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Production of *Neisseria meningitidis* Transferrin-Binding Protein B by Recombinant *Bordetella pertussis*

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Neisseria meningitidis **serogroup B infections are among the major causes of fulminant septicemia and meningitis, especially severe in young children, and no broad vaccine is available yet. Because of poor immunogenicity of the serogroup B capsule, many efforts are now devoted to the identification of protective protein antigens. Among those are PorA and, more recently, transferrin-binding protein B (TbpB). In this study, TbpB of *N. meningitidis* was genetically fused to the N-terminal domain of the *Bordetella pertussis* filamentous hemagglutinin (FHA), and the *fha-tbpB* hybrid gene was expressed in *B. pertussis* either as a plasmid-borne gene or as a single copy inserted into the chromosome. The hybrid protein was efficiently secreted by the recombinant strains, despite its large size, and was recognized by both anti-FHA and anti-TbpB antibodies. A single intranasal administration of recombinant virulent or pertussis-toxin-deficient, attenuated *B. pertussis* to mice resulted in the production of antigen-specific systemic immunoglobulin G (IgG), as well as local IgG and IgA. The anti-TbpB serum antibodies were of the IgG1, IgG2a, and IgG2b isotypes and were found to express complement-mediated bactericidal activity against *N. meningitidis*. These observations indicate that recombinant *B. pertussis* may be a promising vector for the development of a mucosal vaccine against serogroup B meningococci.**

Serogroup B *Neisseria meningitidis* is responsible for fulminant septicemia and is a common cause of pyogenic meningitis. In addition to sporadic outbreaks, large epidemics of serogroup B meningococcal disease occur in many parts of the world (7). Therefore, the development of a vaccine against *N. meningitidis* serogroup B remains a high priority worldwide. Unlike other serogroups for which the capsular polysaccharides constitute efficacious vaccines, the serogroup B capsule is poorly immunogenic in humans (2). Therefore, attention has been turned to protein antigens in an attempt to use them for serogroup B meningococcal vaccine development. Among them, the transferrin-binding proteins (Tbp), especially TbpB, appear to be particularly promising. TbpA and TbpB constitute the major components of meningococcal receptors for human transferrin (hTf) (for a review, see reference 6). TbpB is a surface-exposed lipoprotein expressed during iron starvation and plays a critical role in *N. meningitidis* iron uptake from iron-loaded hTF (5). It has been shown previously to be protective in animals after passive or active immunization (1, 9, 24), and humans mount bactericidal antibody responses to TbpB upon meningococcal infections (3). Complement-mediated bactericidal activity of immune sera is considered to be a major mechanism of protection by preventing the multiplication of the microorganism after its passage through the upper respiratory tract epithelium and invasion of the bloodstream (34).

In modern vaccine development, strong emphasis is laid on mucosal delivery systems (25). In particular, the intranasal (i.n.) route holds promise for the induction of protective immune responses, since it is able to elicit both strong local and strong systemic immune responses. i.n. vaccination may therefore be of particular interest against respiratory tract infections, such as those caused by *N. meningitidis* (11, 14). However, efficacious mucosal vaccination generally requires multiple high-dose regimens (47). We have recently developed recombinant *Bordetella pertussis* strains able to induce mucosal (38) and systemic (33) antibody responses against heterologous antigens after a single i.n. administration. Interestingly, and in contrast to other live bacterial vectors, attenuated *B. pertussis* strains induced stronger systemic antibody responses than did their virulent parent strains (32, 33).

In this study, we have thus used recombinant *B. pertussis* to present TbpB at its surface, utilizing the highly efficient secretion mechanism of the *B. pertussis* filamentous hemagglutinin (FHA) (for a review, see reference 26). The recombinant strain was found to induce anti-TbpB antibody responses, both in the bronchoalveolar lavage fluids (BALF) and in the sera of mice inoculated with a single i.n. dose. In addition, the serum antibodies expressed bactericidal activity in an in vitro complement-mediated bactericidal activity assay.

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MATERIALS AND METHODS

Strains, plasmids, culture conditions, and DNA manipulations. The *B. pertussis* strains and plasmids used in this study are listed in Table 1. *B. pertussis* growth conditions were as described previously (15). The *Bordetella* strains were transformed by electroporation at 12.5 kV/cm, 600 Ω, and 25 μF. *N. meningitidis* B16B6 (B:2a:P1.2) and M982 (B:9:P1.9) were obtained from C. Frasch and grown in Mueller-Hinton medium at 37°C in a 10% CO_2-enriched atmosphere.

TABLE 1. *B. pertussis* strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Reference or source
Strains		
BPSM	Sm^r Nal^r Tohama I derivative	31
BPGR4	BPSM derivative lacking *fhaB*	27
BPRA	BPSM derivative lacking *ptx*	4
BPIC1	BPSM derivative producing Fha44-TbpB/M982	This study
BPIC3	BPRA derivative producing Fha44-TbpB/B16B6	This study
BPIC4	BPSM derivative expressing Fha44-TbpB/B16B6	This study
Plasmids		
pTG4710	pARA13 derivative containing *tbpB*/B16B6	45
pTG4714	pARA13 derivative containing *tbpB*/M982	45
pTG6705	pTG4710 derivative with *tbpB* mutations (R185–200)	45
pTG6706	pTG4710 derivative with *tbpB* mutations (R306–321)	45
pBG4	pBBR122 derivative with the 5′ end of *fhaB* (*fha44*)	39
pRIT13202	pUC8 derivative containing *fhaB*	10
pIC-20H	ColE1-based cloning vector	28
pUC18	ColE1-based cloning vector	48
pNFNT	pBG4 derivative with 5′ end of *tbpB*/B16B6	This study
pNFCT	pBG4 derivative with 3′ end of *tbpB*/B16B6	This study
pNFT/B16B6	pBG4 derivative with *tbpB*/B16B6	This study
pNFT/M982	pBG4 derivative with *tbpB*/M982	This study
pIC5	pIC-20H derivative with *fha44-tbpB*/M982	This study
pIC6	pIC5 derivative with 2.4-kb *Bam*HI *fhaB* fragment	This study
pIC7	pUC18 derivative with 2.4-kb *Bam*HI *fhaB* fragment	This study
pIC8	pIC7 derivative with *fha44-tbpB*/B16B6	This study

To induce the production of TbpB, the meningococcal strains were grown for 4 h in the presence of 30 μM ethylene diaminodiorthohydroxyphenyl acetic acid (Sigma, St. Louis, Mo.). The anti-*N. meningitidis* bactericidal activities of the mouse sera were tested as described previously (9). Bactericidal titers are expressed as the highest dilution of serum which killed ≥50% of the bacteria, compared to the complement-only control. All DNA manipulations were performed as described previously (43). Southern blot analyses were performed using nonradioactive DNA probes labeled with digoxigenin-dUTP (Boehringer Mannheim Corp., Mannheim, Germany) according to the instructions of the supplier.

Construction of plasmids and recombinant *Bordetella*. The TbpB-encoding sequences of *N. meningitidis* strains B16B6 and M982 were amplified by PCR from clones pTG4710, pTG6705, pTG6706, and pTG4714 (45), using the oligonucleotides AAAGATCTCTGGGTGGCGGCGGCAGTTCG and AAAGATCTTCGCACCGAATACCACCGATGCTT (for strain B16B6) and TCAGATCTCTGGGCGGCGGCGGCAGTTTCGAT and GCAGATCTGCTGTTGGCGTTTCGCACCGAATACC (for strain M982), respectively, each containing a *Bgl*II site (underlined). The amplified DNA encompassed nucleotides 151 to 1864 for strain B16B6 and 151 to 2232 for strain M982 (21).

The PCR products were digested by *Bgl*II and cloned into the *Bam*HI site of pBG4. The resulting plasmids, named pNFNT, pNFCT, pNFT/B16B6, and pNFT/M982, were introduced into *B. pertussis* BPGR4 (27). pNFT/B16B6 and pNFT/M982 encode the full-length TbpB from strains B16B6 and M982, respectively, whereas pNFNT and pNFCT encode the N-terminal and C-terminal portions of TbpB from strain B16B6, respectively. The latter two plasmids contain the 5′ *Bgl*II fragment amplified from pTG6706 and the 3′ *Bgl*II fragment amplified from pTG6705, respectively.

To insert the B16B6 *tbpB* gene into the *B. pertussis* chromosome at the *fhaB* locus, the 2.4-kb *Bam*HI fragment from pRIT13202 was first introduced into the *Bam*HI site of pUC18, yielding pIC7. This plasmid was further digested by *Eco*RI to introduce the 4.5-kb *Eco*RI fragment from pNFT/B16B6, generating pIC8. This plasmid was then introduced into *B. pertussis* BPRA and BPSM to generate strains BPIC3 and BPIC4, respectively, by allelic exchange. To insert the M982 *tbpB* gene into the *fhaB* gene on the *B. pertussis* chromosome, the 4.9-kb *Eco*RI fragment from pNFT/M982 was first inserted into the *Eco*RI site of pIC-20H, generating pIC5. The 2.4-kb *Bam*HI fragment from pRIT13202 was then inserted into the *Bam*HI site of pIC5, yielding pIC6. This plasmid was inserted into the *B. pertussis* BPSM chromosome by allelic exchange at the *fhaB* locus, yielding strain BPIC1. The proper insertion of the *fha-tbpB* hybrid genes was analyzed by Southern blotting.

Protein analysis, immunodetection, and transferrin binding assays. For the detection of the Fha44-TbpB hybrid proteins, the culture supernatants or whole-cell lysates of the various *Bordetella* strains were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 8% polyacrylamide gels (20). The proteins were stained with Coomassie blue or electrotransferred onto polyvinylidene difluoride membranes and stained with rat (37) or chicken (13) anti-Fha44 antibodies or rabbit polyclonal (45) or mouse monoclonal (511F8; Aventis Pasteur) anti-TbpB antibodies in phosphate-buffered saline (PBS) containing 0.1% Tween and 1% bovine serum albumin (BSA), followed by alkaline phosphatase (AP)-conjugated anti-immunoglobulin G (IgG; Sigma Chemical Co.) and AP substrate.

For transferrin binding assays, the polyvinylidene difluoride membrane was incubated for 1 h at 37°C in blocking buffer (50 mM Tris-HCl, 2 mM $CaCl_2$, 90 mM NaCl, pH 7.4) containing 50 mg of BSA/ml and then incubated at room temperature for 1 h in the presence of 100 μg of hTf (Sigma)/ml. After being washed in blocking buffer containing 5 mg of BSA/ml, the membrane was incubated for 1 h at 37°C with polyclonal goat anti-hTf antibodies (Sigma) diluted 1/1,000, washed with blocking buffer, and incubated with AP-conjugated anti-goat IgG-AP (Sigma) diluted 1/5,000 and then with AP substrate. Because of a slight anti-TbpB reactivity, the anti-hTf antiserum was incubated with purified TbpB before use.

Purified TbpB was provided by Transgène (Strasbourg, France). FHA and Fha44 were purified from *B. pertussis* culture supernatants by heparin-Sepharose chromatography (30).

Antibody responses. The mouse colonization assay was performed as described previously (38). After i.n. administration of 5×10^6 CFU of the various *B. pertussis* strains, IgA and IgG responses in the BALF and sera were analyzed using enzyme-linked immunosorbent assays (ELISAs) at the indicated time points. Immulon 3 flat-bottomed 96-well plates (Dynatech Laboratories, Chantilly, Va.) were coated with 50 μl of PBS containing 10 μg of purified FHA, Fha44, or TbpB/ml. After blocking with PBS containing 0.1% Tween, 1% BSA, and 4% fetal calf serum, 50 μl of BALF or sera was added in twofold serial dilutions. The plates were then incubated overnight at 4°C, and biotinylated goat anti-IgA, anti-IgG, anti-IgG1, anti-IgG2a, or anti-IgG2b antibodies (Amersham France, Les Ulis, France) were added for 90 min at 37°C at a 1/500, 1/1,000, 1/5,000, 1/12,000 or 1/12,000 dilution, respectively. Anti-IgA and anti-IgG antibodies were detected by the addition of peroxidase-conjugated streptavidin (Amersham) at a 1/1,000 dilution and 1 mg of *o*-phenylenediamine (Sigma)/ml in 0.1 M citrate buffer (pH 5.5) containing 0.03% H_2O_2. The reaction was stopped by the addition of 2 M HCl, and the optical density was measured at 492 nm. Results

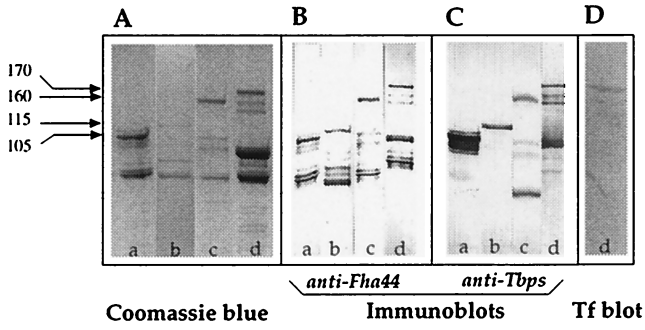

FIG. 1. Production and secretion of Fha44-TbpB hybrid proteins by *B. pertussis*. Fifty-microliter samples of unconcentrated culture supernatants from *B. pertussis* BPGR4 containing pNFNT (lanes a), pNFCT (lanes b), pNFT/B16B6 (lanes c), or pNFT/M982 (lanes d) were analyzed by SDS-PAGE and Coomassie blue staining (A), by immunoblotting using rat anti-Fha44 (B) or rabbit anti-TbpB (C) antibodies directed against TbpB from either strain B16B6 (lanes a to c, panel C) or strain M982 (lane d, panel C), and by hTf binding overlay assays (D). The molecular masses of the chimeras are given in kilodaltons in the left margin.

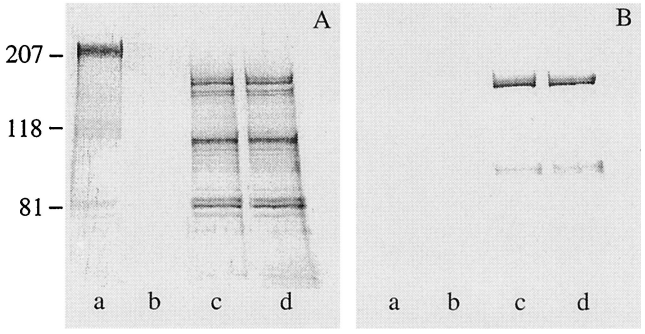

FIG. 2. Production and secretion of the hybrid protein Fha44-TbpB/B16B6 by *B. pertussis*. Twenty microliters of unconcentrated culture supernatants from *B. pertussis* BPSM (lanes a) or BPGR4 (lanes b) and from recombinant strains BPIC3 (lanes c) or BPIC4 (lanes d) was analyzed by immunoblotting using anti-Fha44 IgY (A) or monoclonal anti-TbpB/B16B6 (B) antibody 511F8. The numbers at left are molecular masses in kilodaltons.

are expressed as titers, defined as the highest dilution of BALF and sera yielding an optical density two and three times, respectively, above the values obtained for the mice infected with BPSM or BPRA.

RESULTS

Production of chimeric TbpB-FHA proteins in *Bordetella*. Previous studies have shown that the highly efficient secretion mechanism of FHA can be used to present heterologous antigens by *B. pertussis* (38). Subsequently, we have found that a truncated derivative of FHA, named Fha44, that contains the N-proximal secretion determinants (16) was secreted even more efficiently than was full-length FHA (37). We therefore used Fha44 as a carrier for TbpB. Since TbpBs are relatively large proteins (65 to 90 kDa) and adopt tight folding (45) and because a globular conformation may not be compatible with FHA-mediated secretion (13), we constructed hybrid proteins that contained either the full-length TbpB from *N. meningitidis* B16B6 or N-terminal or C-terminal portions thereof. The full-length TbpB ranged from residue 2 of the mature protein to residue 569, whereas the N-terminal and C-terminal portions comprised amino acids 2 to 133 and 217 to 569, respectively. These proteins were named Fha44-TbpB, Fha44-TbpBN, and Fha44-TbpBC, respectively.

The various TbpB-encoding sequences were amplified by PCR and fused to the Fha44-encoding sequence present in pBG4, a plasmid able to replicate in *B. pertussis*. The pBG4 derivatives were then introduced into *B. pertussis* BPGR4, a strain which carries a chromosomal deletion of the FHA structural gene *fhaB* (27). Unconcentrated culture supernatants of the recombinant bacteria grown to late exponential phase were then analyzed by SDS-PAGE and immunoblotting. As shown in Fig. 1A, polypeptides of the expected sizes (105 kDa for Fha44-TbpBN, 115 kDa for Fha44-TbpBC, and 160 kDa for Fha44-TbpB), as well as smaller polypeptides, were readily detected. Immunoblot analyses using anti-Fha44 (Fig. 1B) or anti-TbpB antiserum (Fig. 1C) confirmed that these proteins contain both Fha44 and TbpB epitopes. Most of the smaller polypeptides reacted only with the anti-Fha44 antibodies, indicating that they lack the TbpB domain and therefore most likely represent N-terminal proteolytic breakdown products of the chimeras. These results indicate that the Fha44 carrier protein is able to mediate highly efficient secretion of large passenger proteins, such as the N-terminal or C-terminal portions of TbpB, and even the full-length TbpB. In addition, the hybrid proteins could also be detected at the bacterial cell surface (data not shown).

To determine whether the TbpB secretion was specific for the B16B6 strain, we also fused full-length TbpB from *N. meningitidis* M982 to Fha44. This protein is considered to represent a quite distinct antigenic group of TbpB proteins (40). As shown in Fig. 1, lanes d, TbpB from strain M982 was secreted as efficiently as that from strain B16B6. In addition, the M982 hybrid protein was able to bind to hTf (Fig. 1D), indicating that at least its N-terminal domain is properly folded in the *B. pertussis* culture supernatant, since hTf binding requires correct folding of this domain (39, 45).

Construction of *B. pertussis* producing chromosomally encoded Fha44-TbpB. Unfortunately, the plasmid-borne genes were readily lost during colonization of the mouse respiratory tract by the recombinant *B. pertussis*. We therefore inserted the *fha44-tbpB*/B16B6 hybrid gene into the *B. pertussis* chromosome. It was inserted by allelic exchange into the *fhaB* locus of virulent *B. pertussis* BPSM and of attenuated *B. pertussis* BPRA, resulting in strains BPIC4 and BPIC3, respectively. BPRA is a strain from which the genes encoding pertussis toxin (PTX), the major *B. pertussis* virulence determinant, have been deleted (4). This strain is strongly attenuated in the mouse respiratory infection model (18, 33). Immunoblot analyses of unconcentrated culture supernatants of these strains revealed the presence of polypeptides immunoreactive with both the anti-Fha44 and anti-TbpB antibodies that correspond to the Fha44-TbpB hybrid molecules of the expected size (Fig. 2). These results indicate that high levels of production and secretion of the hybrid protein can be obtained, even when only a single copy of the gene is inserted in the bacterial chromosome.

Anti-TbpB antibody responses. To evaluate the anti-TbpB antibody responses elicited by i.n. administration of the recom-

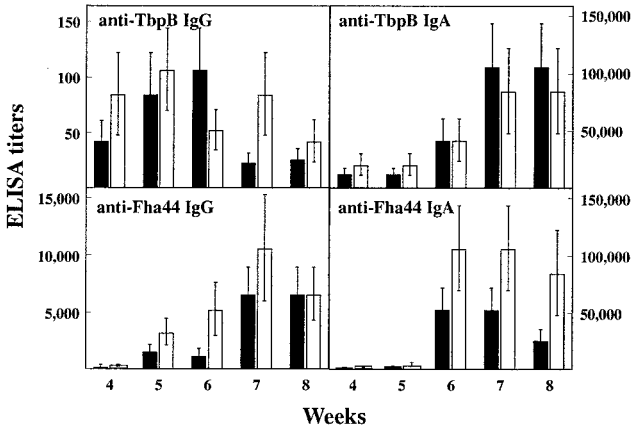

FIG. 3. Anti-TbpB and anti-Fha44 antibody responses in BALF after i.n. administration of *B. pertussis* BPIC3 or BPIC4. Groups of three 4-week-old female OF1 mice were infected i.n. with 5×10^6 CFU of *B. pertussis* BPIC3 (black bars) or BPIC4 (white bars). Infections with similar amounts of *B. pertussis* BPRA or BPSM served as negative controls. At the indicated time points, the mice were sacrificed, BALF were harvested, and anti-TbpB and anti-Fha44 IgG and IgA titers were estimated by ELISA. Results are expressed as means ± standard deviations.

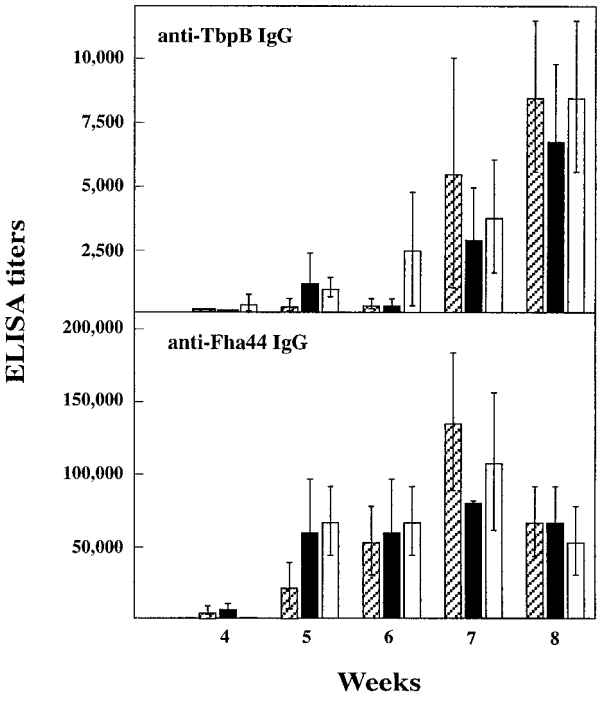

FIG. 4. Anti-TbpB and anti-Fha44 serum antibody responses after i.n. administration of *B. pertussis* BPIC1, BPIC3, or BPIC4. Groups of three 4-week-old female OF1 mice were infected i.n. with 5×10^6 CFU of *B. pertussis* BPIC1 (hatched bars), BPIC3 (black bars), or BPIC4 (white bars). Infections with similar amounts of *B. pertussis* BPRA or BPSM served as negative controls. At the indicated time points, the mice were bled by retro-orbital plexus puncture, and anti-TbpB and anti-Fha44 serum IgG titers were estimated by ELISA. Results are expressed as means ± standard deviations.

binant *B. pertussis* strains, OF1 mice received a single i.n. administration of 5×10^6 CFU of BPIC3 or BPIC4. The colonization kinetics of the recombinant strains, as measured by the numbers of CFU in the lungs, roughly followed those of the parental strains. The PTX-deficient strains (BPRA and BPIC3) were able to colonize but were cleared slightly faster than were the virulent strains BPSM and BPIC4, consistent with previously reported studies (18, 33). Anti-TbpB and anti-Fha44 IgA and IgG responses were analyzed by ELISA in both BALF and sera at different time points. Both anti-TbpB IgG and anti-TbpB IgA, as well as anti-Fha44 IgG and IgA, were readily detected in BALF after a single i.n. administration of BPIC3 or BPIC4 (Fig. 3). Anti-TbpB IgG was detected as early as 4 weeks after administration and declined at week 7, whereas anti-TbpB IgA and the anti-Fha44 antibodies (IgG and IgA) were mostly seen 6 weeks after infection. Attenuation of the *B. pertussis* strain through the deletion of the PTX genes did not appear to influence significantly the antibody responses in BALF.

When serum antibody responses against TbpB and Fha44 were measured, anti-TbpB and anti-Fha44 serum IgG were found to be induced 5 to 7 weeks after a single i.n. administration of either recombinant strain (Fig. 4), whereas no detectable anti-Fha44 or anti-TbpB IgA was found (data not shown).

Bactericidal antibodies against *N. meningitidis* induced by recombinant *B. pertussis*. Complement-mediated bactericidal activity of antibodies is a major defense mechanism against *N. meningitidis* in humans (34). In mice, this activity is associated with antigen-specific IgG2a. We therefore analyzed the isotypic profiles of the anti-TbpB serum antibodies after i.n. administration of BPIC3 and BPIC4. As illustrated in Fig. 5, anti-TbpB IgG1, IgG2a, and IgG2b were induced after infection with either strain. The TbpB-specific IgG2a and IgG2b titers were quite high as soon as 5 weeks after infection, whereas the specific IgG1 titers were low after 5 weeks but increased four- to sevenfold at week 8. Bactericidal titers for antisera were as follows: preimmune serum, <8; anti-TbpB/B16B6 antiserum (hyperimmune serum obtained from rabbits against TbpB isolated from strain B16B6), 512; anti-*B. pertussis* BPSM antiserum, <8; and anti-*B. pertussis* BPIC4 antiserum (collected 8 weeks after i.n. administration of *B. pertussis* BPIC4), 256. These data show that the mouse antisera containing high levels of anti-TbpB/B16B6 IgG2a can kill *N. meningitidis* B16B6 through complement-mediated lysis.

Lack of cross-reactivity among two different TbpBs of different isotypes. TbpB proteins display molecular heterogeneity (22, 29, 41), and on the basis of TbpB cross-reactivity, major isotypes have been identified previously (40). The two main isotypes are represented by strains B16B6 and M982. To assess the specificity of the anti-TbpB antibodies induced after i.n. administration of recombinant *B. pertussis*, the anti-TbpB/B16B6 antibodies were tested by ELISA on TbpB from strain M982. No reactivity was detected. In addition, when mice were infected with *B. pertussis* BPIC1, a strain that produces the *N. meningitidis* M982 TbpB fused to Fha44, anti-TbpB/M982 antibodies were induced with kinetics similar to those induced by BPIC3 and BPIC4 (Fig. 4). However, these antibodies did not recognize TbpB from strain B16B6, demonstrating the specificity of the anti-TbpB antisera.

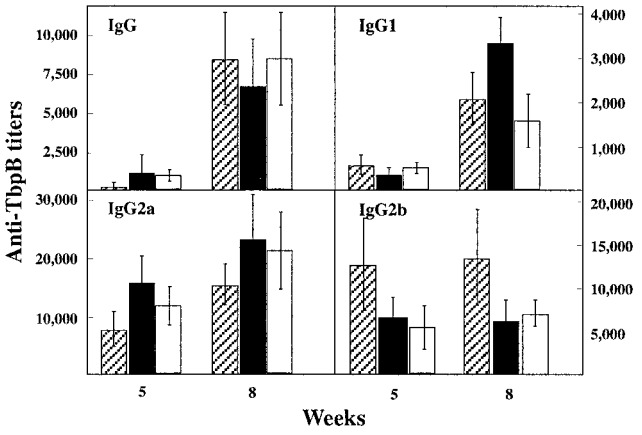

FIG. 5. Anti-TbpB serum IgG isotype profiles elicited after i.n. administration of *B. pertussis* BPIC1, BPIC3, or BPIC4. Groups of three 4-week-old female OF1 mice were infected i.n. with 5×10^6 CFU of *B. pertussis* BPIC1 (hatched bars), BPIC3 (black bars), or BPIC4 (white bars). Five and eight weeks later, the sera were analyzed by ELISA for the presence of specific anti-TbpB IgG, IgG1, IgG2a, and IgG2b isotypes. Results are expressed as means ± standard deviations.

DISCUSSION

The development of efficacious single-dose vaccines to be delivered mucosally constitutes one of the priorities of many health institutions worldwide, including the World Health Organization. Most efforts have so far been devoted to oral delivery of vaccine formulations. However, this route usually requires large quantities of antigen and several administrations to achieve protective immunity, probably due to poor uptake and substantial degradation of the antigens in the proteolytic and acidic environment of the stomach before they can reach the induction sites. The i.n. route may therefore constitute an attractive alternative, perhaps particularly well adapted to induce protective immunity against respiratory pathogens. In this study, we have used genetically engineered *B. pertussis* for the presentation of the protective antigen TbpB of *N. meningitidis* serogroup B to the respiratory mucosa. *N. meningitidis* serogroup B represents one of the major causes of severe childhood meningitis and septicemia, often with a fatal outcome.

TbpB was fused to the N-terminal 80-kDa fragment of FHA, named Fha44, and efficiently secreted into the culture supernatant of recombinant *B. pertussis*. Previous studies have indicated that Fha44 contains the secretion determinants of FHA, and here we demonstrate that it is sufficient for high-level secretion of even quite large (68 to 87 kDa for the two TbpB proteins used here) passenger proteins. In addition, the recombinant Fha44-TbpB hybrid protein was able to bind hTf in an overlay assay, indicating that the TbpB portion is highly structured, which is essential for its interaction with hTf. We have previously shown that disulfide bond formation and the resulting globular fold of passenger proteins may be incompatible with secretion via FHA through the outer membrane of *B. pertussis* (13). If this were a general rule, it would imply that the recombinant TbpB in this study acquires its three-dimensional structure only after it has reached the outer surface of the microorganism. The secretion of FHA requires the presence of an outer membrane accessory protein named FhaC (46). Recent evidence strongly suggests that this protein forms pores in the outer membrane, the size of which may be compatible with the passage of proteins in an extended conformation (17).

A single i.n. administration of the recombinant *B. pertussis* strain elicited the production of anti-TbpB antibodies both in BALF and in the sera of mice. This is in contrast to previous studies with recombinant *B. pertussis* producing the *Schistosoma mansoni* glutathione *S*-transferase (Sm28GST) fused to full-length FHA (38). In these studies, anti-Sm28GST antibodies were found only in the BALF of mice after a single i.n. administration. Anti-Sm28GST serum antibodies could be found only after subsequent boosting with the purified protein (32). However, anti-Sm28GST serum antibodies were readily induced after a single i.n. administration when the hybrid protein was produced by an attenuated *B. pertussis* strain, deficient for PTX production (33), suggesting that the attenuation increased the immunogenicity of the bacteria producing the hybrid protein. In contrast to these observations, we found here that the absence of PTX had no significant effect on the levels of anti-TbpB serum antibodies induced by *B. pertussis* that produce the Fha44-TbpB chimera.

Several reasons might account for this difference. The amounts of Fha44-TbpB produced by *B. pertussis* were substantially higher than those of FHA-Sm28GST. High levels of Fha44-TbpB were readily found in unconcentrated culture supernatants, whereas only minute amounts of FHA-Sm28GST could be found even in highly concentrated culture supernatants (38). In the latter case, the recombinant protein remained essentially cell associated. This may be due to the intrinsic properties of the passenger proteins. Sm28GST is an intracellular protein in *S. mansoni* and has been generally found to be difficult to secrete in heterologous hosts (19), whereas TbpB crosses two membranes in its natural meningococcal environment. In addition, Fha44, the carrier protein to which TbpB was fused, is more efficiently secreted by *B. pertussis* than is FHA, the Sm28GST carrier (37), and it may be worthwhile to compare the two carrier proteins fused to the same passenger protein.

In addition to the levels of heterologous antigen secretion, the nature of the carrier protein (Fha44 or full-length FHA) may also conceivably affect the immune responses with respect to the influence of PTX. FHA is the major adhesin of *B. pertussis* (for a review, see reference 26). It contains at least three distinct binding sites, two of which are missing in Fha44. A carbohydrate-binding site is located in the C-terminal half of FHA and is believed to interact with glycoprotein ligands at the surface of ciliated epithelial cells (35). Full-length FHA, but not Fha44, also contains an Arg-Gly-Asp site known to be involved in the interaction of *B. pertussis* with alveolar macrophages via binding to the β_2 integrin CR3 (36). Using CR3 as the port of entry into macrophages results in increased survival of *B. pertussis* within these cells, which very likely influences antigen presentation by macrophages. Recent observations indicate that the action of PTX has an impact on antigen presentation by professional phagocytes (unpublished observations). PTX catalyzes ADP-ribosylation of Giα proteins (44) involved in signal transduction, some of which play a major role in antigen presentation by macrophages. A link between the interaction of FHA with CR3 via the Arg-Gly-Asp site and the PTX-mediated ADP-ribosylation of macrophage Giα may

perhaps explain the differences in immune responses observed between passenger proteins fused to Fha44 and those fused to FHA. These hypotheses warrant further investigation.

The anti-TbpB serum antibody isotypes induced by the recombinant *B. pertussis* strains were mixed, including IgG1, IgG2a, and IgG2b. These antibodies also expressed bactericidal activity against *N. meningitidis*, suggesting that i.n. administration with the TbpB-producing *B. pertussis* may result in protective immunity, since the major antimeningococcal protective mechanism in children is based on the presence of bactericidal antibodies (34).

TbpB is present in all clinical isolates of all meningococcal serogroups. It is even present in other neisserial species, such as *Neisseria gonorrhoeae* (8). Therefore, anti-TbpB vaccines may be widely cross-protective among all the serogroups. However, considerable antigenic and sequence heterogeneity among meningococcal TbpBs has been described elsewhere (22, 23, 29, 41, 42), and different TbpBs possess both strain-specific and cross-reactive epitopes. Based on the presence of mutually exclusive epitopes, the TbpB proteins can be divided into two major isotypes, I and II, represented by the B16B6 TbpB and the M982 TbpB, respectively. Polyclonal anti-TbpB antibodies cross-react with TbpB proteins within each family but not between the two families (40). As shown here, this is also the case when the two proteins are administered i.n. by recombinant *B. pertussis*. To provide protection against the full array of meningococcal strains, at least two *B. pertussis* strains may therefore have to be administered simultaneously, or preferably, they may have to be produced within the same strain. However, the strain specificity and the cross-reactivity of TbpB epitopes appear to be species dependent. Although mice generate essentially strain-specific anti-TbpB antibodies, patients recovering from natural infection produce cross-reactive anti-TbpB antibodies, as evidenced by immunoblot analyses (3, 12). It will therefore be interesting to investigate whether recombinant *B. pertussis* producing only one TbpB protein induces cross-reactive immunity against all *N. meningitidis* serogroups after a single i.n. administration in humans. However, before human studies can be initiated, the recombinant *B. pertussis* vectors have to be properly attenuated. Although the deletion of the *ptx* genes has been shown to strongly attenuate *B. pertussis* in the mouse model (18, 33), work to further attenuate *B. pertussis* is ongoing.

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