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Production of Nontypeable *Haemophilus influenzae* HtrA by Recombinant *Bordetella pertussis* with the Use of Filamentous Hemagglutinin as a Carrier

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***Bordetella pertussis*, the etiologic agent of whooping cough, is a highly infectious human pathogen capable of inducing mucosal and systemic immune responses upon a single intranasal administration. In an attenuated, pertussis toxin (PTX)-deficient recombinant form, it may therefore constitute an efficient bacterial vector that is particularly well adapted for the delivery of heterologous antigens to the respiratory mucosa. Filamentous hemagglutinin (FHA) has been used as a carrier to present foreign antigens at the bacterial surface, thereby inducing local, systemic, and protective immune responses to these antigens in mice. Both full-length and truncated (Fha44) forms of FHA have been used for antigen presentation. To investigate the effect of the carrier (FHA or Fha44) on antibody responses to passenger antigens, we genetically fused the HtrA protein of nontypeable *Haemophilus influenzae* to either FHA form. The *fha-htrA* and Fha44 gene-*htrA* hybrids were expressed as single copies inserted into the chromosome of PTX-deficient *B. pertussis*. Both chimeras were secreted into the culture supernatants of the recombinant strains and were recognized by anti-FHA and anti-HtrA antibodies. Intranasal infection with the strain producing the FHA-HtrA hybrid led to significantly higher anti-HtrA and anti-FHA antibody titers than those obtained in mice infected with the Fha44-HtrA-producing strain. Interestingly, the *B. pertussis* strain producing the Fha44-HtrA chimera colonized the mouse lungs more efficiently than the parental, Fha44-producing strain and gave rise to higher anti-FHA antibody titers than those induced by the parental strain.**

The mucosal delivery of vaccines has several advantages over systemic delivery. Consequently, the development of efficient mucosal vaccine delivery systems, including live attenuated or commensal bacterial vaccines, has attracted much attention over the last decades. However, most efforts have so far been devoted to the oral route, although the oral delivery of vaccine formulations requires large quantities of antigen and repeated administrations to be effective. The nasal route may represent an interesting alternative (23), as it avoids the encounter with an acidic and proteolytic environment as well as the competition with commensal microorganisms present in the digestive tract.

Bordetella pertussis, the etiologic agent of whooping cough, has recently been used as a live vector to deliver foreign antigens to the respiratory mucosa in laboratory models (29). As a highly contagious agent, it colonizes the human respiratory tract very efficiently and induces both local and systemic immune responses. The deletion of the genes encoding pertussis toxin (PTX), the major virulence factor of *B. pertussis* (24), has led to a highly attenuated strain, as evidenced by a strong reduction in lung inflammation and lymphocytosis (18). In this genetically attenuated form, *B. pertussis* is able to induce strong protection against respiratory challenges with virulent strains when given by the nasal route in a single dose (31). Interestingly, strains impaired in the ability to produce active PTX induced a stronger serum antibody response against filamentous hemagglutinin (FHA) in mice after intranasal (i.n.) administration than did virulent strains (31).

FHA is one of the major *B. pertussis* adhesins, and it is both exposed on the surface and secreted by the microorganism (25). It is a 230-kDa protein that is able to induce high levels of mucosal and systemic antibodies upon infection by *B. pertussis* in both humans (14) and mice (3). Heterologous antigens have been genetically fused to FHA and thereby exposed at the *B. pertussis* surface or secreted into the extracellular milieu (7, 30, 36, 37). The *Schistosoma mansoni* glutathione *S*-transferase (Sm28GST) fused to full-length FHA and produced by a *B. pertussis* strain lacking PTX has been shown to induce a strong anti-Sm28GST serum antibody response after a single i.n. administration of the attenuated recombinant strain (31). More recently, a truncated form of FHA, corresponding to the N-terminal, 80-kDa half of the mature protein and named Fha44, has been used as a carrier for the transferrin-binding protein B (TbpB) from *Neisseria meningitidis* (7) because Fha44 is produced in much higher amounts and is more efficiently secreted by *B. pertussis* than full-length FHA (35). As expected, the genetic fusion of TbpB to Fha44 resulted in much more production and secretion of the hybrid protein (7) than those induced by a fusion of Sm28GST to full-length FHA, which was barely detectable in the culture supernatants of the recombinant strains (36). The Fha44-TbpB-producing *B. pertussis* strain induced serum anti-

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body responses against both Fha44 and TbpB after i.n. administration (7).

For this study, we engineered *B. pertussis* strains to produce HtrA from nontypeable *Haemophilus influenzae* (NTHI) fused to either Fha44 or full-length FHA in order to investigate the effect of the carrier protein on the immunogenicity of the passenger antigen. HtrA was used as a model antigen because it is a naturally secreted monomeric protein produced by NTHI, in contrast to previously used antigens, which were either cytosolic or part of multimeric structures in their natural hosts. NTHI is a major cause of otitis media in young children and of lower respiratory tract infections in adults, with recurrent episodes of the disease (19, 32), and i.n. immunization has been demonstrated to be an effective means of reducing the colonization of NTHI in the nasal tract (20, 21). HtrA is a stress response protein with serine protease activity that belongs to the σ^E-dependent family of heat shock proteins (6). It is well conserved among NTHI strains and has been shown to elicit partial protection in infant rat and chinchilla models (5, 26), which makes this protein an attractive candidate for a subunit vaccine. HtrA has been identified as a virulence factor in *Legionella pneumophila*, *Salmonella enterica* serovar Typhimurium, *Yersinia enterocolitica*, and *Brucella abortus* (9, 17, 22, 33). However, the role of HtrA in the pathogenesis of NTHI remains to be determined.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *B. pertussis* strains used for this study are listed in Table 1. They were all derived from a PTX-deficient Tohama I derivative named BPRA (4) and were grown on Bordet-Gengou agar (Difco, Detroit, Mich.) supplemented with 1% glycerol, 20% defibrinated sheep blood, and 100 μg/ml streptomycin (Sigma Chemical Co., St Louis, Mo.) at 37°C for 72 h. Liquid cultures of *B. pertussis* were incubated as described previously (27) in Stainer-Scholte medium containing 1 g/liter heptakis(2,6-di-*o*-methyl) β-cyclodextrin (Sigma). For cell adherence assays, exponentially growing *B. pertussis* was inoculated at an optical density at 600 nm of 0.15 in 2.5 ml of Stainer-Scholte medium supplemented with 65 μCi/ml L-[^{35}S]methionine plus L-[^{35}S]cysteine (NEN, Boston, Mass.) and then was grown for 24 h at 37°C. The bacteria were then washed three times with phosphate-buffered saline (PBS) and resuspended in RPMI 1640 (Gibco, Grand Island, N.Y.) at the desired density.

***B. pertussis* mutant constructs.** *B. pertussis* BPSA85, BPSA199, and BPSA167 were obtained by allelic exchange as described by Stibitz (40), using the pJQmp200rpsL18 (34) derivatives pAS28 (to produce the Fha44-HtrA hybrid), pAS86 (to produce the FHA-HtrA hybrid), and pAS65 (to produce the Fha44-HtrA* hybrid, a protein in which the HtrA RGD sequence was changed to RAD [see below]) to replace the *fhaB* gene of BPRA with DNAs encoding Fha44-HtrA, FHA-HtrA, and Fha44-HtrA*, respectively.

pAS28 was obtained by inserting the 4,085-bp SalI fragment from pAS23 into SalI-digested pJQmp200rpsL18. pAS23 was the result of the insertion of the 1,469-bp BamHI fragment from pAS22 into BamHI-digested pAS3. pAS3 was a derivative of pBR328 (39) digested with EcoRI and BamHI, into which the EcoRI-BglII fragment of *fhaB* was inserted. This plasmid was then digested with BamHI and religated to delete the internal BamHI fragment within the *fhaB* gene. pAS22 was a pUC18 derivative in which the PCR product corresponding to the mutated HtrA coding sequence from NTHI strain 33 was inserted into the BamHI site. This PCR product was amplified from pBRT7H91A (26) by use of the oligonucleotides 5′-CGCGGATCCACTTTGCCAAGTTTTGTTTCG GAA-3′ and 5′-GCGGGATCCCTTATCGATTTGATGGCCCATTAA-3′ and then digested with BamHI prior to insertion into pUC18. pAS86 resulted from the insertion of the 3,255-bp BamHI-ScaI fragment from pAS85 into BamHI-digested pJQmp200rpsL18. pAS85 was obtained by inserting the BamHI fragment from pAS56 into BglII-digested pAS76. pAS76 was a pUC18 derivative into which the 3,192-bp XhoI-digested fragment of *fhaB* was inserted. pAS56 resulted from the insertion of a PCR product into PCRIItopo (Invitrogen). This PCR product was generated by the use of pBRT7H91A and the oligonucleotides 5′-TATAAGGATCCCAATGACTTTGCCAAGTTTTGTT-3′ and 5′-TATAA GGATCCCTTGCACTAATAAATAGAAATTAC3′. pAS65 was the result of the insertion of the 3,815-bp SalI fragment from pAS64 into SalI-digested pJQmp200rpsL18. pAS64 was obtained by inserting the BamHI fragment from pAS60 into BamHI-digested pAS3. pAS60 resulted from the insertion of a PCR product into PCRIItopo. This PCR product was generated by the use of pBRT7H91A and the oligonucleotides 5′-CGCGGATCCACTTTGCCAA GTTTTGTTTCGGAA-3′ and 5′-GCAGATTATTGCACTAATAAATAGA AATTACTGTCA**G**CTCG-3′. A mutation corresponding to a change of the RGD sequence to RAD was generated by a C→G point mutation (shown in bold).

TABLE 1. *B. pertussis* strains used for this study

B. pertussis strain	Relevant phenotype	Reference
BPRA	FHA$^+$ ΔPTX	4
BPSA85	Fha44-HtrA$^+$ ΔPTX	This study
BPSA87	Fha44$^+$ ΔPTX	2
BPSA167	Fha44-HtrA*$^+$ ΔPTX	This study
BPSA199	FHA-HtrA$^+$ ΔPTX	This study

Immunodetection of recombinant proteins. Culture supernatants or whole-cell lysates of the various *Bordetella* strains were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 8% polyacrylamide gels. The proteins were electrotransferred onto polyvinylidene difluoride membranes and then incubated with a chicken anti-Fha44 (12) or guinea pig anti-HtrA (26) antibody or with a mixture of monoclonal (F1, F2, and F5) anti-FHA antibodies (8) in PBS containing 0.1% Tween and 1% bovine serum albumin, followed by alkaline phosphatase-conjugated goat anti-chicken, -guinea pig, or -murine immunoglobulin G (IgG; Sigma), respectively, and the alkaline phosphatase substrate.

Antigens. FHA was purified from *B. pertussis* BPRA by heparin-Sepharose chromatography, as described previously (28). Purified HtrA was kindly provided by S. Loosmore (Aventis-Pasteur, Toronto, Canada).

Cells and growth conditions. The human pulmonary epithelial cell line A549 (ATCC CL-185) was cultured in RPMI medium containing sodium penicillin G (1,000 U/ml), streptomycin (50 μg/ml) (Gibco), 2 mM L-glutamine (Gibco), and 10% heat-inactivated fetal calf serum (Gibco), using uncoated tissue culture flasks and 24-well plates. The murine alveolar macrophage cell line MH-S (ATCC CRL-2019) was propagated in uncoated tissue culture flasks and 24-well plates in the same RPMI-based medium as that described above, supplemented with 1.5 g of sodium bicarbonate/liter, 4.5 g of glucose/liter, 10 mM HEPES, 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol. Cells were detached mechanically by scraping.

Cell adherence assay. Cells (2×10^5 per well) were cultured for 2 days in 24-well plates. The cells were then washed once with RPMI medium before the addition of 4×10^6 ^{35}S-labeled bacteria per well and incubation for 1 h 30 min at 37°C in 5% CO_2. After three washes with RPMI medium to remove nonadherent bacteria, the cells were lysed with 0.5% sodium dodecyl sulfate. The radioactivity in the whole-cell lysates was quantified by liquid scintillation counting. Quadruplicate experiments were performed independently three times.

Intranasal infection. *B. pertussis* grown on BG agar was suspended in sterile PBS and adjusted to a concentration of approximately 2.5×10^7 CFU/ml for colonization studies or 2.5×10^8 CFU/ml for immunogenicity studies. Infections were performed by the i.n. route as described before (1), using 9-week-old BALB/c mice (Iffa Credo, L'Arbresle, France). For colonization studies, the lungs were aseptically removed at the indicated time points and homogenized in PBS. Serial dilutions of individual lung homogenates were plated onto BG agar, and the numbers of CFU were determined after 3 to 4 days of incubation at 37°C. Four to 8 mice per time point and per group of mice were assessed. All animal studies were carried out under the guidelines of the Institut Pasteur de Lille animal study board.

Antibody detection. The levels of antibodies to HtrA and FHA were measured with enzyme-linked immunosorbent assays (ELISAs). Microtiter plates (Maxisorp; Nunc) were coated with 50 μl of 0.05 M carbonate buffer (pH 9.6; Sigma) containing 5 μg/ml purified antigen. After blocking of the samples with PBS containing 0.1% Tween and 1% bovine serum albumin, 50 μl of serum was added in twofold serial dilutions. The plates were incubated for 2 h at 37°C, and goat anti-mouse total IgG-horseradish peroxidase or biotinylated goat anti-IgA with a conjugate (Amersham, Les Ulis, France) was added at a 1/4,000 or 1/6,000 dilution, respectively. For anti-IgA detection, peroxidase-conjugated streptavidin (Amersham) was added at a 1/4,000 dilution. The ELISAs were developed by using ABTS [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid); Boehringer Mannheim, Germany] according to the manufacturer's specifications. The results

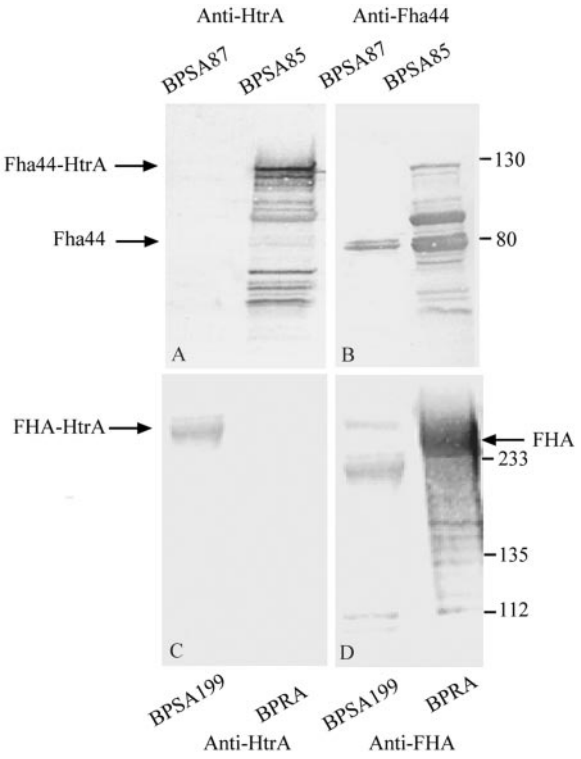

FIG. 1. Production and secretion of Fha44-HtrA and FHA-HtrA by *B. pertussis*. Twenty microliters of unconcentrated culture supernatant from *B. pertussis* BPSA85 (A and B), BPSA87 (A and B), or BPRA (C and D) or 20 μl of 10-fold-concentrated culture supernatant from BPSA199 (C and D) was analyzed by immunoblotting using anti-HtrA (A and C), anti-Fha44 (B), or anti-FHA (D) antibodies. The sizes of the molecular mass markers, expressed in kDa, are given on the right.

are expressed as the highest dilutions of sera yielding an absorbency at 405 nm three times above the control values.

Statistical analysis. The results were analyzed by use of the unpaired Student *t* test. Differences were considered significant at *P* values of <0.05.

RESULTS

Production of chimeric Fha44-HtrA and FHA-HtrA by recombinant *B. pertussis*. The 46-kDa HtrAH91A antigen was fused either to full-length FHA by use of the unique BglII site of the *fhaB* gene or to the C terminus of Fha44, a truncated form corresponding to the N-terminal 80-kDa portion of mature FHA (35). Since HtrA expresses proteolytic activity (26), we chose the enzymatically inactive mutant form HtrAH91A to avoid potential interference of the proteolytic activity with its immunogenicity. The chimeric proteins were named FHA-HtrA and Fha44-HtrA, respectively. The constructs were introduced by double homologous recombination into the chromosomal *fhaB* locus of PTX-deficient *B. pertussis* BPRA.

The production of the chimeric proteins by the recombinant *B. pertussis* strains was analyzed by immunoblotting of culture supernatants. As shown in Fig. 1, polypeptides of the expected sizes (approximately 130 kDa for Fha44-HtrA and 270 kDa for FHA-HtrA) were detected by using an anti-HtrA, anti-Fha44, or anti-FHA antibody, indicating that these chimeric proteins contained both HtrA and Fha44-FHA epitopes. Several smaller polypeptides were also detected, most likely corresponding to proteolytic breakdown products of the chimeras. In addition, the chimeras were also detected at the bacterial cell surface by immunoblotting (data not shown). These results indicate that both FHA and Fha44 are competent carrier proteins allowing *B. pertussis* to secrete the 46-kDa HtrA protein. However, the supernatant from the culture of FHA-HtrA-producing *B. pertussis* was concentrated 10-fold in order to detect the FHA-HtrA chimera, whereas the Fha44-HtrA hybrid protein was readily detected in unconcentrated supernatants. In contrast, there were approximately equal amounts of cell-associated Fha44-HtrA and FHA-HtrA (not shown), indicating that Fha44 is a more efficient carrier protein than full-length FHA with respect to the level of secretion.

Lung colonization by recombinant *B. pertussis*. To study the ability of the recombinant *B. pertussis* strains to colonize the respiratory tract, we infected mice i.n. with BPSA85 or BPSA199 and compared the colonization profiles to those of the parental strains. The colonization profile observed with the FHA-HtrA-producing strain BPSA199 was very similar to that observed with the corresponding parental strain, BPRA, indicating that the fusion of HtrA to full-length FHA does not alter the ability of *B. pertussis* to colonize the lungs of mice (Fig. 2A). In contrast, the recombinant strain BPSA85, which produced Fha44-HtrA, colonized mice significantly more efficiently than the parental Fha44-producing strain BPSA87 (Fig. 2B). This result indicates that the fusion of HtrA to the C terminus of Fha44 overcomes the previously described defect in colonization of a *B. pertussis* strain producing Fha44 instead of FHA in a PTX-deficient background (2). Adherence studies using the human pulmonary epithelial cell line A549 (Fig. 3A) or the murine

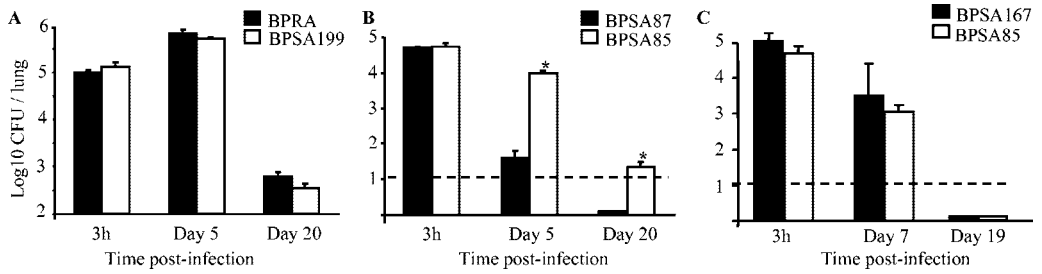

FIG. 2. Lung colonization by *B. pertussis*. BALB/c mice were infected i.n. with approximately 5×10^5 CFU of *B. pertussis* BPRA (black bars in panel A), BPSA199 (white bars in panel A), BPSA87 (black bars in panel B), BPSA85 (white bars in panels B and C), or BPSA167 (black bars in panel C). At the indicated times after challenge, the mice were sacrificed, and the viable bacteria present in the lungs were counted. Four mice from each group were analyzed per time point. The dashed lines in panels B and C represent the limit of bacterial counts. *, $P < 0.05$.

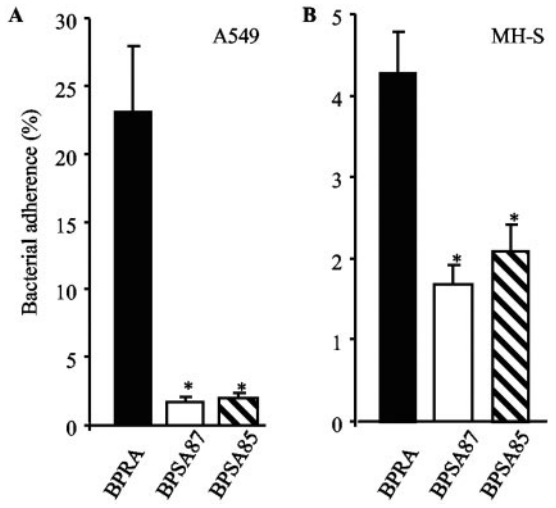

FIG. 3. In vitro adherence of *B. pertussis*. Human pulmonary epithelial A549 cells (A) and murine alveolar macrophage-like MH-S cells (B) were incubated with ^{35}S-labeled *B. pertussis* BPRA (black bars), BPSA87 (white bars), or BPSA85 (hatched bars) for 1 h 30 min at a multiplicity of infection of 20. After the cells were washed, adherence was estimated by scintillation counting. The results are expressed as percentages of cpm relative to the cpm present in the inoculum. The data represent averages and standard deviations for quadruplicate experiments. *, $P < 0.05$, relative to the BPRA values.

macrophage-like cell line MH-S (Fig. 3B) showed that the Fha44-HtrA-producing strain BPSA85 adhered as poorly as the Fha44-producing strain BPSA87, indicating that the fusion of HtrA to Fha44 does not enhance its adhesive properties for these cells.

Antibody response upon infection with recombinant *B. pertussis*. To evaluate the immune responses induced by the recombinant strains, we infected mice i.n. twice at a 2-month interval with approximately 5×10^6 CFU of the recombinant strain BPSA85 or BPSA199 or the corresponding parental strain BPSA87 or BPRA. Two weeks after the last administration, sera were collected and analyzed for the presence of anti-HtrA and anti-FHA antibodies. A significant serum antibody response was raised against HtrA upon infection with either recombinant strain (Fig. 4A). However, the anti-HtrA IgG titers obtained from mice infected with the FHA-HtrA-producing strain BPSA199 were significantly higher than those obtained from mice infected with the Fha44-HtrA-producing strain.

Significant serum anti-FHA antibody responses were obtained from mice infected with the Fha44-HtrA-producing strain BPSA85 (Fig. 4B). In contrast, and as previously observed (2, 37), no anti-FHA IgG was detected in the sera of mice infected with the corresponding parental strain, BPSA87, indicating that the addition of HtrA to Fha44 enhances the immunogenicity of Fha44. This observation is in contrast to what was observed when HtrA was fused to full-length FHA. The anti-FHA antibody response obtained from mice infected with the FHA-HtrA-producing strain BPSA199 was significantly lower than that obtained from mice infected with the parental counterpart, BPRA (Fig. 4C).

Role of the RGD sequence of HtrA in immune responses. HtrA contains an RGD sequence (26) whose role has not yet been investigated, although the RGD sequences of several microbial proteins have been implicated in several important biological functions (38). FHA also contains an RGD motif, which may be involved in epithelial colonization (15). This motif is not present in Fha44. To investigate whether the RGD motif from HtrA complements the FHA RGD deficiency in Fha44-HtrA, we produced a mutated Fha44-HtrA chimera by introducing a Gly→Ala change into the HtrA RGD sequence. The resulting chimera was named Fha44-HtrA*, and the recombinant PTX-deficient *B. pertussis* strain producing Fha44-HtrA* was named BPSA167. The lung colonization profile for

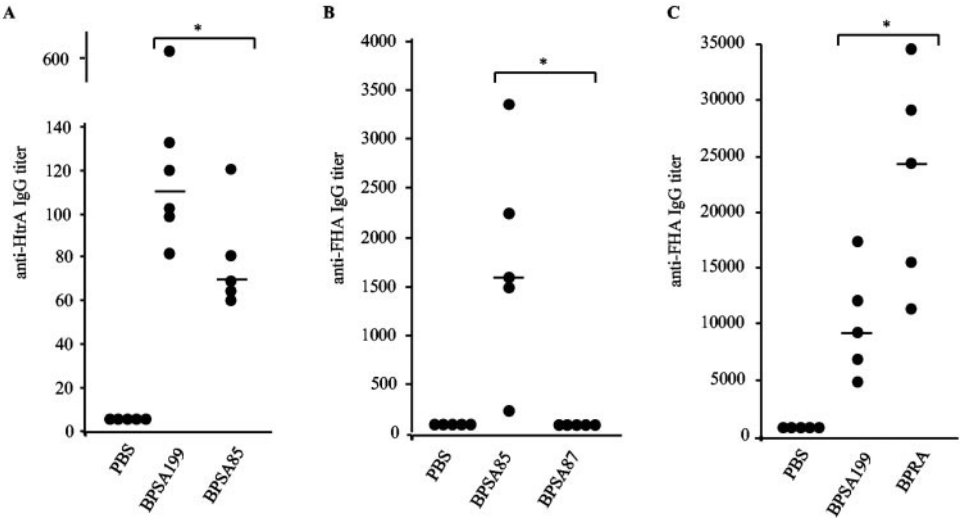

FIG. 4. Anti-HtrA and anti-FHA antibody responses in serum after i.n. administration of *B. pertussis*. Groups of five or six mice were infected twice at a 2-month interval with 5×10^6 CFU of *B. pertussis* BPSA85, BPSA87, BPSA199, or BPRA or were given PBS, as indicated. Two weeks after the second administration, the mice were bled by retro-orbital puncture, and anti-HtrA (A) and anti-FHA (B and C) IgG titers in sera were individually estimated by ELISAs. The titers represent the highest dilutions of sera corresponding to a value equal to three times the control value. The bars represent medians. *, $P < 0.05$.

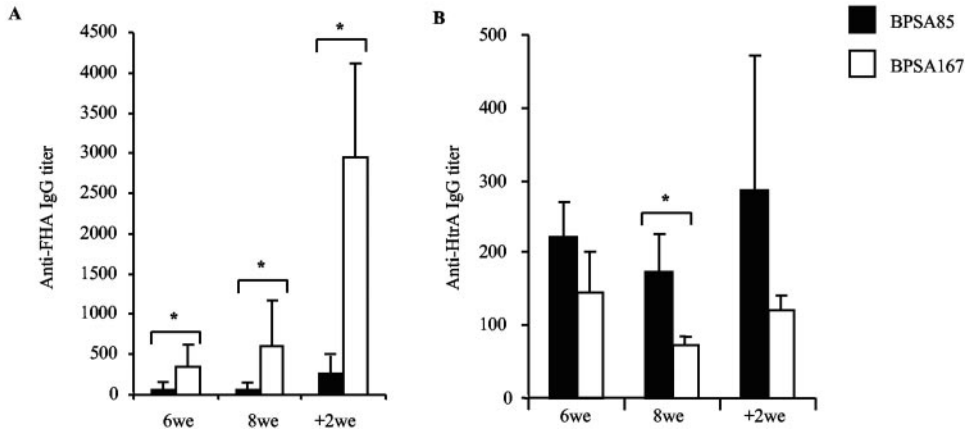

FIG. 5. Anti-HtrA and anti-FHA antibody responses in serum after i.n. administration of recombinant *B. pertussis*. Groups of eight mice were infected twice at a 2-month interval with 5×10^6 CFU of BPSA85 (black bars) or BPSA167 (white bars). Sera were collected 6 and 8 weeks after the first infection and 2 weeks after the booster, as indicated, and anti-FHA (A) and anti-HtrA (B) IgG titers were estimated by ELISAs using individual sera. *, $P < 0.05$.

this recombinant strain was similar to that obtained with the Fha44-HtrA-producing strain BPSA85 (Fig. 2C), indicating that the HtrA RGD sequence plays no role in the colonization efficacy of the recombinant *B. pertussis* strains.

However, when the effect of the Gly→Ala change on antibody responses was investigated, the anti-FHA antibody response was greatly enhanced by this substitution. This was already evident 6 weeks after the first administration and was even stronger 2 weeks after the second administration. In contrast, the mutation had no strong effect on the anti-HtrA antibody response (Fig. 5). A statistically significant difference in the anti-HtrA responses of the BPSA85- and BPSA167-infected groups was found for only one time point. These results indicate that the RGD sequence present in the HtrA portion of the Fha44-HtrA chimera is able to modulate the anti-FHA antibody response.

DISCUSSION

Attenuated recombinant *B. pertussis* has been used successfully to induce local and systemic antibody responses to a variety of foreign antigens after a single i.n. administration (7, 31, 36). For the induction of these immune responses, the heterologous antigens were genetically fused to either full-length or truncated FHA. Although FHA is among the largest bacterial proteins known, it is nevertheless one of the most efficiently secreted proteins from gram-negative bacteria (25). This property, together with its ability to induce strong local and systemic immune responses in *B. pertussis*-infected humans and in mice, has prompted the use of FHA as a carrier of foreign antigens to be presented at the surfaces of *B. pertussis* cells or to be secreted into the extracellular milieu. When chimeric genes are expressed from single copies stably inserted into the *B. pertussis* chromosome, i.n. infection with recombinant strains has been shown to result in the induction of immune memory (30, 37), protection against challenge (31), or the generation of bactericidal antibodies (7).

To investigate the potential effect of the carrier protein on the immunogenicity of the passenger antigen, we engineered *B. pertussis* strains that produce the same heterologous antigen, HtrA from NTHI (strain 33), fused to either full-length FHA or Fha44. To avoid potential effects of the protease activity of HtrA on the immune response, we used a mutated form (HtrAH91A) in which the active-site histidine was replaced with alanine (5, 26). HtrAH91A was genetically fused to FHA and Fha44, and the chimeric constructs were introduced into the chromosomal *fhaB* locus of *B. pertussis* BPRA, a strain that lacks PTX. Both chimeras were detected in the culture supernatants of the recombinant strains, although Fha44-HtrA was secreted at a much higher level than FHA-HtrA. These results demonstrate that both Fha44 and full-length FHA are able to carry passenger proteins of up to 46 kDa into the extracellular milieu. This is in contrast to the very poor production and secretion of a FHA-Sm28GST chimera (36). Since HtrA and Sm28GST were inserted at exactly the same site in FHA, these observations indicate that the production/secretion efficiency of FHA-derived hybrid proteins largely depends on the nature of the passenger protein. The rules that govern the production/secretion efficiency are not known. However, it has been shown that FHA secretion requires the interaction of the FHA secretion domain with the channel-forming outer membrane protein FhaC (16) and that the folding of a passenger protein into a globular conformation prior to FhaC-mediated export is incompatible with secretion (12). When secretion is hampered, both the FHA moiety and the passenger domain are readily degraded in *B. pertussis*, providing an explanation as to why disulfide-containing passenger proteins, such as the cholera toxin B subunit (12) and fragment C of tetanus toxin (37), are poorly secreted when fused to FHA or Fha44 and are found only in small cell-associated amounts. However, neither HtrA nor Sm28GST contains disulfide bonds, indicating that additional properties of the passenger proteins may strongly influence their ability to be produced and secreted via the FHA secretion machinery.

We have previously described that PTX and FHA exert redundant functions during infection of the mouse respiratory tract (1). PTX- and FHA-deficient strains colonize the respiratory tracts of mice nearly as well as the isogenic wild-type strains. However, when both PTX and FHA are lacking, col-

onization is strongly impaired. The replacement of FHA with Fha44 in a PTX-deficient background did not restore the ability of the strain to colonize the respiratory tract at wild-type levels (2). Surprisingly, the fusion of HtrA to Fha44 in a PTX-deficient background partially restored the ability of the strain to colonize mouse lungs. This observation suggests that HtrA may at least partially replace some of the functional roles of FHA during colonization of the mouse respiratory tract and may thus act as a colonization factor. HtrA is a heat shock protein. Although heat shock proteins are usually intracellular, some may become surface exposed under certain physiological conditions, and these have been shown to serve as adhesion factors in pathogenic bacteria (10, 11, 13). A potential signal peptide has been identified in HtrA from NTHI, suggesting that it is secreted through the inner membrane of the bacterium and, perhaps, at least partially surface exposed in response to stress (26). As such, it has been suggested that it could act as an attachment factor under certain circumstances (26). Alternatively, the fusion of HtrA may have restored functionality to Fha44, possibly by altering its conformation. This hypothesis is supported by the increase in Fha44 immunogenicity after its fusion to HtrA (see Fig. 5). However, the Fha44-HtrA hybrid was not able to functionally replace full-length FHA in cell adherence assays using epithelial and macrophage-like cell lines, the two cell types that are known to play a role in *B. pertussis* adherence in vivo. Fha44-HtrA-producing *B. pertussis* adhered as poorly to A549 and MH-S cells as did Fha44-producing *B. pertussis* or bacteria that totally lack FHA (2). Although these cell lines are often used to study the in vitro attachment mechanisms of *B. pertussis* to host cells (1, 2), it is possible that the potential adherence activities of HtrA in the context of *B. pertussis* infection of mice may actually involve other cell types or host structures that have yet to be identified. Alternatively, it is possible that the colonization-promoting activity of HtrA depends on functions of the protein other than adherence functions. A role of the protease activity can be ruled out in this context, as care was taken to use an HtrA derivative that is devoid of its natural protease activity. The additional function(s) of HtrA that may be involved in colonization therefore remains unknown at present.

i.n. infection by the *B. pertussis* strain producing the FHA-HtrA protein gave rise to a level of anti-HtrA serum antibodies that was significantly higher than that obtained upon infection with the Fha44-HtrA-producing strain, despite the fact that the Fha44-HtrA chimera was produced and secreted in substantially larger amounts than FHA-HtrA. This observation is in agreement with previous studies showing that although the Sm28GST-containing chimera was very poorly produced and secreted by recombinant *B. pertussis* (36), high levels of anti-SmGST serum antibodies were observed (31). These results suggest that the immunogenicity of the chimera depends on domains of FHA that are missing in Fha44 and that may express adjuvant activities. Alternatively, the carrier may have an effect on the conformation of the passenger protein, which may influence its immunogenicity. In addition, since the FHA-HtrA-producing strain colonized mice significantly better than the Fha44-HtrA-producing strain, the difference in immunogenicity may be a reflection of the difference in colonization between the two recombinant strains.

As previously reported (2), infections with *B. pertussis* strains producing and secreting Fha44 instead of FHA did not result in the induction of significant levels of anti-FHA antibodies. However, when the Fha44-HtrA chimera was produced instead of Fha44, anti-FHA antibodies could readily be detected in the sera of infected mice. Similar results were obtained with a Fha44-TbpB chimera (7), indicating that the fusion of a passenger protein at the C terminus of Fha44 enhances the immunogenicity of the FHA moiety of the hybrid protein. Since two very different passenger proteins had similar effects on the immunogenicity of the carrier protein, it is likely that they induced a conformational change of the carrier, leading to increased immunogenicity. However, in addition, HtrA contains an RGD site that may potentially modulate immune responses via interactions with integrins on antigen-presenting cells (38). A *B. pertussis* strain producing a Fha44-HtrA hybrid protein in which the RGD site of HtrA was altered induced a much higher anti-FHA antibody response than *B. pertussis* producing unaltered Fha44-HtrA, whereas the level of anti-HtrA antibodies was not affected in a dramatic way. These observations point to a complex interplay between the interaction of RGD sequences from surface proteins with their receptors and other functional domains of the proteins. The identification of the cellular and molecular elements involved in this interplay will provide exciting new research areas to help us understand host-pathogen relationships and the induction of specific immune responses.

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