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Tetanus toxin fragment C-specific priming by intranasal infection with recombinant *Bordetella pertussis*

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Abstract

As an alternative to parenteral administration, mucosal administration offers several advantages including the ease of administration, safety and the ability to induce mucosal immunity. As a first step towards nasal administration of important childhood vaccines, we have previously developed attenuated *Bordetella pertussis* strains able to protect mice against pertussis upon nasal vaccination. Since pertussis vaccines are generally combined with tetanus and diphtheria vaccines, we constructed recombinant *B. pertussis* strains producing the non-toxic protective tetanus toxin fragment C (TTFC). TTFC was genetically fused to the N-terminal domain of the *B. pertussis* filamentous haemagglutinin. The hybrid gene was introduced into *B. pertussis* both on a multi-copy replicative plasmid and as a single copy inserted into the chromosome of a pertussis toxin-producing strain and a toxin-deficient attenuated strain. The hybrid protein was secreted by the recombinant strains. However, the recombinant multi-copy plasmid was unstable in vivo, and immunisation could only be carried out with the strains containing the single-copy chromosomal integration. Both the toxin-producing and the toxin-deficient recombinant *B. pertussis* strains were able to prime mice for the production of anti-TTFC serum antibodies upon intranasal administration, suggesting the feasibility of using recombinant attenuated *B. pertussis* for the development of combined childhood vaccines. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Tetanus toxin fragment C; Recombinant *Bordetella pertussis*; Intranasal vaccination

1. Introduction

Mucosal surfaces are the entry sites of most pathogenic micro-organisms responsible for respiratory, digestive or urogenital infections. Therefore, inducing, or at least priming, immune responses at mucosal surfaces seems to be a logical thing to do. Nevertheless, most currently available vaccines are administered by parenteral routes, although mucosal vaccination offers several advantages over parenteral vaccination. Due to the common mucosal immune system, immune cells stimulated at one mucosal surface can disseminate to other mucosal sites [1]. In addition, mucosal immunisation can also induce high systemic immune responses. Mucosal vaccines are easy to administer to large populations, reduce the need for trained personnel and eliminate the risk of contamination through the use of needles required for parenteral vaccination.

However, most antigens given by mucosal routes induce at best low immune responses due to their rapid degradation, especially in the gastro-intestinal tract, and due to the induction of mucosal tolerance [2]. Efficient adjuvants are, therefore, needed to enhance the immunogenicity of mucosally delivered antigens. The most powerful mucosal adjuvants studied so far are bacterial enterotoxins, such as cholera toxin or heat-labile enterotoxin of *Escherichia coli* [3]. However, despite considerable efforts to dissociate toxicity from adjuvanticity through site-directed alterations of these toxins, there is still concern about residual toxic activities, especially when these adjuvants are given by the nasal route [4].

The use of live microbial vaccine carriers, especially attenuated pathogens, constitutes an attractive alternative. Many pathogenic micro-organisms are known to colonise mucosal surfaces of the host efficiently and to induce strong immune responses. These properties have been exploited to engineer several bacterial species into potentially useful vaccine carriers for mucosal immunisation (for a review see [5]). Numerous studies using diverse antigens in various animal models have shown that the intranasal route seems

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to be particularly advantageous [6]. Antigens delivered intranasally avoid the proteolytic and acidic environment of the stomach which is encountered by orally-administered antigens. The respiratory tract is less colonised by commensal micro-organisms than the gut, thereby decreasing interference with vaccine strain up-take through ecological competition.

However, compared to live vectors adapted for the gastro-intestinal tract, relatively few vectors have been developed that are especially adapted for the respiratory tract [7]. We have recently assessed the potential of *Bordetella pertussis* to be used for the intranasal delivery of protective antigens. *B. pertussis* is the etiological agent of whooping cough, is very contagious and has long been known to elicit both strong humoral [8] and cellular [9] immune responses in humans. Heterologous antigens have been produced in *B. pertussis* using a system in which the *B. pertussis* adhesin filamentous haemagglutinin (FHA) serves to carry the passenger antigens to the bacterial surface [10] or into the extracellular milieu [11]. In addition, attenuated strains of *B. pertussis*, lacking pertussis toxin, a major virulence factor of the pathogen [12], have been found to induce strong protection against pertussis when given by the nasal route in a single dose [13]. Current efforts in our laboratory are devoted to further attenuate *B. pertussis* by altering the genes responsible for the production of the other *B. pertussis* toxins.

Since pertussis vaccines are generally given in combination with at least tetanus and diphtheria vaccines, we wanted to investigate whether a recombinant *B. pertussis* strain could be engineered to express tetanus toxin fragment C (TTFC), the immunogenic, non-toxic portion of tetanus toxin, which constitutes the protective antigen against tetanus. We, therefore, fused TTFC to the secretion determinant of FHA as a hybrid protein produced in recombinant *B. pertussis*. Here, we show that intranasal administration of this recombinant strain efficiently primes mice for the induction of anti-TTFC serum antibodies when they were subsequently boosted with purified, non-adjuvanted TTFC by the nasal route.

2. Materials and methods

2.1. 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

Table 1
Strains and plasmids

Strain or plasmid	Relevant features	Reference or source
Strains		
B. pertussis		
BPSM	Sm^r Nal^r Tohama I derivative	[36]
BPGR4	BPSM derivative; 10 kb *Eco*RI deletion of *fhaB*	[28]
BPRA	BPSM derivative; deletion of the gene encoding pertussis toxin	[37]
BPNR23	BPSM derivative with the gene encoding TTFC fused to *fha44*	This work
BPNR19	BPRA derivative with the gene encoding TTFC fused to *fha44*	This work
BPSA89	BPSM derivative with *fha44* instead of *fhaB*	[Alonso et al., unpublished data]
E. coli		
XL1-Blue	F′::Tn10 *pro*A^+ B^+ *lac*I^q delta (*lac*Z)*M15*/*recA1 endA1 gyrA96* (Nalr) *thi hsdR17* (rk^- mK^+) *supE44 relA1 lac*	Stratagene
MC1061	F^- *araD*139 delta(*ara–leu*)7696 *galE*15 *galK*16 delta(*lac*)X74 *rps*L (Strr) *hsdR2*(rk^- mk^+) *mcrA mcrB1*	ATCC
S17-1	*pro, hdsR, res, mod, recA*, RP4-2Tc::Mu, Km::Tn7	[21]
Plasmids		
Cloning vectors		
pAS32	2835 bp *Eco*RI–*Bam*HI and 1075 bp *Bam*HI–*Bgl*II fragments of mature FHA gene in pBR328. Ap^r	[Alonso et al., unpublished data]
pJQ200mp18-rpsl	Suicide vector for *B. pertussis*. Gm^r	[38]
pMEC118	1494 bp *Bam*HI–*Bam*HI fragment of pMEC68 coding for TTFC in pAS32. Ap^r	This work
pMEC125	4106 bp *Sal*I–*Sal*I fragment of pMEC118 in pJQ200mp18-rpsl; suicide vector for *B. pertussis*. Gm^r	This work
Expression vectors		
pBG4	2835 bp *Eco*RI–*Bam*HI fragment containing the 5′ end of *fhaB* (*fha44*) in pBBR122. Kan^r	[26]
pMEC4	1368 bp *Nco*I–*Hin*dIII fragment coding for TTFC in Pgit032. Ap^r	[Reveneau et al., unpublished data]
pMEC68	1494 bp *Bam*HI–*Bam*HI fragment coding for TTFC in pBG4, leading to a *fha44*::*ttfC* fusion gene. Kan^r	This work

grown at 36°C on Bordet Gengou agar [14] supplemented with 1% glycerol and 20% defribrinated sheep blood (BG) or in modified Stainer–Scholte medium containing 2,6-*O*-dimethyl-β-cyclodextrin at 1 g/l [15,16]. Antibiotics were used at the following concentrations: kanamycin, 25 μg/ml; streptomycin, 100 μg/ml; gentamycin, 10 μg/ml. Recombinant *E. coli* was selected with kanamycin (25 μg/ml), ampicillin (100 μg/ml) or gentamycin (25 μg/ml). All DNA manipulations were carried out in *E. coli* XL1-Blue as described by Sambrook et al. [17].

2.2. Construction of recombinant B. pertussis strains

To obtain pMEC68, a 1494 bp fragment coding for TTFC and terminating with a stop codon was amplified by PCR from pMEC4 (Table 1) using the oligonucleotides 5′-GCGGATCCGCATTTTCTTATTCTAAAAATCTGG-3′ and 5′-GCGGATCCGGCACCTACTGGTAAAACGG-3′, digested with *Bam*HI and cloned into the *Bam*HI site of pBG4 (Table 1). This fragment contains 1368 bp corresponding to the TTFC-encoding gene and additional 126 bp vector-derived DNA at the 3′ end of the TTFC-encoding DNA. The *Bam*HI site of pBG4 is located at the 3′ end of *fha44*. The resulting plasmid, pMEC68, was transferred by electroporation into *B. pertussis* BPGR4, a strain lacking *fhaB*, the structural gene of FHA. The *B. pertussis* transformants were selected on BG agar medium containing kanamycin and screened by PCR on colonies, as described in [18]. The presence and the integrity of the constructs introduced into *B. pertussis* were determined by retro-electroporation into *E. coli* MC1061 [19], restriction analyses and DNA sequencing.

B. pertussis BPNR19 and BPNR23 were obtained by double homologous recombination as described by Stibitz [20]. To construct these strains, the 1846 bp *Bam*HI fragment of *fhaB* in pAS32 was first replaced by the above described PCR fragment digested with *Bam*HI to yield pMEC118. A 2835 bp *Sal*I fragment was then isolated from pMEC118 and cloned into the *Sal*I site of pJQ200mp18-rpsl. The resulting plasmid harboring a fragment coding for TTFC flanked by two regions of *fhaB*, was named pMEC125 and introduced into *E. coli* S17-1 [21]. This strain was then conjugated with *B. pertussis* BPRA or BPSM to allow for the insertion of the TTFC-encoding construct into the *fhaB* locus. The first recombination event was selected on BG agar containing gentamycin, and the second recombination event was selected on streptomycin. The streptomycin-resistant, gentamycin-sensitive BPRA and BPSM cells, named BPNR19 and BPNR23, respectively, were screened by immunoblotting for the production of a Fha44–TTFC chimera and the absence of the mature FHA.

2.3. Protein extraction and immunoblotting

For the detection of the Fha44–TTFC hybrid proteins, the culture supernatants of the *B. pertussis* strains were concentrated by precipitation with trichloroacetic acid (6% final concentration) and 0.4 mM deoxycholate. The amounts of total proteins present in the extracts were determined using a Bradford assay [22] with bovin gamma globulin as a standard, and the proteins were subjected to SDS-PAGE [23] using a 10% polyacrylamide gel. Proteins were transferred onto nitrocellulose membranes by electroblotting. The blots were probed with goat anti-TTFC polyclonal serum diluted at 1:100 (kindly provided by Innogenetics, Ghent, Belgium) or anti-Fha44 IgY [24] and developed using alkaline phosphatase-conjugated secondary antibodies (Promega, Madison, WI, USA) diluted at 1:7000 or 1:5000.

2.4. B. pertussis colonization of the mouse respiratory tract

The *B. pertussis* strains were grown for 48 h at 37°C on BG agar supplemented with streptomycin. The bacteria were then scraped off the plates and resuspended at 2.5×10^8 CFU/ml in sterile phosphate-buffer saline (PBS). Groups of 24, 4-week-old OF1 mice (Iffa Credo, L'Arbesle, France) were anesthetised with 100 μl of a cocktail [25] containing 20% Imalgene 1000 (Merial, Lyon, France), 0.5 mg of valium per ml (Roche, Neuilly-sur-Seine, France) and 62.5 μg of atropine per ml (Aguettant Laboratory, France) in PBS. Bacterial suspension (20 μl) (corresponding to 5×10^6 CFU) was then instilled into the nostrils of the mice. Infected mice were sacrificed by cervical dislocation 3 h after intranasal infection or at the indicated time points. Four mice were analysed per time point. The lungs of the sacrificed mice were removed aseptically and homogenised individually in PBS as described in [10]. Serial dilutions of the lung homogenates were then plated onto BG agar supplemented with streptomycin, and CFUs were counted after 3–5 days of incubation at 37°C.

2.5. Serum antibody determination

Serum samples were taken by retro-orbital plexus puncture and were stored at −20°C until assayed for the presence of specific antibodies. Bronchoalveolar lavage fluids were harvested as described in [10]. The levels of Fha44- and TTFC-specific antibodies in sera and bronchoalveolar lavage fluids were determined by enzyme-linked immunoabsorbent assays (ELISAs). Microtiter plates Immulon III (Dynatech Laboratories, Mclean, VA, USA) were coated for 1 h at 37°C with 100 μl per well of 2 μg/ml recombinant TTFC (rTTFC, Boehringer Mannheim, Germany) and then incubated overnight at 4°C. rTTFC was diluted in 0.1 M carbonate/bicarbonate buffer (pH 9.5). After one wash with PBS containing 0.1% Tween 20, the plates were incubated with 3% bovine serum albumin (BSA; Sigma, St. Louis, MO, USA) in PBS for 1 h at 37°C. Samples were diluted by two-fold serial dilution starting from 1:50 (for sera) or 1:2 (for bronchoalveolar lavage fluids) in PBS containing 1% BSA. The plates were then incubated at room temperature for 2 h and washed three times with PBS containing

0.1% Tween before the addition of biotin-labelled goat anti-mouse IgG (Southern Biotechnology, Birmingham, AL, USA) diluted in PBS containing 1% BSA and 0.1% Tween. Anti-mouse IgG was used at a 1:10,000 dilution. After incubation for 1 h at room temperature and three washes in PBS containing 0.1% Tween, the streptavidin–horseradish peroxidase conjugate (Amersham, Buckinghamshire, UK) was added at a 1:2000 dilution in PBS containing 0.1% Tween for 30 min at room temperature. A volume of 100 μl of 0.2 M Na_2HPO_4, 0.1 M citrate buffer (pH 5.5) containing 0.2% H_2O_2 and 1 mg/ml of *o*-phenylenediamine (Sigma, St. Louis, MO, USA) was added to each well, and the plates were incubated at 37°C for 30 min. The reaction was stopped by the addition of 50 μl of 2N HCl. The absorbance was measured at 490 nm with an Elx800GUV automated microplate reader (Bio-Tek Instruments, Inc., Vinooski, VT). End-point titers were defined as the highest dilution that gave an absorbency three times higher than background for serum samples, using the Kc4 program (Kinetical for windows-Bio-Tek Instruments, Inc., Vinooski, VT). The results are expressed as means ± S.E.M. The levels of anti-Fha44 antibodies were determined by ELISA using purified Fha44, as described by Alonso et al. [unpublished results].

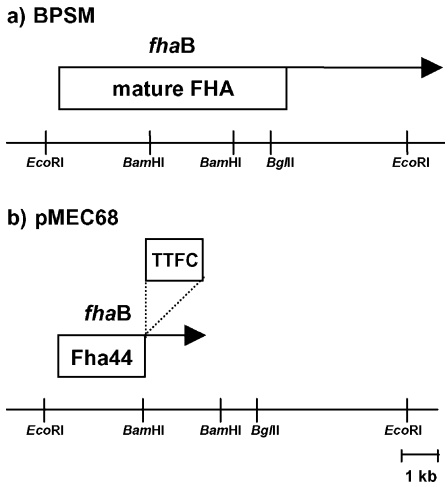

Fig. 1. Physical map of the *fhaB* locus in BPSM and in BPGR4(pMEC68). The arrows represent the lengths and directions of the open reading frames. The relevant restriction sites of this locus are shown in the top line. (a) BPSM contains the wild-type *fhaB* locus coding for a 370 kDa precursor protein that includes the 230 kDa portion of the mature form indicated by the open box. (b) pMEC68 contains the hybrid gene encoding Fha44–TTFC, as indicated by the open boxes labelled Fha44 and TTFC, respectively.

3. Results

3.1. Expression of the chimeric fha44–ttfC gene in B. pertussis

To express the *ttfC* gene in *B. pertussis*, the export machinery of FHA was used in order to produce the recombinant protein in a secreted form. Since Fha44, an 80 kDa N-terminal fragment of FHA, contains the complete secretion determinant and is even better secreted by *B. pertussis* than full-length FHA [26], TTFC was fused to Fha44 in *B. pertussis* BPGR4(pMEC68). The genetic construct contained the sequence coding for the 792 first amino acids of FHA followed by the sequence encoding the 456 residues of TTFC and the termination codon (Fig. 1). This construct was engineered into a replicative plasmid, based on the pBBR1 replicon [27], which was then introduced into *B. pertussis* BPGR4, a strain from which the chromosomal *fhaB* gene had been deleted [28].

The recombinant bacteria were grown to late exponential phase, and 20-fold concentrated culture supernatants were analysed by immunoblotting using anti-Fha44 and anti-TTFC polyclonal antibodies. As shown in Fig. 2, the anti-TTFC antibodies reacted with an approximately 85 kDa protein (Fig. 2B, lane 4), not present in the culture supernatants of non-recombinant *B. pertussis* (not shown) or *B. pertussis* BPGR4(pBG4) which produces Fha44 (Fig. 2B, lane 3). The anti-Fha44 antibodies recognise several proteins in the supernatants of both BPGR4(pBG4) and BPGR4(pMEC68). The two major bands detected by the anti-Fha44 antibodies in the supernatants of the BPGR4(pBG4) cultures were also detected in the BPGR4(pMEC68) culture supernatants (Fig. 2A). The band at 65 kDa corresponds to a typical breakdown product of Fha44 [26]. In addition, the BPGR4(pMEC68) supernatant also contained a 85 kDa protein recognised by the anti-Fha44 antibodies (Fig. 2A, lane 2). This protein corresponds to that recognised by the anti-TTFC antibodies (Fig. 2B, lane 4), indicating that BPGR4(pMEC68) produced and secreted a chimeric protein containing both Fha44 and TTFC epitopes. However, the apparent size of the 85 kDa chimera was smaller than expected (130 kDa), indicating that the protein had undergone proteolytic breakdown. Since the two lower anti-Fha44 reactive bands were not recognised by the anti-TTFC antibodies, they most

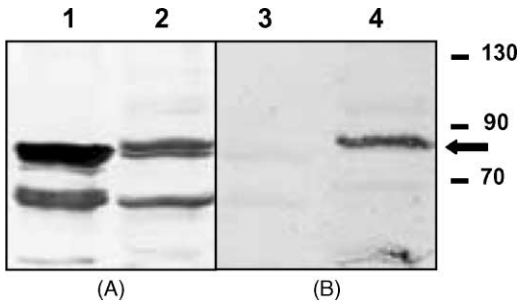

Fig. 2. Immunoblot analysis of the Fha44–TTFC chimera produced by *B. pertussis* containing pMEC68. Concentrated culture supernatants of BPGR4(pBG4) (lanes 1 and 3) or BPGR4(pMEC68) (lanes 2 and 4) were analysed by 10% SDS-PAGE and immunoblotting using anti-Fha44 IgY (A) or anti-TTFC polyclonal antiserum (B). Approximately 20 μg of total protein was loaded in each lane. The sizes of the molecular mass markers are given in kDa in the right margin.

likely correspond to molecules that had lost the TTFC part. In contrast, the upper, 85 kDa band was recognised by both anti-Fha44 and anti-TTFC antibodies, indicating that the protein corresponding to that band was truncated at either or both sides. Occasionally, additional smaller anti-TTFC reactive proteins could be detected in the BPGR4(pMEC68) culture supernatants (not shown), confirming proteolytic cleavage.

Together, these results indicate that Fha44 can be used to carry heterologous proteins such as TTFC into the culture supernatants. However, upon intranasal infection, recombinant pBBR1 derivatives are rapidly lost, and therefore, the immune response against Fha44 and TTFC could not be determined after infection with the recombinant *B. pertussis* strain. To stabilise the genetic constructs, they were, thus, inserted into the chromosome by homologous recombination at the *fhaB* locus, yielding strains BPNR19 and BPNR23. These two strains differ from each other by the production of pertussis toxin, as BPNR19 was derived from a pertussis toxin-deficient strain, whereas BPNR23 was derived from a toxin-producing wild-type strain.

Immunoblot analyses of the two strains indicated that both BPNR19 (Fig. 3) and BPNR23 (not shown) produced and secreted proteins recognised by the anti-Fha44 antibodies (Fig. 3A, lane 2), and that the supernatants of both strains contained a 85 kDa major protein recognised by the anti-TTFC antibodies (Fig. 3B, lane 4). However, as in the case of BPGR4(pMEC68) an anti-TTFC antibody reactive protein of the expected size (130 kDa) could not be detected.

3.2. Lung colonisation by recombinant B. pertussis

To study the ability of BPNR23 and BPNR19 to colonise the respiratory tract, OF1 mice were infected intranasally

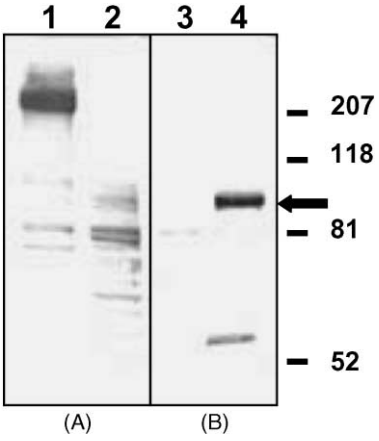

Fig. 3. Immunoblot analysis of the Fha44–TTFC chimera produced by *B. pertussis* BPNR19. Concentrated culture supernatants of BPRA (lanes 1 and 3) or BPNR19 (lanes 2 and 4) were analysed by 10% SDS-PAGE and immunoblotting using anti-Fha44 IgY (A) or anti-TTFC polyclonal antiserum (B). Approximately 20 μg of total protein was loaded in each lane. The sizes of the molecular mass markers are given in kDa in the right margin.

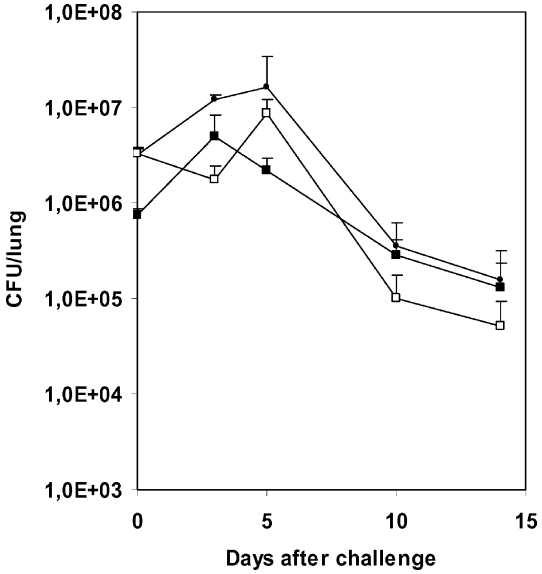

Fig. 4. Colonisation by *B. pertussis*. OF1 mice were infected intranasally with 5×10^6 CFU of *B. pertussis* BPNR23 (solid circles), BPNR19 (open squares) and BPSA89 (solid squares). The lungs were harvested at indicated time points, and total CFUs were counted after plating serial dilutions of the lung homogenates onto BG agar.

with a suspension containing 5×10^6 CFU of either strain or of BPSA89, a strain producing Fha44 instead of FHA. The total CFUs in the lung homogenates were measured 3 h, and 3, 5, 10 and 14 days after infection. As shown in Fig. 4, the number of CFUs in the lungs of mice infected with BPNR23 or BPNR19 increased during the first 5 days and decreased slowly over the next 10 days.

3.3. TTFC-specific priming after infection with recombinant B. pertussis

Mice were infected by the nasal route with 5×10^6 CFU of BPNR19, BPNR23 or BPSA89. Control mice received 20 μl PBS by the nasal route. Three months later, the mice received a second identical administration. After an additional 3 months, the mice were boosted intranasally with 2 μg of purified TTFC. Anti-Fha44 and anti-TTFC antibodies in sera were measured over time, after the first and second administrations and after the boost with purified TTFC. No anti-Fha44 IgG response was detected in mice infected with either *B. pertussis* strain, neither before nor after the TTFC boost (data not shown).

No anti-TTFC antibodies were detected in mice infected with BPSA89 with or without TTFC boost, nor in the control mice that had received PBS and were subsequently boosted with TTFC (Fig. 5). In contrast, significant amounts of anti-TTFC antibodies were detected in the sera of mice that were primed with either BPNR19 or BPNR23 and subsequently boosted with TTFC. These antibodies were only detected after the TTFC boost, indicating that neither BPNR19, nor BPNR23 induced a detectable

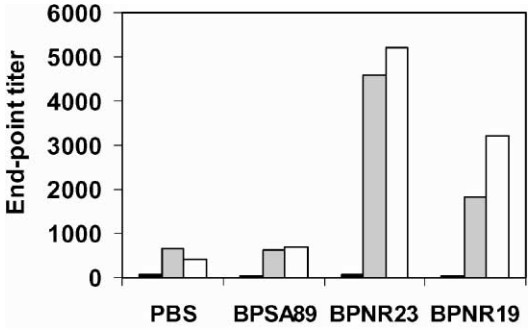

Fig. 5. Serum anti-TTFC antibody responses following intranasal administration with recombinant *B. pertussis* and intranasal boost with TTFC. OF1 mice were infected intranasally with 5×10^6 CFU of *B. pertussis* BPNR23, BPNR19 or BPSA89. Control mice received PBS. Three months later, the mice received a second identical administration. Each group was intranasally boosted with 2 μg of purified TTFC. Sera of five mice in each group were collected 1 week after the second administration (solid bars), and 1 (grey bars) and 2 weeks (white bars) after TTFC boost and pooled before antibody detection.

primary response, but that both were able to efficiently prime the mice by the nasal route. The mice primed with BPNR23 produced a slightly higher anti-TTFC antibody response than mice infected with BPNR19, indicating that, in contrast to previous studies using full-length FHA instead of Fha44 to display heterologous antigens [13], the presence of pertussis toxin did not appear to diminish the immune response upon infection with the recombinant *B. pertussis*. None of the mice produced detectable anti-TTFC or anti-Fha44 antibodies in the bronchoalveolar lavage fluids.

4. Discussion

Childhood vaccination would benefit tremendously from the possibility to immunise mucosally. It would certainly increase the compliance, because non-invasive immunisation is less painful than injection and because it is associated with less risk of contamination occasionally encountered through the use of needles. In addition, the ease of mucosal vaccination over parenteral immunisation makes it less dependent on trained medical personnel. One of the most widely used childhood vaccines is the diphtheria–tetanus–pertussis (DTP) vaccine. Full immunisation against these three diseases requires several vaccine doses and boosters. In addition, this vaccine has met with typical problems of compliance over the last 20 years.

In order to address some of these problems, we have initiated efforts to develop novel DTP vaccines that can be administered by the nasal route. This approach is based on the use of live attenuated *B. pertussis* that we have previously shown to provide protection against challenge after a single intranasal administration [13]. As an initial attempt to add the tetanus valence to the attenuated live pertussis vaccine, we evaluated here the potential of the N-terminal 80 kDa fragment of FHA, named Fha44, to target TTFC into the supernatant of *B. pertussis*, in order to induce an immune response against TTFC after intranasal administration of the recombinant *B. pertussis* strain. Although infection with the recombinant strain did not induce detectable titers of anti-TTFC antibodies in the serum, it was able to prime mice for a serum response against TTFC after an intranasal boost with the purified protein.

Since vaccination should at least induce priming, such that a secondary immune response is rapidly mounted as soon as the actual pathogen is encountered, these results constitute a promising first step towards the development of a novel nasal DTP vaccine. However, since tetanus toxin is in itself a potent antigen, there is certainly room for improvement. Compared to other antigens [11], the fusion of TTFC to Fha44 resulted in rather low levels of extracellular TTFC production, especially when the hybrid gene was introduced as a single copy into the *B. pertussis* chromosome. The level of expression was somewhat higher when the hybrid gene was expressed from a multi-copy plasmid, such as pMEC68. However, due to instabilities of this plasmid in the absence of selective pressure, the immune response against TTFC could not be assessed after immunisation with a *B. pertussis* strain containing pMEC68. In addition to low levels of expression, the chimeric Fha44–TTFC protein was rather unstable as it was secreted into the culture supernatant. Whether the hybrid gene was expressed from a multi-copy plasmid or from a single copy in the chromosome, most of the anti-TTFC reactive proteins were smaller than expected from the calculated molecular weight.

It is possible that some of these problems are related to the mechanism of secretion by the FHA secretory machinery. Although proteins larger than TTFC have been efficiently secreted through the FHA secretion pathway [11], and the size may, thus, not be a problem, proteins containing disulfide bonds have recently been shown to be difficult to secrete [29]. The FHA secretion mechanism requires an outer membrane accessory protein named FhaC [30], which is believed to form pores in the outer membrane allowing the secretory protein to reach the outer surface of *B. pertussis* [31]. However, secretion of FHA through FhaC most likely occurs in an extended conformation, and the addition of a globular domain, such as the B subunit of cholera toxin, to Fha44 hampers secretion [29]. Disruption of the cholera toxin B subunit disulfide bond improved secretion. It is, therefore, likely that the globular structure of TTFC [32] containing several cysteines, some of which being involved in disulfide bond formation, strongly interferes with secretion. The mutational alterations of the cysteines in TTFC or the use of *B. pertussis dsbA* mutants deficient in their ability to catalyse disulfide bonds may improve the secretion of the Fha44–TTFC chimera, but will most likely destroy the protective epitopes of TTFC. Future research will, therefore, be directed towards the identification of alternative ways to produce TTFC in attenuated *B. pertussis*, hoping that higher

levels of production will lead to stronger immune responses after intranasal administration.

The absence of serum antibodies against TTFC after a primary immunisation with the recombinant *B. pertussis* strain is reminiscent of previous results obtained with a recombinant *B. pertussis* strain producing the *Schistosoma mansoni* Sm28GST antigen fused to FHA [10]. Similar to the results described here, intranasal administration with Sm28GST-producing *B. pertussis* did not induce serum antibodies against the passenger protein, but was able to prime mice against the foreign antigen [33]. However, in contrast to what is observed here, the deletion of the pertussis toxin gene from the Sm28GST-producing *B. pertussis* increased the immunogenicity of the foreign antigen to an extent that a single intranasal immunisation with the attenuated recombinant *B. pertussis* strain resulted in high levels of serum antibodies against Sm28GST and in protection against parasite challenge [13]. Deletion of the pertussis toxin gene from the TTFC-producing *B. pertussis* strain did not result in a detectable increase in the anti-TTFC immune response. One important difference between the two types of constructs lies in the carrier protein. Whereas Sm28GST was fused to full-length FHA, TTFC was fused to Fha44, the truncated N-terminal portion of FHA. Although Fha44 contains the entire secretion determinant and is secreted even better than full-length FHA [26], important functional sites present in FHA are missing in Fha44 [34]. It is possible that some of these functional sites are involved in the immunogenicity of FHA and proteins fused to FHA, although at present it is difficult to speculate which additional structure of FHA might be responsible for the difference in immunogenicity of the passenger protein. We have previously described that full-length FHA expresses adjuvant activity for antigens that are co-delivered with it by the intranasal route [35]. In addition, we found recently that Fha44, when produced by *B. pertussis* instead of full-length FHA, totally lacks immunogenicity upon intranasal infection of the corresponding *B. pertussis* strain [Alonso et al., unpublished data], confirming that the two molecules differ substantially in their immunogenic properties. Future studies will certainly address this important issue. We hope that this will shed new light on the mechanism of interaction of *B. pertussis* with the immune system of the host and ultimately help to optimise attenuated *B. pertussis* for the presentation of protective heterologous antigens.

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