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Orally Administered Microencapsulated *Bordetella pertussis* Fimbriae Protect Mice from *B. pertussis* Respiratory Infection

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Fimbriae from *Bordetella pertussis* have been encapsulated in poly(lactide-co-glycolide) microparticles of a size appropriate for uptake by the immune inductive tissues of the gastrointestinal tract. Mice were immunized by oral gavage with a single dose of 10 μg of microencapsulated fimbriae. The resulting immune responses were compared with those resulting from intraperitoneal injection of mice with equivalent amounts of fimbriae adsorbed onto alhydrogel. The examination of serum and mucosal secretions, collected over a 6-week period, for specific antifimbrial antibodies clearly demonstrated that only orally immunized animals mounted measurable immune responses in external secretions. Six weeks after immunization, all immunized animals were protected against intranasal challenge with live *B. pertussis*.

Successful oral vaccination could revolutionize human immunization programs worldwide. Oral vaccines would be delivered to the largest mucosal surface in the body, the gastrointestinal tract, with its associated lymphoid tissue, thereby accessing the common mucosal immune system. Despite this obvious advantage, only two generally used human oral vaccines are licensed, Sabin oral polio and *Salmonella typhi*, all others being delivered by injection.

There are, of course, major problems to be overcome in the development of oral vaccines. Orally administered vaccines are digested and excreted, very little practical progress has been made towards the development of mucosal (oral) adjuvants, and attempts to compensate by the addition of large quantities of antigen have resulted in immunological tolerance. However, microencapsulation technologies, which have for years been used for the delivery of drugs to humans, are now being considered for the delivery of oral vaccines.

One of these encapsulation matrices, poly(lactide-co-glycolide) (PLG), offers a number of real advantages as a vehicle for vaccine delivery. The polymer is totally biocompatible (52), is unaffected by passage through the stomach (11), and can stabilize entrapped biomolecules (29). Recent studies using experimental animals have shown that highly efficient delivery of antigen is possible with this system (51) and that the polymer acts as a depot for antigen which, depending on the composition, allows a sustained or pulsed release of antigen to the immune system (37, 39). Accurate sizing of the PLG microparticles during manufacture facilitates their uptake after oral delivery by modified epithelial cells, M cells (1), which transport the microparticles to the Peyer's patches (35), thus presenting concentrated vaccine to the site of mucosal immune stimulation (11). Orally delivered antigen can not only elicit a vigorous disseminated mucosal immunity but also stimulate circulating antibody (6, 11). Further, experimental animals immunized by PLG-entrapped antigens produce both humoral and cellular immunity (24) and can in some instances be protected from subsequent infection (31). The demonstrable flexibility of this system for the delivery of complex antigens (25), combinations of antigens (50), antigens and adjuvants (16), and recombinant and genetic vaccines (7) makes the system one of the most exciting areas of future vaccine design.

We have previously reported the successful induction of systemic immunity and protection in mice by using *Bordetella pertussis* fimbrial proteins encapsulated in PLG microparticles (23). We have now extended these studies to investigate the immune responses to microencapsulated fimbriae delivered by the oral route. The findings with this approach to vaccine delivery are discussed in the context of the current literature on microencapsulated antigens.

MATERIALS AND METHODS

Reagents. Poly(DL-lactide-co-glycolide) (Medisorb 5050DL, low IV; 52:48 ratio of lactide to glycolide; molecular weight, 50,000 to 70,000) was a generous gift from Medisorb Technologies International L.P. (Cincinnati, Ohio). Dichloromethane (high-performance liquid chromatography grade) was obtained from Fisons plc (Loughborough, United Kingdom), polyvinyl alcohol (88% hydrolyzed; molecular weight, 13,000 to 23,000) was from Aldrich Chemical Company (Poole, United Kingdom), and alhydrogel was from Superfos Biosector a/s (Vedbæk, Denmark).

Peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) and anti-mouse IgM and IgG were purchased from Jackson ImmunoResearch Labs (West Grove, Pa.), and peroxidase-conjugated anti-mouse IgA was obtained from Sigma Chemicals (Poole, United Kingdom). Charcoal agar plates containing 10% (vol/vol) defibrinated horse blood and 40 mg of cephalexin per liter were prepared by Difco Laboratories (East Molesey, United Kingdom).

Fimbriae were purified from *B. pertussis* (Wellcome 28 strain [serotype 1,F2,3]) by repeated ammonium sulfate precipitation as previously described (44). Image analysis of Coomassie blue-stained gels indicated that fimbrial preparations were 98 to 99% pure, and Western blotting (immunoblotting) of fimbrial preparations with polyclonal sera from mice immunized with sodium dodecyl sulfate (SDS)-treated fimbriae revealed only one major band corresponding to fimbrial protein (25a).

Preparation of challenge stocks of *B. pertussis*. *B. pertussis* challenge stocks were prepared by hydration of lyophilized bacteria (Wellcome 28 strain) and inoculation onto charcoal agar plates supplemented with 10% (vol/vol) defibrinated horse blood and 40 mg of cephalexin per liter. After 48 h of incubation at 35°C, bacteria were scraped off, inoculated onto fresh charcoal agar plates, and incubated at 35°C for a further 24 h. Bacteria recovered from the second plates were dispersed in 1% (wt/vol) Casamino Acids, and the turbidity of the suspension was measured at 550 nm. On the assumption that an optical density at 550 nm of 0.1 represents a concentration of 4×10^8 organisms per ml, the suspension was adjusted to a concentration of 2×10^7 organisms per ml (subsequently confirmed by counting viable organisms) and used immediately for intranasal challenge as described below.

Formulation of fimbriae in PLG microspheres. Fimbriae showed no obvious

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contamination when examined by SDS-polyacrylamide gel electrophoresis and were subsequently microencapsulated by the (water-in-oil)-in-water solvent extraction technique (14). Briefly, 3 ml of a 16.67% (wt/vol) solution of PLG polymer in dichloromethane was emulsified at 9,000 rpm with 0.3 ml of a 5-mg/ml aqueous solution of fimbriae by using a homogenizer fitted with a standard emulsor screen (Silverson Machines Ltd., Chesham, United Kingdom). This emulsion was immediately added to 60 ml of an aqueous solution of 8% (wt/vol) polyvinyl alcohol and emulsified as described above. The resulting double emulsion was dispersed rapidly in 1 liter of water, and the microparticles were collected by centrifugation, washed three times in distilled water, and lyophilized. Microparticles containing no protein were prepared as controls.

Characterization of microparticles. The size distribution of suspended microparticles was determined with a Coulter Counter Channelyzer that was calibrated with latex beads. The efficiency of fimbrial encapsulation was determined by protein assay after dissolution of a known weight of microparticles as previously described (19, 23). Encapsulation of protein was expressed as a percentage of the total particle weight. The rate of protein release from microparticles was determined as before (23). The effects of microencapsulation on the denaturation of fimbriae were determined by enzyme-linked immunosorbent assay (ELISA), as described previously (23), with monoclonal antibodies which are specific for conformational (Agg2A and Agg3A) or linear (MM4) epitopes and with rabbit antiserum R326, raised against an 18-mer fimbrial peptide.

Immunization protocols. Experimental groups of female NIH-Porton mice (6 to 8 weeks old; 10 animals per group) were immunized with a single standardized dose of 10 μg of fimbrial protein given as follows: (i) 6 mg of PLG microparticles suspended in 0.5 ml of phosphate-buffered saline (PBS), injected intraperitoneally (i.p.); (ii) 25% (wt/vol) suspension of alhydrogel in 0.5 ml of PBS, injected i.p.; or (iii) 6 mg of PLG microparticles suspended in 0.5 ml of 0.1 M sodium bicarbonate solution, given by oral gavage. Control groups received either 0.5 ml of unformulated fimbriae in 0.1 M sodium bicarbonate, given by oral gavage (control group 1), or 6 mg of PLG microparticles without entrapped protein, suspended in PBS or 0.1 M sodium bicarbonate, and injected i.p. or given by oral gavage (control group 2).

Collection and preparation of samples. Prior to immunization and at 4 and 6 weeks postimmunization (but before challenge with live bacteria), blood and samples of mucosal secretions were taken from all animals to test for the presence of specific antibodies.

(i) Stools. Freshly voided fecal pellets were collected from individual animals and stored in Eppendorf tubes at −70°C. Prior to assays, pellets were resuspended in PBS (50 μl per pellet), vigorously vortexed, and incubated at 20°C for 15 min. Samples were revortexed and centrifuged at 1,000 × *g* for 5 min (9). The supernatants were diluted with blocking buffer (see below) and used immediately for the assays.

(ii) Vaginal washes. Washes were performed by inserting a fine-tipped Liquipette into the vagina and flushing three times with 50 μl of sterile saline. Washes were stored in Eppendorf tubes at −70°C. Samples were diluted with blocking buffer prior to the assays.

(iii) Saliva. Saliva samples (40 μl) were collected in capillary tubes following i.p. injection of the mice with 0.1 ml of a 1-mg/ml aqueous solution of pilocarpine. Care was taken to collect the first flush of saliva in all cases. Samples were transferred to Eppendorf tubes and stored at −70°C until the assays, when dilutions of samples were made in blocking buffer.

(iv) Sera. Mice were anesthetized by ether inhalation, and 50 μl of blood was collected in capillary tubes by retro-orbital puncture. Capillaries were sealed with Cristaseal and blood was allowed to clot at 20°C for 30 min. Capillaries were then centrifuged for 10 min in a hematocrit centrifuge, and the serum samples were transferred to Eppendorf tubes. Samples were stored at −70°C until required, when initial dilutions of sera were made in blocking buffer.

Determination of specific antifimbrial responses. ELISA plates (96 wells) (Maxisorb; Nunc) were coated with 500 ng of native fimbriae per ml in PBS (50 μl per well) for 16 h at 4°C. Following washing in PBS containing 0.05% (vol/vol) Tween 20 (PBST), plates were blocked with 100 μl of PBST containing 4% (vol/vol) newborn calf serum and 1% (wt/vol) bovine serum albumin (blocking buffer) per well for 1 h at 37°C. Plates were washed three times with PBST, twofold serial dilutions of samples in blocking buffer were established across the plates (50 μl per well), and the plates were incubated for 16 h at 4°C. After another washing as described above, specifically bound immunoglobulins were detected with 50 μl of peroxidase-conjugated anti-mouse IgA (1:500), IgG (1:5,000), or IgM (1:5,000) per well by incubation for 30 min at 37°C. Plates were washed with PBST, and tetramethylbenzidine, soluble form (TMBlue; Intergen-CDP, Milford, Mass.) (50 μl per well), was added. Color development was stopped after 15 min by the addition of 1 M H_2SO_4 (50 μl per well), and A_{450} was measured. Endpoint titers were determined for each sample titration by using Hill kinetics, with a cutoff point derived from the mean (plus 2 standard deviations) of each animal's preimmune value.

Intranasal challenge of mice with *B. pertussis*. Six weeks after immunization, mice were challenged intranasally with *B. pertussis*. After light anesthesia, 50 μl of bacterial suspension (approximately 10^6 bacteria) was pipetted onto the nostrils and allowed to be inhaled (44). Mice were sacrificed 7 days postchallenge, at which time lungs and tracheas were removed. Individual tissues were homogenized in 10 ml of 1% (wt/vol) Casamino Acids with a Silverson homogenizer, and the viable organisms in homogenates were assessed by plating out serial dilutions onto charcoal agar plates supplemented with defibrinated horse blood and cephalexin as described above. Plates were read after incubation at 35°C for 6 days.

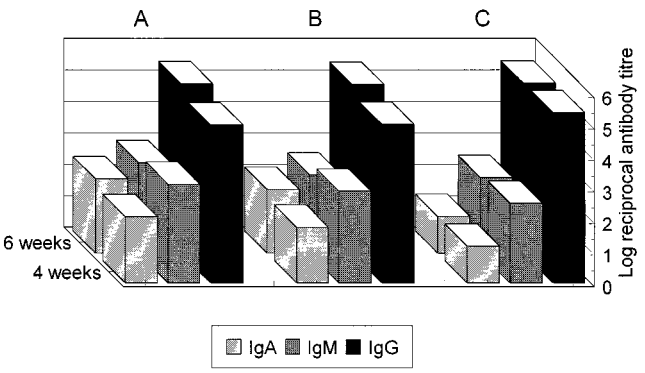

FIG. 1. Antigen-specific antibodies in sera of immunized mice. Antifimbrial responses in sera of mice were determined by ELISA of samples taken 4 and 6 weeks postimmunization with either alhydrogel-formulated fimbriae injected i.p. (A), microencapsulated fimbriae injected i.p. (B), or microencapsulated fimbriae given by oral gavage (C). Log reciprocal endpoint titers (using preimmune sera to determine cutoff values) were measured for individual animals, and the mean for each group of animals is presented. Antigen-specific antibodies were detected in the sera of all animals in each experimental group. No specific antibodies were detected in the sera of control mice.

RESULTS

Characterization of microparticles and encapsulated fimbriae. Following dissolution of weighed samples of microparticles in a solution of SDS in 0.1 M NaOH, the amount of protein encapsulated in microparticles (expressed as a percentage per unit weight of microparticles) was found to be 0.16%. This translated to an efficiency of encapsulation of approximately 52%. The rate of release of fimbrial protein from a known weight of microparticles, expressed as a percentage of total encapsulated protein and plotted against time, was found to follow approximately linear kinetics with a half-life of 30 days. The mean diameter of the particles was 2.04 μm, with 90% of microparticles having diameters within the narrow range of 0.8 to 5.3 μm. Resuspension of lyophilized microparticles was readily achieved with no evidence of aggregation.

Fimbriae released from microparticles by hydrolysis in sterile PBS containing 0.05% (wt/vol) azide at 37°C were assayed to determine whether they were denatured by the encapsulation process. Both monoclonal antibodies which recognize conformational epitopes (Agg2A and Agg3A) bound strongly to native and released fimbriae, while both antibodies which recognize linear epitopes (MM4 and R326) bound only weakly to native and released fimbriae (data not shown). These data demonstrate that, as was the case in the preparation of the larger microparticles (23), the method described above caused little denaturation of fimbriae.

Assessment of immunogenicity using microencapsulated fimbriae. (i) Parenteral immunization. No specific antibodies were detectable in any external secretions from parenterally immunized animals irrespective of the method of formulation. A single i.p. administration of microencapsulated fimbriae elicited a high level of antigen-specific serum IgG which doubled in titer between 4 and 6 weeks postimmunization (Fig. 1B). Consistent with a primary systemic immune response, IgM levels fell between the 4- and 6-week points, while IgA levels, though low compared with IgG and IgM, rose over the same period. A single i.p. injection of fimbriae formulated with alhydrogel as an adjuvant elicited a very similar pattern of serum

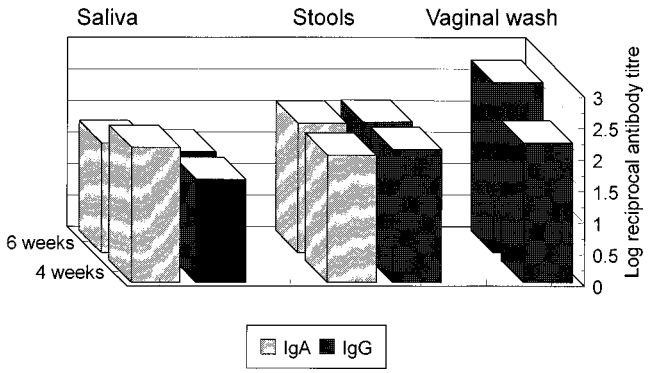

FIG. 2. Antigen-specific antibodies in external secretions. Antifimbrial responses in the external secretions of mice, 4 and 6 weeks after oral immunization with microencapsulated fimbriae, were determined by ELISA. Log reciprocal endpoint titers (using preimmune sera to determine cutoff values) were measured for individual animals, and the mean for each group of animals is presented. Antigen-specific IgA and IgG were detected in external secretions from all animals immunized orally with microencapsulated fimbriae, with the exception of IgA in vaginal washes (see text). No specific antibodies were detected in comparable samples of external secretions from mice immunized parenterally with fimbriae formulated in microparticles or alhydrogel or in samples from control mice.

responses (Fig. 1A). IgA and IgG levels rose between the 4- and 6-week points, while IgM levels fell. As previously observed, i.p. injection of alhydrogel-adsorbed fimbriae elicited an overall stronger immune response than administration of fimbriae encapsulated in PLG microparticles. The i.p. injection of microparticles without entrapped protein (control group 2) failed to elicit any detectable immune response in serum or external secretions (data not shown).

(ii) Oral immunization. A single dose of microencapsulated fimbriae in bicarbonate buffer, administered to mice by oral gavage, elicited substantial immune responses in external secretions distant from the site of immunization, as well as a substantial systemic immune response. Neither soluble fimbriae (control group 1) nor microparticles without entrapped protein (control group 2) elicited any immune response when administered orally to mice (data not shown).

From 4 weeks postimmunization, specific antifimbrial IgA and IgG were detectable in saliva and stool samples, but only antigen-specific IgG was measurable in vaginal washes (Fig. 2). IgM was not detected in any of these samples throughout this study. Antigen-specific IgA levels apparently fell between 4 and 6 weeks in saliva samples but were maintained in stool samples collected at the same time points. In both saliva and stools, antigen-specific IgG levels were constant at 4 and 6 weeks but rose significantly in vaginal washes over this period. In the orally immunized animals high titers of serum IgG (>1:256,000) were maintained between 4 and 6 weeks (Fig. 1C), but serum IgM levels fell over the same time period, and specific IgA was detectable only at the lowest serum dilution used (1:15).

Protection against challenge with live *B. pertussis*. The susceptibility to infection of control and immunized groups of mice was assessed following intranasal instillation of live *B. pertussis*. Viable bacteria in tracheas and lungs of immunized animals were counted. Control animals were highly susceptible to infection with live *B. pertussis*, while all three groups of immunized animals were protected (Table 1), as demonstrated by the significant reduction (>95%) in the numbers of viable organisms recovered from the tracheas and lungs of all three groups of immunized mice compared with control mice.

DISCUSSION

We have previously reported that a single 10 μg dose of either alhydrogel-formulated or PLG-encapsulated *B. pertussis* fimbriae is capable of inducing a vigorous protective immunity when delivered parenterally (23). The primary objective of the present study was to extend this investigation to assess the efficacy of microencapsulated fimbriae following a single oral administration. The results presented here convincingly show the effectiveness of microencapsulation as a means of delivering antigens by the oral route and the ability of orally administered microencapsulated fimbriae to elicit specific systemic and mucosal immune responses.

Mounting evidence now points to the general applicability of stimulating immunity by delivery of antigens to mucosal surfaces (28, 29, 45, 48, 53). Several studies have specifically investigated the effects of administering acellular pertussis vaccine components directly to the upper respiratory tract of mice, either as free antigen (4, 43) or as microencapsulated formulations (2, 3, 46). Both approaches have resulted in the stimulation of both local mucosal immunity and serum immunoglobulins (2–4, 43, 46); however, the latter were detected at titers significantly lower than are typically observed when the antigen is given parenterally (3). In all of these studies, animals were protected against challenge with live *B. pertussis*, but, as yet, an unequivocal correlation between the presence of secretory antibodies and protection has not been established. Although the inhibition of bacterial adhesion to HeLa cells was shown to correlate with the presence of anti-filamentous hemagglutinin antibodies in bronchoalveolar lavage samples from mice immunized intranasally with filamentous hemagglutinin, the possibility that these antibodies were not produced by local antibody-secreting cells but instead transudated from serum into the lungs cannot be excluded (3). Interestingly, no evidence for the elicitation of a disseminated mucosal response following intranasal administration with *B. pertussis* antigens has been presented (2–4, 43, 46), suggesting that this route of administration may not be appropriate for inducing immunity against all pathogens and that immunization of mucosae at one of the components of the common mucosal immune system may be a prerequisite for the induction of extended secretory immune responses (28).

Far less attention has been paid to the delivery of antigens to the gut. This is because antigens must be protected from degradation and digestion in the gut, plus the fact that the rapid elimination of antigens tends to make this route inefficient (36). More recently, however, microencapsulated antigens have been effectively and efficiently delivered orally, resulting in the development of local (26, 32, 50) and disseminated (6,

TABLE 1. Comparison of recoveries of viable bacteria from mice after intranasal challenge with live *B. pertussis* fimbriae

Formulation and immunization route[a]	No. of CFU recovered[b]	
	Tracheas	Lungs
Alhydrogel adjuvant, i.p.	1.8×10^3 (0.7×10^3)	2.0×10^4 (0.2×10^4)
Microencapsulatation, i.p.	1.8×10^3 (0.4×10^3)	7.2×10^3 (0.2×10^3)
Microencapsulatation, oral	7.7×10^2 (2.6×10^2)	7.2×10^3 (2.5×10^3)
Soluble fimbriae, oral	7.0×10^4 (1.1×10^4)	6.5×10^5 (0.5×10^5)
Microparticles, oral or i.p.	7.0×10^4 (1.1×10^4)	6.5×10^5 (0.5×10^5)

[a] Mice were immunized with 10 μg of fimbriae, either adsorbed onto alhydrogel or microencapsulated. Control mice were immunized with soluble fimbriae or microparticles without entrapped protein.

[b] Values were obtained at 6 weeks postimmunization and are reported as means with standard errors of the means in parentheses.

10, 11, 13, 24, 31, 32, 38, 42) mucosal immune responses, systemic immune responses (6, 10, 11, 13, 24, 31, 32, 38, 42), and protection against challenge with relevant pathogens (26, 31, 42, 50). Although different immunization regimens, such as multiple oral dosing (26, 42, 50,) and combinations of oral and parenteral administrations (13, 25, 31), have been necessary to achieve these outcomes, our present study suggests that a single oral dose is sufficient with this particular antigen and suggests that the formulation is important.

In all previous studies relating to the successful induction of immunity following oral delivery of antigens, the biochemical and physical characteristics of the antigen have been crucial. Either the protein has had a specific binding activity, like that of bacterial toxins and their corresponding subunits (17, 54) which direct the molecule to the gut epithelial cells, or the antigen has been particulate in nature (for example, see references 8, 18, 20, 29, 30, and 34). The uptake of particles from the lumen of the gut is understood to be mediated by specialized epithelial cells (M cells) (33, 49) which are concentrated into specialized morphologically distinct regions of the lamina propria termed Peyer's patches (40). Analysis of the mechanism of particle uptake by M cells in the mouse gut has clearly shown that this is restricted to materials with diameters less than or equal to 10 μm (13, 33, 41). The ability of M cells to retain phagocytosed microparticles has been studied by using PLG microspheres loaded with dyes. Particles with diameters of 5 to 10 μm were withheld, while their smaller counterparts (<5 μm) were rapidly distributed and were detectable in the spleen within a matter of days (10–12). Even more rapid uptake and greater enhancement of the immune response has now been reported for submicroparticles (nanoparticles) (21, 22). Certain parallels between the functions of M cells and those of macrophages can be drawn. Studies of the immune responses following subcutaneous injection of mice with antigen entrapped in 1- to 10-μm-diameter or 20- to 50-μm-diameter PLG microparticles suggest that macrophages behave similarly to M cells with respect to the uptake of particulate antigens (14). Although both populations of microparticles induced immune responses, the smaller microparticles were more immunogenic, as the larger microparticles remained at the site of injection while the smaller microparticles were rapidly phagocytosed and distributed. These observations suggest that two mechanisms of immune stimulation operate, a depot effect resulting from slow antigen release at the site of delivery and a more vigorous stimulation resulting from efficient intracellular delivery of high concentrations of antigen to antigen-presenting cells and subsequent presentation to T cells (14). Thus, the roles of M cells and macrophages in immune stimulation, when presented with microencapsulated antigens, may be very similar.

The induction of antifimbrial antibodies following oral immunization with purified microencapsulated *B. pertussis* fimbriae has been studied and compared with corresponding results from parenteral immunization (Fig. 1 and 2). As expected, specific antibodies of all three isotypes were detected in serum following i.p. injection of microencapsulated antigen (Fig. 1B). However, equally vigorous responses accompanied the oral delivery (Fig. 1C). Neither soluble fimbriae nor microparticles without entrapped fimbriae elicited detectable immune responses in serum or in mucosal secretions. Thus, the observed responses are the specific result of the presentation to the immune inductive tissues of fimbriae entrapped in microparticles.

The patterns of immunoglobulins in secretions from mucosal surfaces are, however, much more difficult to interpret (Fig. 2). Undoubtedly, oral immunization elicited a disseminated mucosal response, which was detectable as specific antibodies of the IgA isotype present in saliva and stools at levels higher than those detected in serum (Fig. 1C). The possibility that these higher antibody levels arise from transudation of serum antibodies can, therefore, be eliminated, but the presence of IgG in all samples examined confuses the picture with respect to other reports (45). Whereas the detection of immunoglobulins in serum samples uses well-established techniques, the corresponding measurement of mucosally derived antibodies relies on samples obtained by a variety of procedures. External secretions which have been used for these analyses include saliva (5), tears (28, 29), colostrum (28, 29), and stools, which are reported to contain secretions representative of intestinal mucosal secretions (9). However, samples obtained by invasive techniques such as tracheal or lung lavage (47), gut lavage (15), and vaginal washes (27) are also used. We have attempted to minimize the possibility of contamination by blood by concentrating on the use of saliva, stools, and carefully derived vaginal washes for this study.

The presence of IgG in mucosal secretions could be the result of high serum IgG titers (Fig. 1C). However, the identification of IgG-secreting cells in populations of lymphocytes prepared from the gut and genitourinary mucosae suggests that IgG subclass antibodies may originate directly from B lymphocytes located in the mucosae (27).

The secretion of IgG at mucosal surfaces may also be a direct response to the antigen itself. The powerful mucosal adjuvant cholera toxin subunit B is reported to induce IgG secretion at the gut and in saliva and vaginal washings following oral immunization of mice with cholera toxin subunit B-keyhole limpet hemocyanin conjugates. Oral immunization with keyhole limpet hemocyanin alone elicited primarily IgA (27). In the present study, the combination of the potent immunogenicity of pertussis fimbriae and the optimizing of a formulation for oral delivery may be the cause of the relatively high IgG levels in external secretions.

Finally, protection against challenge with live *B. pertussis* bacteria was successfully demonstrated with a single oral dose of microencapsulated fimbriae (Table 1) at levels comparable to those previously obtained by us with equivalent amounts of microencapsulated antigen delivered i.p. (23). However, parenteral administration of PLG-encapsulated vaccines is likely to be of limited usefulness, for both children and adults, because it involves the injection of a bulky matrix which is designed to reside at or close to the injection site for extended periods. In this respect, the oral route is much more acceptable. This demonstration that orally administered microencapsulated fimbriae elicits not only disseminated mucosal immune responses but also systemic immune responses which are comparable to those elicited by i.p. injection of encapsulated fimbriae or fimbriae with alhydrogel as an adjuvant proves the potential for using PLG as a slow release vehicle for orally delivered vaccines. It is now important to optimize both the uptake of microparticles and the vaccine dose.

Although a number of studies have demonstrated the capacity to elicit immune responses by enteral or parenteral immunization with PLG-microencapsulated antigen (3, 25, 31, 32, 42, 46, 50), few studies have used relevant antigens which would allow the demonstration of protection (3, 31, 32, 46). Recent reports have clearly highlighted the potential for acellular pertussis vaccine components, individually (3) or in combination (46), to elicit protection when administered by the intranasal route. By contrast, the present report is the first to describe the induction of protective immunity by the oral delivery of a pertussis antigen. Thus, our studies extend the literature on protection studies with animals immunized with

microencapsulated vaccine antigens in general and, more specifically, demonstrate by using *B. pertussis* fimbriae that a single oral dose can confer protection at a remote mucosal surface.

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