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Induction of mucosal immune responses against a heterologous antigen fused to filamentous hemagglutinin after intranasal immunization with recombinant *Bordetella pertussis*

(heterologous expression/28-kDa *Schistosoma mansoni* glutathione *S*-tranferase)

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ABSTRACT **Live vaccine vectors are usually very effective and generally elicit immune responses of higher magnitude and longer duration than nonliving vectors. Consequently, much attention has been turned to the engineering of oral pathogens for the delivery of foreign antigens to the gut-associated lymphoid tissues. However, no bacterial vector has yet been designed to specifically take advantage of the nasal route of mucosal vaccination. Herein we describe a genetic system for the expression of heterologous antigens fused to the filamentous hemagglutinin (FHA) in *Bordetella pertussis*. The *Schistosoma mansoni* glutathione *S*-transferase (Sm28GST) fused to FHA was detected at the cell surface and in the culture supernatants of recombinant *B. pertussis*. The mouse colonization capacity and autoagglutination of the recombinant microorganism were indistinguishable from those of the wild-type strain. In addition, and in contrast to the wild-type strain, a single intranasal administration of the recombinant strain induced both IgA and IgG antibodies against Sm28GST and against FHA in the bronchoalveolar lavage fluids. No anti-Sm28GST antibodies were detected in the serum, strongly suggesting that the observed immune response was of mucosal origin. This demonstrates, to our knowledge, for the first time that recombinant respiratory pathogens can induce mucosal immune responses against heterologous antigens, and this may constitute a first step toward the development of combined live vaccines administrable via the respiratory route.**

The mucosal surfaces are the port of entry of most infectious agents and constitute, therefore, the first line of the host defense against many pathogens. Consequently, the mucosal immune system has attracted much attention over the last decade with the aim of developing efficient mucosal vaccine delivery systems. The gut-associated immune system has been most extensively explored, and several oral vaccine vehicles including live vectors, generally derived from oral pathogens, have been developed for the presentation of heterologous antigens (1).

An alternative route of mucosal immunization is the intranasal administration of vaccines. This route presents several advantages over the oral route. It avoids the encounter of the antigens with the acidic and proteolytic environment of the stomach. On a dose basis, nasal administrations appear, therefore, to be more effective than oral administrations (2). Since the respiratory tract is less colonized by commensal microorganisms than the gut, live vaccine vectors will meet less competition to establish themselves. Although there is evidence for the existence of a common mucosal immune system (3), local exposure to an antigen appears to induce higher levels of specific antibodies at site of exposure than at distant sites (4). Thus, efficient mucosal immunity against respiratory pathogens may be best achieved by the nasal administration of vaccines. While considerable effort has been devoted to the development of live vectors for the colonization of the gastrointestinal tract, no vector has so far been designed to specifically colonize the respiratory tract.

In this study, we explored the potential of *Bordetella pertussis*, the etiologic agent of whooping cough, to serve as a live antigen delivery system for the nasal route, since *B. pertussis* very efficiently colonizes the human respiratory tract. This property is most likely related to the numerous virulence-associated adhesins this organism produces. One of them is the filamentous hemagglutinin (FHA), the major adhesin that expresses at least three distinct binding activities (for review, see ref. 5). FHA is also a potent immunogen, capable of inducing high levels of mucosal and systemic antibodies of long persistence after natural infection in humans (6, 7) or experimental infection in mice (8).

To assess whether the intranasal administration of recombinant *B. pertussis* can induce a mucosal immune response against heterologous antigens, we chose the *Schistosoma mansoni* glutathione *S*-transferase (Sm28GST) as a model antigen and genetically fused it to FHA. The gene encoding this antigen has been cloned and sequenced (9), and protection in humans against the pathology caused by a massive egg output of *S. mansoni* has been correlated with the presence of IgA antibodies against Sm28GST (10). Immunogenicity of the Sm28GST coupled to fragment C of tetanus toxin produced in a live attenuated *Salmonella* vaccine strain has been recently demonstrated after oral immunization (11), whereas oral or nasal administration of purified Sm28GST alone did not induce a significant immune response. In addition, the respiratory tract may represent an important site of elimination of schistosomulae (12), since the life cycle of *S. mansoni* has an important lung stage. For these reasons, we felt that Sm28GST was a good model antigen, and herein we demonstrate that a genetic fusion of Sm28GST to FHA is capable of inducing specific IgA and IgG in bronchoalveolar lavage fluids (BALF) after a single intranasal administration of recombinant *B. pertussis*.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Growth Conditions, and DNA Manipulations. The bacterial strains and plasmids used in this study are listed in Table 1. All *B. pertussis* strains were grown at 36°C on Bordet–Gengou agar (18) supplemented with 1%

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Abbreviations: BALF, bronchoalveolar lavage fluids; cfu, colony-forming unit(s); FHA, filamentous hemagglutinin; MALT, mucosa-associated lymphoid tissue; Sm28GST, *Schistosoma mansoni* 28-kDa glutathione *S*-transferase.

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Table 1. Bacterial strains and plasmids

Strain/plasmid	Relevant phenotype/genotype	Source/ref.
Strains		
B. pertussis		
BPSM	Tohama I-derivative, resistant to streptomycin and nalidixic acid	13
BPGR4	*fhaB* deletion mutant	14
BPGR5	BPSM-derivative with entire Sm28GST fused with FHA	This study
BPGR6	BPSM-derivative with truncated Sm28GST fused to FHA	This study
BPGR60	BPSM-derivative with entire modified Sm28GST fused to FHA	This study
E. coli		
JM109	e14$^-$(*mcrA*), Δ(*lac-proAB*), *thi-1*, *gyrA96*, *endA1*, *hsdR17* (rk^-mk^+), *relA1*, supE44, *recA1*, [F' *traD36*, *lacIqZΔM15*, *proAB*]	Stratagene
S17-1	*pro*, *hdsR*, *res*, *mod*, *recA*, RP4-2Tc::Mu, Km::Tn7	15
Plasmids		
pRIT13202	*Eco*RI fragment of *fhaB* in pUC8	16
pUC8-A	*Bgl*II Sm28GST fragment (0.68 kb) in pRIT13202	This study
pUC8-F	*Bgl*II–*Bcl*I Sm28GST fragment (0.5 kb) in pRIT13202 deleted of its 0.1-kb *Bgl*II–*Bcl*I fragment	This study
pUC7-28	*Bam*HI Sm28GST fragment (0.64 kb) in pUC7	This study
pUC7-28*	*Pst*I–*Sal*I mutagenized Sm28GST fragment (0.28 kb) in pUC7-28	This study
pUC8-928*	*Bam*HI Sm28GST fragment (0.64 kb) of pUC7-28* in pRIT13202	This study
pGR5	5′ end of *bvgA/S* and 3′ end of *fhaB* in pSS1129	14
pGR53	*Eco*RI fragment of pUC8-A in pGR5	This study
pGR54	*Eco*RI fragment of pUC8-F in pGR5	This study
pGR540	*Eco*RI fragment of pUC8-928* in pGR5	This study

glycerol and 20% defibrinated sheep blood (BG) or in modified Stainer–Scholte medium containing 2,6-*O*-dimethyl-β-cyclodextrin at 1 g/liter (19, 20). Antibiotics were used at the following concentrations: streptomycin, 100 μg/ml; gentamycin, 10 μg/ml. *Escherichia coli* strains were grown in LB broth (21), and antibiotic-resistant *E. coli* strains were selected with ampicillin (100 μg/ml) and/or gentamycin (25 μg/ml).

Restriction enzymes, T4 DNA ligase, and other DNA modifying enzymes were purchased from Boehringer Mannheim, New England Biolabs, or Amersham and were used as recommended by the suppliers. All DNA manipulations were performed under standard conditions as described by Sambrook *et al.* (21). Polymerase chain reactions (PRCs) were performed on a Perkin–Elmer thermal cycler model 480. Chromosomal DNA was isolated from *B. pertussis* as described by Hull *et al.* (22). Southern blot analyses were performed using nonradioactive DNA probes labeled with digoxigenin-dUTP according to the instructions of the supplier (Boehringer Mannheim).

Construction of Recombinant *B. pertussis* Strains. To construct *B. pertussis* BPGR5, a 0.68-kb fragment harboring the Sm28GST-encoding cDNA was amplified by PCR from TG10 (R. J. Pierce, personal communication) using the oligonucleotides 5′-TAAGATCTCCATGGCTGGCGAGCAT-3′ and 5′-TAAGATCTCCGAGCTTTCTGTTG-3′, digested with *Bgl*II, and cloned into the *Bgl*II site of *fhaB* in pRIT13202 (16) to yield pUC8-A. The 10.68-kb *Eco*RI fragment from pUC8-A was then inserted into the *Eco*RI site of pGR5, a pSS1129-derivative harboring the 5′ and 3′ *fhaB* flanking regions (14). The resulting plasmid, named pGR53, was transferred into *E. coli* S17–1 (15). This strain was then conjugated with *B. pertussis* BPGR4, a strain lacking *fhaB*, and two successive homologous recombination events were selected as described (14). The *B. pertussis* exconjugant strains were then screened by colony blot hybridization with a probe corresponding to the 0.68-kb *Bgl*II fragment harboring the Sm28GST cDNA. One of the positive clones was further examined by Southern blot analysis to ensure that the hybrid gene was correctly inserted into the *fhaB* locus. This strain was named BPGR5.

To construct *B. pertussis* BPGR6, the above described PCR fragment was digested with *Bgl*II and *Bcl*I, and the resulting 0.5-kb fragment was inserted into pRIT13202 digested with *Bgl*II and *Bcl*I to yield pUC8-F. The 10.4-kb *Eco*RI fragment was then isolated from pUC8-F and cloned into the *Eco*RI site of pGR5. The resulting plasmid was named pGR54 and introduced by conjugation into *B. pertussis* BPGR4 via *E. coli* S17–1, as described above.

To construct *B. pertussis* BPGR60, the Sm28GST cDNA was first mutagenized to replace the TGC triplet coding for Cys-140 by AGC coding for Ser. To mutagenize this TGC triplet, the Sm28GST cDNA was amplified by PCR using the oligonucleotides 5′-TAAGGATCCCCATGGCTGGCGAG-CATATCAAG-3′ and 5′-CCTGTCGACCCTTTCAGA-GATTCGCTGATCATATTGAG-3′. The amplified DNA fragment was then digested with *Pst*I and *Sal*I, and the resulting 0.28-kb fragment was used to replace the 0.28-kb *Pst*I–*Sal*I fragment of pUC7–28, a pUC7-derivative containing the Sm28GST cDNA as a 0.64-kb *Bam*HI fragment. This fragment was generated by PCR using the oligonucleotides 5′-TAAGGATTCCCCATGGCTGGCGAG-CATATCAAG-3′ and 5′-TAAGGATCCCGAAGGGAGT-TGCAGGCCTGTT-3′. The entire DNA insert was then sequenced to ensure that the TGC triplet was replaced by AGC, as expected, and that no other alteration had occurred. The 0.64-kb *Bam*HI fragment isolated from the resulting plasmid was inserted into the *Bgl*II site of pRIT13202 to generate pUC8–928*. The 10.64-kb *Eco*RI fragment was then isolated from pUC8–928* and inserted into the *Eco*RI site of pGR5, yielding pGR540. The latter plasmid was introduced into *B. pertussis* BPGR4 by conjugation via *E. coli* S17–1, as described above.

Protein Techniques. For the detection of the FHA–Sm28GST hybrid proteins, the culture supernatants or whole cell lysates of the various *B. pertussis* strains were examined by SDS/polyacrylamide gel electrophoresis on 10% gels as described by Laemmli (23) and by immunoblot analysis using anti-FHA monoclonal antibody 12.1.F9 as described by Delisse-Gathoye *et al.* (16) or anti-Sm28GST rabbit polyclonal antibodies, provided by F. Trottein (Institut Pasteur de Lille) at a 1:200 dilution. The FHA–Sm28GST hybrid proteins were purified from culture supernatants by heparin-Sepharose chromatography as described (24).

***B. pertussis* Colonization of the Mouse Respiratory Tract.** The mouse colonization assay was adapted from the method described by Guiso and Khelef (25). Briefly, *B. pertussis* BPSM or BPGR60 were grown for 48 h at 36°C on BG agar supplemented with streptomycin. The bacteria were then scraped off the plates and resuspended in PBS at 10^8 colony-forming units (cfu)/ml. Twenty-five microliters (2.5×10^6 cfu) of this bacterial suspension was then instilled into each nostril of 4-week-old female OF1 mice under anesthesia. Infected mice were sacrificed by cervical dislocation 3 h after intranasal injection (time 0) or at indicated time points (weeks 1–5). Four

to seven mice were analyzed per time point. The lungs of the sacrificed mice were removed and homogenized individually in phosphate-buffered saline (PBS) using tissue grinders. Serial dilutions of the lung homogenates were then plated onto BG agar supplemented with streptomycin, and cfu were counted after 3–5 days of incubation at 36°C.

Analysis of Mucosal Antibody Responses. After intranasal administration of *B. pertussis* BPGR60, as described above, IgA and IgG responses were analyzed in the BALF using enzyme-linked immunosorbant assays at the indicated time points. Immulon 3 flat-bottom 96-well plates (Dynatech) were coated with 50 μl of PBS containing FHA (10 μg/ml) or recombinant Sm28GST (0.625 μg/ml), provided by Transgene (Strasbourg, France). After blocking with 0.5% gelatin (Sigma) and washing with PBS, 50 μl of BALF was added in 1:2 serial dilutions in PBS. The plates were incubated at 37°C and washed, and biotinylated goat anti-IgA or anti-IgE antibodies (Amersham) or peroxidase-conjugated goat anti-IgG antibodies (Pasteur Diagnostic, Marne la Coquette, France) were added at a 1:1000 and 1:10,000 dilution, respectively. Anti-IgA and anti-IgE were detected by the addition of peroxidase-conjugated streptavidin (Amersham) at a 1:2000 dilution, and *o*-phenylene diamine (Sigma) (1 mg/ml) in 0.1 M citrate buffer (pH 5.5) containing 0.003% H_2O_2. The reaction was stopped by the addition of 2 M HCl, and the absorbance was measured at 492 nm using a Titertek Multiskan MCC microplate reader (Labsystems Group, Les Ulis, France). The concentrations of total and specific IgA and IgG antibodies were estimated by comparison to standard curves established with known amounts of antibodies. The results are expressed as the mean values for each time point.

RESULTS

Construction of Recombinant *B. pertussis* Strains Producing FHA–Sm28GST Hybrid Proteins. The unique *Bgl*II site in *fhaB*, the structural FHA gene, was used for the fusion of the Sm28GST coding sequence. This site is located at approximately two-third of the FHA coding sequence as indicated in Fig. 1*a*. The 220-kDa mature form of FHA is encoded by the 5′ two-thirds of *fhaB* (16). A genetic fusion at the *Bgl*II site, therefore, generates a hybrid molecule containing most of the mature FHA followed by Sm28GST. Translation of this hybrid gene in BPGR5 terminates at the Sm28GST stop codon (Fig. 1*b*), and the corresponding chimeric protein is, therefore, composed of the first 1942 amino acid residues of mature FHA followed by a Ser and the 211 Sm28GST residues, including its initiation Met.

Western blot analyses of whole cell extracts using anti-FHA monoclonal antibody 12.1.F9 (Fig. 2*A*, lane 3) and anti-Sm28GST polyclonal antibodies (Fig. 2*B*, lane 5) showed that the hybrid protein was produced in *B. pertussis* BPGR5 and also revealed several proteolytic breakdown products. Analyses of the culture supernatant of BPGR5 did not allow detection the hybrid protein (Fig. 2*B*, lane 2), suggesting that it was not efficiently secreted.

Since in BPGR5 the translation of the hybrid gene terminates at the Sm28GST stop codon, the C-terminal end of the FHA precursor is not produced. Although this domain is not present in mature FHA, it is important for FHA biogenesis (26). We therefore constructed *B. pertussis* BPGR6. This strain contains the FHA–Sm28GST hybrid gene engineered such that its translation terminates at the stop codon of the FHA precursor (Fig. 1*c*). The corresponding chimeric protein is similar to that encoded by BPGR5, except that only the first 168 Sm28GST residues are fused to the 1942 FHA residues and that the *fhaB* open reading frame continues after codon 1976, which is spliced downstream of the Sm28GST-encoding DNA.

Immunoblot analyses of whole cell extracts of *B. pertussis* BPGR6 revealed polypeptides that react with both the anti-FHA (Fig. 2*A*, lane 4) and anti-Sm28GST antibodies (Fig. 2*B*, lane 4). This FHA–Sm28GST hybrid protein reacted less well with the anti-Sm28GST antibodies than that produced by *B. pertussis* BPGR5, presumably because the C-terminal region of Sm28GST was deleted in BPGR6. This region is known to contain immunodominant epitopes (17). However, in contrast to BPGR5, the hybrid protein could be detected in culture supernatants of BPGR6 after concentration by heparin-Sepharose chromatography (Fig. 2*B*, lane 1). This observation suggests that the chimeric protein was secreted from *B. pertussis* BPGR6 and associated with the cell surface. The improved secretion in BPGR6 compared with BPGR5 may be due to the expression of the C-terminal domain of the FHA precursor. Alternatively, it could be related to the deletion of the C-terminal end of Sm28GST.

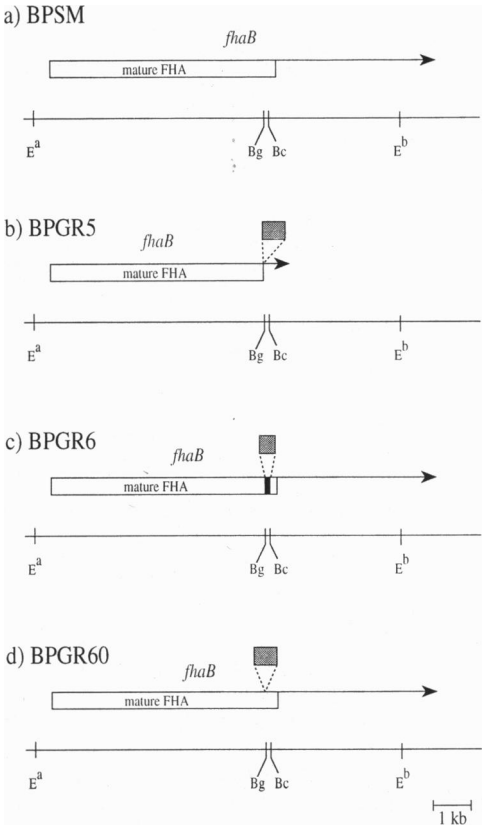

FIG. 1. Physical map of the *fhaB* locus in the various *B. pertussis* strains. The arrow represents the length and direction of the various *fhaB* open reading frames. (*a*) BPSM contains the wild-type *fhaB* locus coding for a 370-kDa precursor protein indicated by the arrow that includes the 220-kDa portion of the mature form indicated by the open box. (*b*) BPGR5 contains the hybrid gene in the *fhaB* locus with the Sm28GST coding sequence (indicated by the stippled box) inserted at the *Bgl*II site. (*c*) BPGR6 contains a truncated Sm28GST sequence (stippled box) inserted between the *Bgl*II and *Bcl*I sites of the *fhaB* locus. The small solid box depicts the resulting truncation in *fhaB*. (*d*) BPGR60 contains the entire modified Sm28GST coding sequence (stippled box) cloned at the *Bgl*II site and containing the TGC to AGC triplet mutation. Bc, *Bcl*I; Bg, *Bgl*II; E^a, first *Eco*RI site; E^b, second *Eco*RI site.

To distinguish between these two possibilities, we constructed BPGR60. In this strain the entire Sm28GST gene was cloned in frame with *fhaB*, such that the hybrid gene was expressed to produce the full-length precursor of FHA (Fig. 1*d*). The corresponding open reading frame codes, therefore, for the 1942 first residues of mature FHA, followed by Pro, the 211 Sm28GST residues, Gly, and residues 1942–3520 of the FHA precursor. In addition, Cys-140 of Sm28GST was replaced by Ser, because this Cys may induce dimerization of Sm28GST by interchain disulfide bond formation (27) that

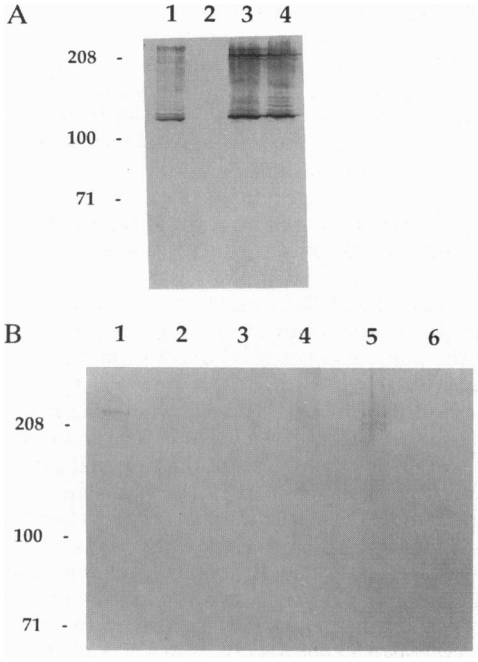

FIG. 2. Immunoblot analyses of *B. pertussis* BPSM, BPGR4, BPGR5, and BPGR6. (*A*) Whole cell lysates of BPSM (lane 1), BPGR4 (lane 2), BPGR5 (lane 3), and BPGR6 (lane 4) were examined by immunoblot analysis using anti-FHA monoclonal antibody 12.1.F9. (*B*) Heparin-Sepharose-purified culture supernatants (lanes 1–3) or whole cell lysates (lanes 4–6) of BPGR6 (lanes 1 and 4), BPGR5 (lanes 2 and 5), and BPSM (lanes 3 and 6) were examined by immunoblot analysis using rabbit anti-Sm28GST polyclonal antibodies. The sizes of the molecular mass markers expressed in kDa are given to the left.

may hamper secretion through the outer membrane, as has been shown for other systems (28, 29).

As shown in Fig. 3 lane 2, this hybrid protein was readily detected in whole cell extracts of *B. pertussis* BPGR60 using anti-Sm28GST antibodies. When the culture supernatant was concentrated by heparin-Sepharose chromatography, the secreted FHA–Sm28GST hybrid protein was detected by both anti-FHA and anti-Sm28GST antibodies, indicating that it was secreted. This suggests that the addition of the C-terminal domain of the FHA precursor rather than the deletion of the C-terminal end of Sm28GST allows for secretion of the chimera into the culture supernatant. However, compared with native FHA from *B. pertussis* BPSM, the secretion of the hybrid protein appeared to be less efficient and was not detected in unconcentrated culture supernatants with the anti-Sm28GST antibodies (Fig. 3, lane 3).

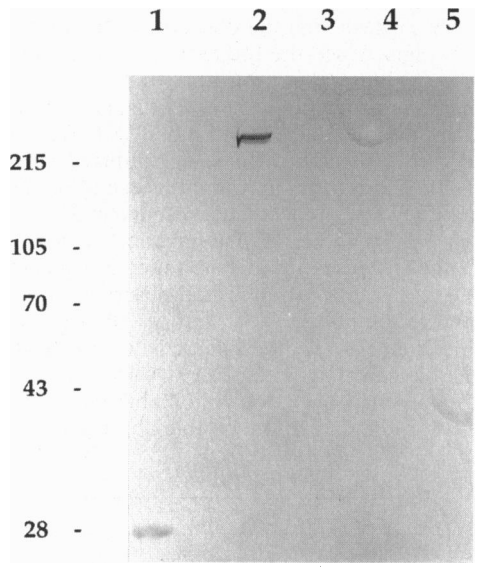

FIG. 3. Immunoblot analysis of *B. pertussis* BPSM and BPGR60. Whole cell extracts (lanes 2 and 4) and culture supernatants (lanes 3 and 5) of BPGR60 (lanes 2 and 3) and BPSM (lanes 4 and 5) were examined by immunoblot analysis using rabbit anti-Sm28GST polyclonal antibodies. Lane 1 contains purified recombinant Sm28GST. The sizes of the molecular mass markers expressed in kDa are given to the left.

Surface Exposure of FHA–Sm28GST. To determine whether the cell-associated hybrid proteins were surface exposed, we used the autoagglutination phenotype of the recombinant *B. pertussis* strains. Menozzi *et al.* (30) have shown that surface exposure of FHA resulted in autoagglutination of *B. pertussis* when grown in the absence of cyclodextrin. We therefore grew BPGR60 in the presence and absence of cyclodextrin. Macroscopical analysis of the culture revealed autoagglutination of BPGR60, indicating that a significant fraction of the FHA–Sm28GST hybrid protein was surface-exposed in this strain. However, in contrast to the nonrecombinant BPSM strain, autoagglutination of BPGR60 was observed regardless of the presence of cyclodextrin, indicating that the fusion of Sm28GST to FHA affected its release into the culture supernatant.

Colonization by *B. pertussis* BPGR60. Kimura *et al.* (31) and Relman *et al.* (32) have demonstrated the crucial role of FHA in colonization of the respiratory tract. Intranasal infection experiments were, therefore, performed in mice to study the ability of *B. pertussis* BPGR60 to colonize the respiratory tract. A suspension containing 5×10^6 BPGR60 or BPSM was administered intranasally to OF1 mice. At 1-week intervals ranging from 0 to 5 weeks after the infection, groups of seven mice were sacrificed, and the total cfu in the lung homogenates were measured. As shown in Fig. 4, the total number of cfu in the lungs of the mice infected by BPGR60 increased during the first week after the infection and then decreased steadily over the next 4 weeks until the majority of bacteria were eliminated. The colonization kinetics of *B. pertussis* BPGR60 was very similar to that of the wild-type strain BPSM (Fig. 4) and was consistent with results obtained by Guiso and Khelef (25). These observations indicate that *B. pertussis* BPGR60 possesses *in vivo* growth rates and colonization abilities similar to those of wild-type *B. pertussis* BPSM, implying that the biological functions of FHA are preserved in BPGR60.

Secretory Immune Responses After Infection with *B. pertussis* BPGR60. As shown in Table 2, a single nasal administration of 5×10^6 *B. pertussis* BPGR60 induced a marked

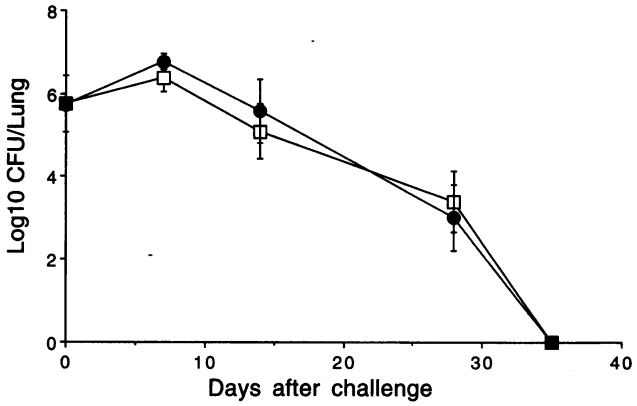

FIG. 4. Colonization by *B. pertussis* BPGR60 and BPSM. OF1 mice were infected intranasally with 5×10^6 cfu of *B. pertussis* BPGR60 (solid circles) or BPSM (open squares). The lungs were harvested at the time points indicated, and total cfu were counted after plating the lung homogenates on BG agar.

increase in total IgA and IgG in BALF after 4 weeks. The elevated levels remained constant at least until day 49. Specific anti-Sm28GST IgA antibodies in BALF were detectable at day 28 and increased gradually until day 42. This was paralleled by the anti-FHA IgA in BALF. However, although we observed a significant drop in the level of anti-Sm28GST IgA at day 49, the anti-FHA IgA levels remained constant. No anti-Sm28GST IgA was detected in mice that received *B. pertussis* BPSM or up to 100 μg of recombinant Sm28GST, whereas high anti-FHA IgA levels were found in BPSM-infected mice.

Specific anti-FHA and anti-Sm28GST IgG were also found in BALF of mice infected with BPGR60, albeit at lower levels than IgA. The total and specific IgE responses in BALF were also investigated. A weak increase of total IgE was observed 35 days after administration, but we were unable to detect specific IgE directed against Sm28GST or FHA. Analyses of serum antibodies in the same mice indicated no detectable levels of anti-Sm28GST IgG and IgA, although anti-FHA IgG and IgA were detected 42 and 49 days after administration of BPGR60.

These results show that a single nasal administration of *B. pertussis* BPGR60 was able to induce the production of anti-Sm28GST IgA and IgG in the BALF, even in the absence of detectable levels of anti-Sm28GST antibodies in the serum.

DISCUSSION

Mucosal immunization offers several important advantages over parenteral immunization. They include higher efficacy to achieve both mucosal and systemic immunity, minimization of adverse effects, ease of delivery, less interference with maternal antibodies in infants, and perhaps increased effectiveness in the elderly (33). Yet, the vast majority of vaccines are usually administered parenterally. This may be related to the fact that many antigens are poorly immunogenic when given by the mucosal route (34) probably because they fail to interact productively with the mucosa-associated lymphoid tissues (MALT).

Notable exceptions are microorganisms that colonize mucosal surfaces. They are believed to prolong the mucosal exposure of the antigens and thereby to increase the net antigen dose delivered to the MALT. Much attention has been turned to the development of live bacteria, such as *Salmonella typhi*, for the presentation of foreign antigens to the MALT by oral immunization (1). However, oral immunization is complicated by the acidic and proteolytic environment that the vaccines encounter, as well as by the large volumes that are usually required to induce a detectable immune response. Mucosal immunization against typhoid fever requires multiple large doses of *Salmonella typhi* Ty21a, the only licenced live oral vaccine against bacterial diseases.

An interesting alternative to oral vaccines are vaccines that can be delivered by the nasal route. Nasal vaccination against influenza has been successful even in children (35). In addition, nasal administration appears to be significantly more efficacious in inducing antibody responses than oral inoculation, when compared on a dose basis (2). However, although several enteric pathogens have been assessed for their ability to deliver heterologous antigens via the oral route, so far no microorganism that specifically colonizes the respiratory tract has been investigated in that respect.

The members of the genus *Bordetella* are known to very effectively colonize the upper respiratory tract of their hosts. In this study, we show, to our knowledge, for the first time that a single nasal administration of a recombinant *B. pertussis* strain induces a significant mucosal antibody response against the foreign antigen. A genetic fusion of Sm28GST to the C-terminal region of the mature form of the FHA resulted in a hybrid protein that was surface-exposed on *B. pertussis* BPGR60 and, to a lesser degree than natural FHA, partially secreted into the extracellular milieu. Secretion of the chimeric antigen required the expression of the entire *fhaB* gene, including the part that encodes the C-terminal domain of the precursor, which is cleaved off during FHA biosynthesis. Unlike in most live bacterial vectors, where the heterologous genes are expressed from plasmids, this system is based on the integration of the hybrid genes into the bacterial chromosome, thereby stabilizing the constructs even in the absence of selective pressure. A construction that contains the stop codon of the Sm28GST cDNA (*B. pertussis* BPGR5) and, therefore, does not translate the 3′ end of *fhaB* does not appear to secrete the hybrid protein. This observation confirms previous conclusions regarding the importance of the C-terminal domain of the FHA precursor in the secretion of FHA (26).

Although *B. pertussis* BPGR60 was found to secrete some of the chimeric protein, the secretion level was significantly lower than that of natural FHA. This relatively low level of secretion was independent of the position of the Sm28GST cDNA insertion within the *fhaB* gene (unpublished observations) and may, therefore, be related to the intrinsic secretion property of Sm28GST, which has generally proven difficult to secrete in heterologous expression systems (e.g., ref. 36). Therefore, it is anticipated that antigens that naturally cross at least one biological membrane should be secreted at higher levels when genetically fused to FHA.

Nevertheless, nasal administration of *B. pertussis* BPGR60 induced an antibody response against Sm28GST in the BALF, whereas the nonrecombinant isogenic strain BPSM or intranasal inoculation of up to 100 μg of recombinant purified Sm28GST did not. The absence of detectable levels of anti-Sm28GST antibodies in the serum of *B. pertussis* BPGR60-infected mice suggests that the observed antibody response is of mucosal origin. Since the life cycle of *S. mansoni* involves migration to the lungs, the respiratory tract may be one of the sites for immunoprotection against schistosomiasis (12). In addition, the production of anti-Sm28GST IgA has been correlated with protection against egg-induced pathology in humans (10). Therefore, intranasal vaccination against *S. mansoni* may have an impact on parasitemia and on pathology. Preliminary results indicate that infection with *B. pertussis* BPGR60 indeed causes significant reduction in both worm burden and egg output (unpublished observations).

The colonization ability of *B. pertussis* BPGR60 was indistinguishable from that of the isogenic wild-type strain, implying that the biological activities of FHA are preserved in this strain. It is likely that the colonization ability of *B. pertussis* is responsible for the induction of the observed immune re-

Table 2. IgA and IgG levels in BALF after nasal administration of BPGR60

	Day 0	Day 28	Day 35	Day 42	Day 49
Total IgA, μg/ml	110 ± 27	816 ± 37*	780 ± 39*	770 ± 22*	771 ± 25*
Total IgG, μg/ml	19	132	162	182	136
Anti-Sm28GST IgA, ng/ml	ND	730 ± 177	985 ± 637	1157 ± 615	579 ± 51
Anti-Sm28GST IgG, ng/ml	ND	69 ± 48	79 ± 32	129 ± 58	78 ± 42
Anti-FHA IgA, ng/ml	ND	997 ± 284	1744 ± 749	14,244 ± 6428	13,551 ± 6064
Anti-FHA IgG, ng/ml	ND	202 ± 54	379 ± 89	664 ± 399	529 ± 326

Results are expressed as the mean ± SD of BALF from five mice per time point. Total IgG levels have been evaluated in pooled BALF. ND, not detectable. *, $P < 0.001$, indicates a significant increase compared to day 0.

sponse. For other systems it has been proposed that the induction of mucosal immunity via the nasal route depends on the amount of antigen reaching the MALT beyond the nasal cavity (37). Since *B. pertussis* is known to colonize the trachea, BPGR60 most likely carries Sm28GST to the trachea-associated lymphoid tissues. One would expect that higher levels of immune responses might be achieved when better secreted antigens are fused to FHA. This is currently being tested in our laboratory.

Heterologous antigens genetically fused to FHA may also benefit from the various binding activities expressed by FHA (for review, see ref. 5), which include binding to alveolar macrophages via a RGD sequence (38, 39). Although it remains to be investigated whether this RGD sequence or other FHA binding activities play a role in antigen presentation by *B. pertussis*, targeting antigens to mononuclear phagocytes including macrophages would be expected to improve antigen presentation to the T helper cells and hence to facilitate the development of antibody responses. On the other hand, alveolar macrophages have been shown to suppress immune responses in rodents (40, 41). It will therefore be interesting to investigate whether *B. pertussis*, perhaps via binding by FHA, is able to inhibit this suppression.

Although the use of *B. pertussis* as a live vaccine carrier is not directly applicable in the field because of the inherent virulence of this microorganism, it is now feasible to genetically attenuate this organism. Over the past decade many *B. pertussis* virulence genes have been cloned, and the molecular mechanisms of pertussis pathogenesis are beginning to be well understood (42). Attenuated *B. pertussis* strains can thus be developed by the allelic exchange of one or several virulence genes with mutant genes. In addition, genetic attenuation has already been achieved by the development of *aroA* mutants of *B. pertussis* that have been shown to induce protective immunity against challenge with a virulent strain (43).

Live recombinant *B. pertussis* may be particularly interesting for the presentation of antigens to protect against respiratory pathogens. However, since B cells stimulated at one mucosal site can migrate to distal mucosal sites presentation of antigens by *B. pertussis* may also be useful for the production of IgA at sites other than the respiratory tract. In addition, as new vaccines are being developed and immunization schedules become more complex, the use of recombinant bacteria offers the opportunity to combine several protective antigens within the same carrier organism and thereby increases considerably the probability to provide complete coverage of populations to be vaccinated.

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1. Cardenas, L. & Clements, J. D. (1992) *Clin. Microbiol. Rev.* **5,** 328–342.
2. Hirabayashi, Y., Kurata, H., Funato, H., Nagamine, T., Aizawa, C. & Kurata, T. (1990) *Vaccine* **8,** 243–248.
3. McGhee, J. R., Mestecky, J., Dertzbaugh, M. T., Eldridge, J. H., Hirasawa, M. & Kiyono, H. (1992) *Vaccine* **10,** 75–88.
4. Haneberg, B., Kendall, D., Amerongen, H. M., Apter, F. M., Kraehenbuhl, J.-P. & Neutra, M. R. (1994) *Infect. Immun.* **62,** 15–23.
5. Locht, C., Bertin, P., Menozzi, F. & Renauld, G. (1993) *Mol. Microbiol.* **9,** 653–660.
6. Zackrisson, G., Arminjon, F., Krantz, I., Lagergard, T., Sigurs, N., Taranger, J. & Trollfors, B. (1988) *Eur. J. Clin. Microbiol. Infect. Dis.* **7,** 764–770.
7. Zackrisson, G., Lagergard, T., Trollfors, B. & Krantz, I. (1990) *J. Clin. Microbiol.* **28,** 1502–1505.
8. Amsbaugh, D. F., Li, Z.-M. & Sahin, R. D. (1993) *Infect. Immun.* **61,** 1447–1452.
9. Balloul, J. M., Sondermeyer, P., Dreyer, D., Capron, M., Grzych, J. M., Pierce, R. J., Carvallo, D., Lecocq, J. P. & Capron, A. (1987) *Nature (London)* **326,** 149–153.
10. Grzych, J.-M., Grezel, D., Xu, C. B., Neyrinck, J.-L., Capron, M., Ouma, J. H., Butterworth, A. E. & Capron, A. (1993) *J. Immunol.* **150,** 527–535.
11. Kahn, C. M. A., Villarreal-Ramos, B., Pierce, R. J., Riveau, G., de Hormaeche, R. D., McNeill, H. Ali, T., Fairweather, N., Chatfield, S., Capron, A., Dougan, G. & Hormaeche, C. E. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 11261–11265.
12. Wilson, R. A., Coulson, P. S. & Dixon, B. (1986) *Parasitology* **92,** 101–106.
13. Menozzi, F. D., Mutombo, R., Renauld, G., Gantiez, C., Hannah, J. H., Leininger, E., Brennan, M. J. & Locht, C. (1994) *Infect. Immun.* **62,** 769–778.
14. Locht, C., Geoffroy, M.-C. & Renauld, G. (1992) *EMBO J.* **11,** 3175–3183.
15. Simon, R., Priefer, U. & Pühler, A. (1983) *BioTechnology* **1,** 784–791.
16. Delisse-Gathoye, A.-M., Locht, C., Jacob, F., Raaschou-Nielsen, M, Heron, I., Ruelle, J.-L., de Wilde, M. & Cabezon, T. (1990) *Infect. Immun.* **58,** 2895–2905.
17. Wolowczuk, I., Auriault, C., Bossus, M. Boulanger, D., Gras-Masse, H., Mazingue, C., Pierce, R. J., Grezel, D., Reid, G. D. F., Tartar, A. & Capron, A. (1991) *J. Immunol.* **146,** 1987–1995.
18. Bordet, J. & Gengou, O. (1906) *Ann. Inst. Pasteur (Paris)* **20,** 731–741.
19. Stainer, D. W. & Scholte, M. J. (1971) *J. Gen. Microbiol.* **63,** 211–220.
20. Imaizumi, A., Suzuki, Y., Ono, S., Sato, H. & Sato, Y. (1983) *Infect. Immun.* **41,** 1138–1143.
21. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed. (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
22. Hull, R. A., Gill, R. E., Hsu, P., Minshew, B. H. & Falkow, S. (1981) *Infect. Immun.* **33,** 933–938.
23. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
24. Menozzi, F. D., Gantiez, C. & Locht, C. (1991) *FEMS Microbiol. Lett.* **78,** 59–64.
25. Guiso, N. & Khelef, N. (1992) *Zbl. Bakt. Suppl.* **23,** 263–271.
26. Renauld-Mongénie, G., Cornette, J., Mielcarek, N., Menozzi, F. D. & Locht, C. (1996) *J. Bacteriol.* **178,** 1053–1060.
27. Trottein, F., Vaney, M.-C., Bachet, B., Pierce, R. J., Colloc'h, N., Lecocq, J.-P., Capron, A. & Mornon, J.-P. (1992) *J. Mol. Biol.* **224,** 515–518.
28. Klauser, T., Pohlner, J. & Meyer, T. F. (1990) *EMBO J.* **9,** 1991–1999.
29. Klauser, T., Pohlner, J. & Meyer, T. F. (1992) *EMBO J.* **11,** 2327–2335.
30. Menozzi, F. D., Boucher, P. E., Mutombo, R., Gantiez, C., Riveau, G. & Locht, C. (1994) *Infect. Immun.* **62,** 4261–4269.
31. Kimura, A., Mountzouros, K. T., Relman, D. A., Falkow, S. & Cowell, J. L. (1990) *Infect. Immun.* **58,** 7–16.
32. Relman, D. A., Domenighini, M., Tuomanen, E., Rappuoli, R. & Falkow, S. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 2637–2641.
33. Walker, R. I. (1994) *Vaccine* **12,** 387–400.
34. de Aizpurua, H. J. & Russell-Jones, G. J. (1988) *J. Exp. Med.* **167,** 440–451.
35. Anderson, E. L., Newman, F. K., Maassab, H. F. & Belshe, R. B. (1992) *J. Clin. Microbiol.* **30,** 2230–2234.
36. Kremer, L., Baulard, A., Estaquier, J., Content, J., Capron, A. & Locht, C. (1995) *J. Bacteriol.* **177,** 642–653.
37. Yetter, R. A., Lehrer, S., Ramphal, R. & Small P. A. Jr. (1980) *Infect. Immun.* **29,** 654–662.
38. Relman, D., Tuomanen, E., Falkow, S., Golenbock, D. T., Saukkonene, K. & Wright, S. D. (1990) *Cell* **61,** 1375–1382.
39. Ishibashi, Y., Claus, S. & Relman, D. A. (1994) *J. Exp. Med.* **180,** 1225–1233.
40. Holt, P. G., Degebrodt, A., O'Leary, C., Krska, K. & Plozza, T. T. (1993) *J. Exp. Med.* **177,** 397–407.
41. Thepen, T., Van Rooijen, N. & Krall, G. (1989) *J. Exp. Med.* **170,** 499–509.
42. Rappuoli, R. (1994) *Pathogenicity Mechanisms of Bordetella: In Bacterial Pathogenesis of Plants and Animals*, ed. Dangl, J. L. (Springer, Berlin), pp. 319–336.
43. Roberts, M., Maskell, D., Novotny, P. & Dougan, G. (1990) *Infect. Immun.* **58,** 732–739.