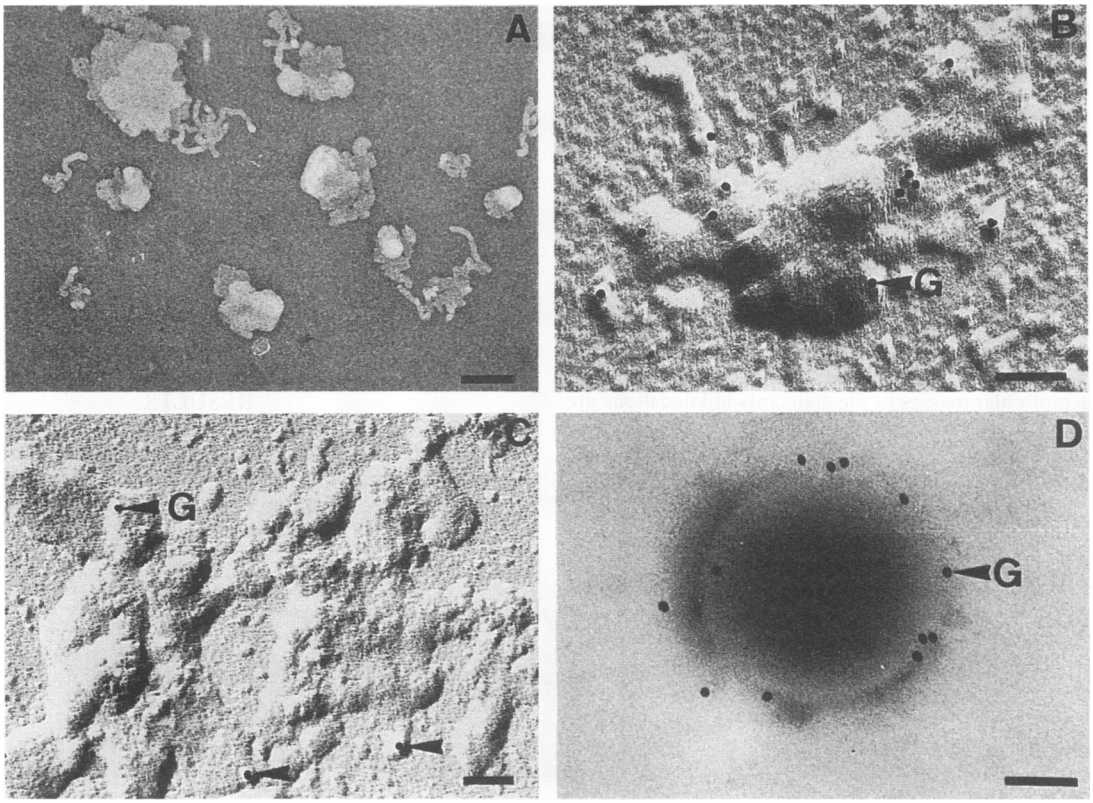

FIG. 3. Electron-microscopic examination of PT-coated liposomes that were negatively stained (A), incubated with polyclonal antibodies against PT and then with protein A-gold complexes and metal shadowed (B), incubated with preimmune serum and then with protein A-gold complexes and metal shadowed (control experiment) (C), or incubated with polyclonal anti-PT antibodies and protein A-gold complexes (postembedding labeling) (D). G, gold particle. Bars, 0.1 μm.

(226 to 262) nm, respectively. These data suggest that the incorporation of FHA and PT into the liposomes does not significantly affect the average diameter of the liposomes.

Western blot analysis of liposomes containing FHA and PT. The presence of FHA and PT in the vesicles was confirmed by Western blot analysis using the P12H3 monoclonal antibody (Fig. 1, lanes 2, 3, and 4) and the cocktail of hybridoma supernatants against PT subunits S1 to 4 (Fig. 1, lanes 2, 5, and 6). No differences in FHA content between free FHA and FHA-coated liposomes (Fig. 1, lanes 3 and 4) were detected. On the other hand, differences between free PT and liposome-incorporated PT were evident (Fig. 1, lanes 5 and 6), suggesting either variations in binding affinity between monoclonal antibodies specific for S1 or S4 and S2 or S3 or preferential incorporation of S1 and S4-S5 subunits in the liposomes. No proteins were detected in the uncoated liposomes (Fig. 1, lane 2).

Immunoelectron-microscopic analysis of liposomes containing FHA and PT. Electron microscopy of the uncoated, FHA-coated, and PT-coated liposomes revealed that the liposomes were multilamellar and varied in size (Fig. 2A, B, and C and 3A). The differences in size were greater than those shown by Coulter analysis. This could be due in part to either storage after synthesis or the methods used for electron microscopy. Moreover, the FHA- and PT-coated liposomes exhibit a morphological appearance different from that of the uncoated liposomes (compare Fig. 2A with B and with 3A), with the presence of protrusions most probably due to the incorporation of FHA and PT into the liposomes. However, the protrusions are not artifacts, as demonstrated by metal shadowing (Fig. 2C and E and 3B). Incubation of the FHA-coated liposomes with anti-FHA polyclonal antibodies and then with protein A-gold complexes resulted in an intensive labeling pattern on the surface of the FHA-coated liposomes (Fig. 2D and E). Incubation with the monoclonal antibodies against FHA revealed less labeling (data not shown). Incubation of the PT-coated liposomes with polyclonal anti-PT antibodies resulted in a labeling pattern much weaker than that for the FHA-coated liposomes (compare Fig. 2D with 3B). Since the label intensity was sometimes only slightly above the background level, we have undertaken to demonstrate by postembedding labeling that PT can be detected on the liposome membrane. The former method allows only for the detection of antigenic reactive sites which stick out of the liposomes but not of those which reside on the inner side of the liposome membrane. In Fig. 3D, a liposome which exhibits a label around the membrane is depicted, demonstrating that PT is incorporated in the external membrane. In control experiments (Fig. 2F and 3C), only a very few gold particles could be detected.

Antibody responses specific for *B. pertussis* FHA and PT in vaccinated mice. Both free FHA and PT and PT- and FHA-coated liposome prototype vaccines elicited specific serum and mucosal antibody responses (Fig. 4 and 5). After oral immunization of mice with a total dose of 12 μg of FHA or PT, administered in three equal doses, both systemic (mainly IgG) and lung secretory (mainly IgG and IgA)

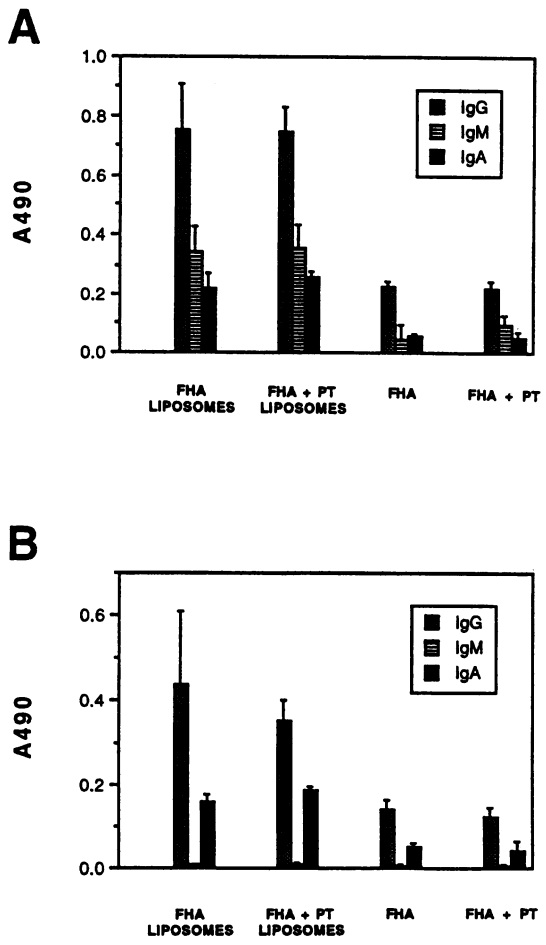

FIG. 4. Levels of anti-FHA specific antibodies in serum (A) and lung wash (B) samples after oral immunization of mice with free FHA and FHA-coated liposomes. Bars, standard deviations.

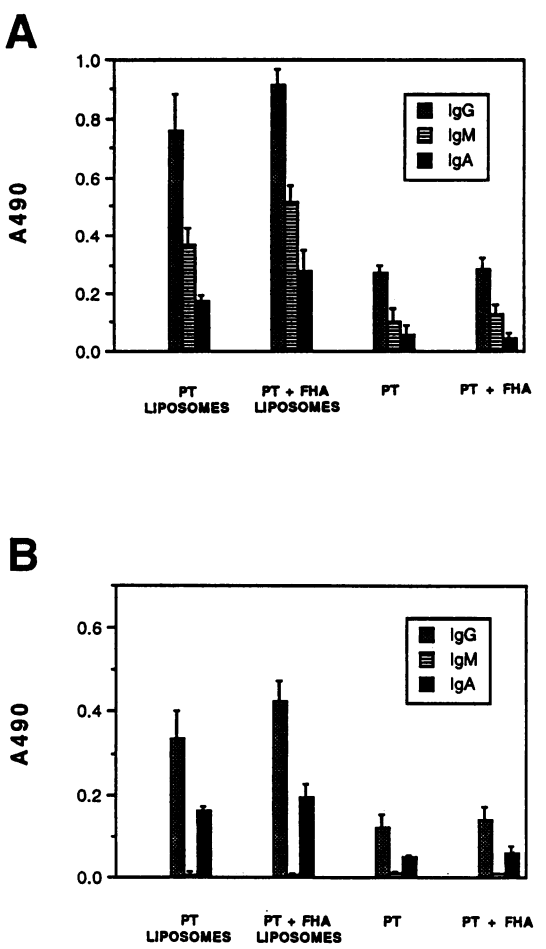

FIG. 5. Levels of anti-PT specific antibodies in serum (A) and lung wash (B) samples after oral immunization of mice with free PT and PT-coated liposomes. Bars, standard deviations.

antibody responses were obtained (Fig. 4A and B). The adjuvanticity of the liposome-based system was confirmed for FHA by the presence of antibody responses in mice immunized with FHA-coated liposomes that were approximately three times higher than the responses in mice immunized orally with free protein (Fig. 4). The differences in antibody levels between mice immunized with FHA-coated liposomes and those immunized with free protein were statistically significant ($P \leq 0.05$). Simultaneous vaccination with FHA and PT, both free and liposome associated, did not affect anti-FHA antibody responses. The levels of FHA-specific IgM detected in lung wash samples were not significant ($P > 0.05$).

Similar results were obtained by immunization with PT (Fig. 5A and B), and as with FHA, coimmunization with both proteins (FHA and PT) gave good anti-PT antibody responses.

DISCUSSION

Current parenteral vaccines against whooping cough are more effective in protecting against clinical pertussis than against infection. In designing new, safer vaccines, however, it would seem appropriate to reconsider the immunization strategy and to take into account the respiratory tract portal of entry of pertussis infection and possible use of vaccines that stimulate efficient responses at the mucosal level which block infection. Secretory IgA and specific IgG (derived from serum by transudation through capillary vessels and being the predominant Ig in the lower respiratory tract) may interfere with the early events of bacterial attachment and colonization and thereby facilitate the eradication of the disease.

The site of antigen processing influences the Ig class profile of the immune response elicited. In fact, the secretory IgA response is typical of antigen administration by oral route (2, 6, 19, 23, 26, 45). The specialized M cells which cover the Peyer's patches pass antigenic material to lymphocytes below the epithelium, where the processed antigens are presented to IgA precursor B cells. These B cells travel via the lymphatic system to the different mucosae and then give rise to IgA-secreting plasma cells. T lymphocytes may also acquire their homing pattern in the Peyer's patches (45).

B. pertussis whole-cell vaccine given orally was demonstrated to be as effective as parenterally administered vaccine (9). We have recently demonstrated specific lung mucosal responses against FHA and the S1 subunit of PT after oral immunization with recombinant *aroA* mutants of *Salmonella* spp. (20, 49). The present work extends these observations by demonstrating that systemic and mucosal immune responses against *B. pertussis* antigens follow oral

immunization with free FHA and PT. The incorporation of FHA and PT into the liposome delivery system resulted in an adjuvant effect with greatly improved antibody responses. The immunizations were administered orally, mimicking the method which could be used in human oral vaccines. Therefore, we cannot rule out partial contributions to the elicited immune responses from other mucosa-associated immune tissues (i.e., nasal, salivary, and/or esophageal) in addition to those of gut-associated tissues.

The liposome system can be used to deliver multiple antigens including the 69-kDa outer membrane protein of *B. pertussis*, which was recently demonstrated to be an important protective antigen (33). *Escherichia coli* strains which express recombinant FHA (21), *Bordetella* sp. strains which overproduce PT (50, 52), and methods for the genetic detoxification of PT (36, 52) are now available and will facilitate the production of high yields of nontoxic, fully antigenic components for liposome-based subunit vaccines. In fact, multivalent vaccines which could replace the old DTP vaccine in infant vaccination regimes can be envisaged, as enhancement of the primary and secondary immune responses has resulted from diphtheria toxoid association with liposomes (1).

The mechanisms which mediate protection against *B. pertussis* infection are not clearly understood. Humoral and cellular responses following parenteral immunization do not always correlate (36). Moreover, cell-mediated immune responses are believed to be important in vivo for protection against whooping cough disease (7, 15). As liposomes are known to induce cell-mediated immunity, it will be of interest to determine whether the delivery systems used in this study induce cell-mediated responses to FHA and PT in addition to the documented antibody responses.

The immune responses elicited following immunization with FHA- and PT-coated liposomes reported in this work suggest the usefulness of this delivery system in the development of oral vaccines against whooping cough. However, the system could be further enhanced by the incorporation of other products known to have adjuvant activity, which may have additive or synergistic effects on secretory-IgA priming (35, 38), and/or administration of vitamin A, which may improve the immune responses after oral vaccination (2). Moreover, other aspects, including the length of the immunity obtained, optimal vaccination schedule, and protection after challenge, must be investigated also.

The use of liposomes as a delivery system for subunit vaccines to induce immune responses distal to the site of entry are very promising. The oral route may reduce or eliminate the most frequent local side effects associated with whooping cough vaccines; then, adjuvanticity may increase both cell-mediated and humoral immune responses. Liposomes are chemically stable, simple to manufacture, and biodegradable; toxicity was not reported after liposomes were used in phase I and II trials (1, 18). Moreover, the inexpensive raw materials used to produce the liposomes will contribute to reduced production costs. The oral administration of vaccines is in any case associated with a major cost saving vis-à-vis parenteral vaccination and is a key aspect of major vaccination programs, particularly in rural settings, where health care delivery is very expensive.

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