INFECTION AND IMMUNITY, July 2004, p. 4052–4060 Vol. 72, No. 7
0019-9567/04/$08.00+0 DOI: 10.1128/IAI.72.7.4052–4060.2004

Mucosal Vaccination against Serogroup B Meningococci: Induction of Bactericidal Antibodies and Cellular Immunity following Intranasal Immunization with NadA of *Neisseria meningitidis* and Mutants of *Escherichia coli* Heat-Labile Enterotoxin

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Received 22 December 2003/Returned for modification 17 February 2004/Accepted 25 March 2004

Conjugated polysaccharide vaccines protect against serogroup C meningococci. However, this approach cannot be applied to serogroup B, which is still a major cause of meningitis. We evaluated the immunogenicity of three surface-exposed proteins from serogroup B *Neisseria meningitidis* (App, NhhA, and NadA) identified during whole-genome sequencing. Mice were immunized intranasally with individual proteins in the presence of wild-type *Escherichia coli* heat-labile enterotoxin (LTwt), LTR72, a partially inactivated mutant, or LTK63, a completely nontoxic mutant, as the adjuvant. Each of the meningococcal proteins induced significant cellular responses; NhhA and NadA induced strong antibody responses, but only NadA induced bactericidal antibody when administered intranasally with mucosal adjuvants. In addition, immunoglobulin A and bactericidal antibodies were detected in the respiratory tract following intranasal delivery of NadA. Analysis of antigen-specific cytokine production by T cells from immunized mice revealed that intranasal immunization with NadA alone failed to generate detectable cellular immune responses. In contrast, LTK63, LTR72, and LTwt significantly augmented NadA-specific gamma interferon, interleukin-4 (IL-4), IL-5, and IL-10 production by spleen and lymph node cells, suggesting that both Th1 and Th2 cells were induced in vivo. The strongest cellular responses and highest bactericidal antibody titers were generated with LTR72 as the adjuvant. These findings demonstrate that the quality and magnitude of the immune responses generated by mucosal vaccines are influenced by the antigen as well as the adjuvant and suggest that nasal delivery of NadA with mucosal adjuvants has considerable potential in the development of a mucosal vaccine against serogroup B meningococci.

Neisseria meningitidis is a major causative agent of bacterial meningitis and fatal septicemia. Infants, young children, and adolescents are most susceptible to infection. Mortality rates among infected individuals are high (around 10%), and death can result in hours, despite treatment with appropriate antibiotics. Furthermore, up to 25% of survivors suffer from neurological sequelae. Five major pathogenic serogroups of *N. meningitidis* have been identified based on the chemical composition of the bacterial capsule (A, B, C, Y, and W135). Capsular polysaccharide vaccines are available against four serogroups (A, C, Y, and W135). Although these are effective in adults, protection is short-lived and they have very little efficacy in children under 18 months of age (32). In late 1999, a second-generation glycoconjugate vaccine was introduced against serogroup C. This vaccine is highly efficacious in all age groups including infants, and since its introduction there has been a 90% decrease in cases of serogroup C disease (27). Similar vaccines against the A, Y, and W135 serogroups are under development (20).

There is currently no licensed commercial vaccine against serogroup B meningococci available in Europe or the United States. Strains from this serogroup are responsible for most cases of *N. meningitidis* infection in Europe, around a third of cases in the United States, and about half of the meningococcal infections found elsewhere in the world (with the exception of sub-Saharan Africa, where serogroup A strains cause more than 90% of meningococcal infections) (5, 13, 28). Vaccines against serogroup B strains have proved difficult to develop. The polysaccharide antigen is poorly immunogenic in humans since it mimics a widely distributed human carbohydrate [$\alpha(2\rightarrow8)$*N*-acetyl neuraminic acid] (4), and attempts to improve its immunogenicity could lead to autoimmunity. Vaccines based on outer membrane vesicles, which contain bacterial surface proteins, have met with some success. Clinical trials have revealed that vaccinated individuals produce bactericidal antibodies, which protect against infection with homologous meningococcal strains, but since *N. meningitidis* species are subject to antigenic variation, they offer no protection against infection with heterologous strains (26). The challenge therefore is to identify novel antigens which are highly conserved

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across a range of virulent group B strains and are capable of inducing bactericidal antibodies, a correlate of protection against *N. meningitidis* (2).

The complete genome sequence of a serogroup B strain of *N. meningitidis* has recently been determined (35). During the course of this work, unassembled fragments of the genome were analyzed to identify novel proteins which were potentially surface exposed or secreted. These proteins were then expressed in *Escherichia coli*, and the purified proteins were screened for immunogenicity in mice. A number of candidate antigens were identified which were surface exposed and induced bactericidal antibodies following parenteral delivery with Freund's adjuvant (23). We selected three of these antigens, App, NhhA, and NadA, with the intention of evaluating their ability to induce a mucosal immune response following intranasal immunization, using mucosal adjuvants that have already been used in humans (21). App (NMB1985 [number for the gene encoding this protein from the *N. meningitidis* MC58 genome sequence]) is a serine protease autotransporter protein which has structural homology with immunoglobulin A (IgA) serine proteases and 76% sequence homology with Hap, an adhesin from *Haemophilus influenzae* (11). The protein has been shown, by immunogold electron microscopy, to be localized at the meningococcal surface. It is also cleaved and secreted by *N. meningitidis* (10). It is highly conserved among disease-associated strains, and there is evidence that it is an adhesin which may be involved in the initial interaction between meningococci and epithelial cells (31). It is recognized by serum from convalescents and carriers of meningococci, suggesting that it is expressed in vivo and is immunogenic in humans (10). NhhA (NMB0992), a putative adhesin, is also highly conserved among virulent meningococci and recognized by convalescent-phase sera (18, 30, 37). The protein is a homolog of Hia, a fibrillar adhesin from *H. influenzae* (33). It is located in the bacterial outer membrane and may form oligomers. NadA (NMB1994) is surface exposed in *N. meningitidis*, forms oligomers, and can bind epithelial cells in vitro. It has predicted structural homology with YadA, an adhesin/invasin from enteropathogenic *Yersinia*, and UspA2, a protein from *Moraxella catarrhalis* which is involved in serum resistance (7). It has been found in approximately 50% of 150 meningococcal strains representing major disease-associated serogroups. However, among a subset of hypervirulent lineages (ET5, ET37, and cluster A4), 100% are positive for NadA. The protein is also recognized by convalescent-phase sera (15).

We examined the immunogenicity of these proteins following mucosal delivery to mice in the presence of *E. coli* heat-labile toxin (LTwt), a potent mucosal adjuvant, or mutant derivatives of LTwt: LTK63, which is completely nontoxic, or LTR72, which retains partial enzyme activity. Mucosal vaccines are easy to deliver and have the potential of conferring protection at the site of infection by inducing local as well as systemic immunity. Our findings demonstrate that intranasal immunization with NadA formulated with LT or LT mutants induced *N. meningitidis*-specific Th1 and Th2 responses, serum IgG, local IgA, and bactericidal antibodies in serum and in the respiratory tract.

MATERIALS AND METHODS

Antigens and adjuvants. The sequences coding for App (amino acids 43 to 1454), NhhA (amino acids 52 to 598), and NadA, (amino acids 1 to 350) were amplified by PCR on chromosomal DNA of meningococcus strain 2996 and cloned into the pET21b$^+$ vector (Novagen, Madison, Wis.). The plasmids were transformed into *E. coli* BL21(DE3) to express App and NhhA as COOH-terminal histidine fusion proteins and NadA as a nonfusion protein. Protein expression was induced at 37C° by adding 1 mM isopropyl-β-D-thiogalactopyranoside at an optical density at 600 nm (OD_{600}) of 0.5 and growing the bacteria for an additional 3 h. Expression was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. App-His and NhhA-His fusion proteins were produced as insoluble inclusion bodies and were solubilized with urea and renatured after purification (6). The fusion proteins were purified by affinity chromatography on an Ni^{2+}-conjugated chelating fast-flow Sepharose 4B resin. NadA (amino acids 1 to 350) was found to be processed and secreted in the culture supernatant of *E. coli* BL21(DE3). The culture supernatant, containing NadA, was filtered through a 0.22-μm-pore-size filter, concentrated seven times by ultrafiltration, and then dialyzed overnight against 20 mM Tris HCl (pH 7.6) at 4°C. The material was cleared by centrifugation at 13,000 × *g* for 20 min and loaded on a Q Sepharose XL column (Pharmacia, Uppsala, Sweden). The starting material was applied to a concentration of 7 mg of resin/ml, washed, and eluted with 400 mM NaCl in 20 mM Tris HCl (pH 7.6). The elution product was then diluted with 5 M NaCl in 20 mM Tris HCl (pH 7.6) to reach a concentration of 1.8 M NaCl and loaded on a phenyl Sepharose 6 Fast Flow column (High Sub; Pharmacia). The protein was eluted with 50 mM NaCl in 20 mM Tris HCl (pH 7.6). To reduce the high endotoxin content, hydroxylapatite ceramic column chromatography (HA Macro.Prep; Bio-Rad Laboratories, Inc., Hercules, Calif.) was subsequently performed. The protein was recovered in the flowthrough of this last step and diafiltrated against phosphate-buffered saline (PBS) (pH 7.4). The lipopolysaccharide content of the antigens was as follows: NhhA, 130 pg/μg; NadA, 113 pg/μg; and App, 405 pg/μg. Mutants of LT, LTK63 and LTR72, were generated by site-directed mutagenesis as described previously (9, 22). Their lipopolysaccharide content was less than 25 pg/mg.

Mice. Female BALB/c mice, 6 to 8 weeks of age (Harlan/Olac, Hull, United Kingdom), were used in all studies. They were allowed food and water ad libitum. They were divided into groups of five or six for experiments, which were performed according to regulations of the European Union, United Kingdom Home Office, or Irish Department of Health and local ethics committees.

Immunization and tissue collection. For intranasal immunizations, groups of five or six animals were lightly anesthetized with fluothane or halothane and 20-μl volumes containing App (5 μg), NhhA (5 μg), or NadA (10 μg) protein together with LTwt (1 μg), LTR72 (1 μg), or LTK63 (1 or 10 μg) in PBS were administered dropwise to the external nares of the mice (10 μl per nostril) using a Gilson pipette. Mice were immunized at 0, 3, and 6 weeks. At 8 weeks, all animals were terminally exsanguinated under fluothane anesthesia or by cervical dislocation and blood, nasal washes, lungs, and spleens were collected onto ice. Serum was separated by centrifugation, and samples were stored at −20°C. Nasal lavage was carried out by flushing the nares three times with a total volume of 0.5 ml of PBS containing 0.5% bovine serum albumin (BSA) and a protease inhibitor cocktail (Complete TM protease inhibitor cocktail; Boehringer Mannheim, Mannheim, Germany). Lungs were removed and homogenized in RPMI medium containing 8% fetal calf serum (Sigma, Poole, United Kingdom) and the protease inhibitor phenylmethylsulfonyl fluoride (0.1 mM; Sigma). Serum, lung homogenates, and nasal lavage samples were also stored at −20°C. Spleens were removed into 5 ml of RPMI medium supplemented with 10% heat-inactivated fetal calf serum (Sigma), 2 mM L-glutamine, 50 U of penicillin/ml, and 50 μg of streptomycin/ml (all from Gibco BRL, Rockville, Md.), and a single-cell suspension was prepared.

Serum IgG assay. Antigen-specific IgG titers were determined by enzyme-linked immunosorbent assay (ELISA). Flat-bottom, 96-well microtiter plates (Corning-Costar, High Wycombe, United Kingdom) were coated with purified App, NhhA, or NadA (3 μg/ml), diluted in PBS, at 4°C overnight. Wells were washed three times with PBS–0.05% Tween 20 (PBS/T) prior to blocking with 100 μl of PBS–1% BSA and 0.05% Tween 20 (PBS/BSA/T) for 1 h at 37°C and then washed a further three times. Serial three- or fivefold dilutions of serum in PBS/BSA/T were added to the plates and incubated at 4°C overnight prior to three washes with PBS/T. Bound IgG antibodies were detected with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Dako, Ely, United Kingdom). After three washes in PBS/T, 50 μl of *o*-phenylenediamine dihydrochloride (OPD) substrate (Sigma) was added. The reaction was stopped after 15 min by adding 50 μl of 3 M H_2SO_4, and the absorbance was measured at 492 nm. For determination of IgG1 or IgG2a titers, bound antibody was incubated with

biotinylated anti-mouse IgG1 or IgG2a (BD Pharmingen, Oxford, United Kingdom). Following incubation for 1 h, plates were washed as above before incubation for a further hour with HRP-conjugated streptavidin. Plates were washed, and OPD substrate was added as above. Antibody titers are expressed as the reciprocal of the dilution that gave an OD of 0.3 above that of the preimmune serum.

IgA in nasal washes. Undiluted nasal-wash samples were added to wells of antigen-coated ELISA plates and serially twofold diluted over a range of 1 to 1/120 in PBS/BSA/T containing a protease inhibitor cocktail (as above). After overnight incubation at 4°C, wells were washed three times. Biotinylated anti-mouse IgA was added (Sigma), and samples were incubated for 1 h at 37°C. Wells were then washed as before, and streptavidin-HRP was added as above. OPD substrate was applied, and absorbance was measured at 492 nm after 15 min. IgA titers are expressed as the reciprocal of the dilution that gave an OD of 0.2 above that of nasal washes from naïve animals.

IgA in lung homogenates. ELISA plates were coated overnight with antigen (3 μg/ml). After blocking with PBS/T containing 10% nonfat dried milk (Marvel), serial twofold or threefold dilutions of lung homogenate samples were added. Bound IgA antibodies were detected with biotinylated rat anti-mouse IgA antibody (1:1,000; BD Pharmingen) followed by streptavidin-HRP (BD Pharmingen) and OPD substrate as described above. Results are expressed as endpoint titers, calculated as described above.

Bactericidal antibody analysis. Complement-mediated bactericidal antibody titers were determined in serum, lung homogenate, and nasal lavage (App and NhhA only) samples as described previously (19). A serogroup B strain of *N. meningitidis*, a B:2b:P1.5-1.2 strain, isolated from a case in the United Kingdom in 1975 (7, 23), with homology to the antigens used for immunization, was used as the antibody target. Pooled baby rabbit serum (CedarLane Laboratories, Hornby, Canada) was used as the complement source. Killing was not observed with buffer or complement alone, with heat-inactivated sera, or with heat-inactivated sera and heat-inactivated complement. Therefore, the killing observed was due to the interaction between specific antibodies and complement. The serum bactericidal antibody titer was defined as the serum dilution resulting in a 50% decrease in CFU per ml after 60 min of incubation of bacteria in the reaction mixture, compared with control CFU per ml at time zero.

Antigen-specific cytokine production. Spleen (2×10^6 cells/ml) or lymph node (1×10^6 cells/ml) cells from immunized mice were cultured in complete RPMI medium at 37°C and 5% CO_2 with antigen (purified *E. coli*-expressed App, NhhA, or NadA; 1 to 50 μg/ml), medium only, or phorbol myristate acetate (PMA) (Sigma; 20 ng/ml) and anti-mouse CD3 (BD PharMingen; 1 μg/ml). Supernatants were collected after 72 h, and cytokine concentrations were determined by two-site ELISA using antibody pairs specific for interleukin-4 (IL-4), IL-5, gamma interferon (IFN-γ) (BD PharMingen), or IL-10 (Duo-Set; R&D Systems, Minneapolis, Minn.).

Statistical analysis. Antibody and cytokine responses were compared by one-way analysis of variance (ANOVA). Where significant differences were found, the Tukey-Kramer multiple comparisons test was used to identify differences between individual groups. In cases when standard deviations were significantly different between groups, a nonparametric test (Kruskal-Wallis test with Dunn's multiple comparison posttest) was used to assess significance.

RESULTS

Serum IgG responses in immunized mice. We assessed the immunogenicity of three novel proteins from *N. meningitidis* following intranasal delivery, with LTwt or LT mutants as adjuvants, in BALB/c mice. Previous studies had indicated that LT and LTR72 were effective adjuvants for intranasal immunization at a dose of 1 μg per mouse, whereas a higher dose of LTK63 is required to induce a comparable adjuvant effect. In our first experiments, App and NhhA (5 μg per mouse) were tested in parallel using LTwt and LTR72 at a dose of 1 μg per mouse and LTK63 at 1 or 10 μg per mouse. Based on results obtained from these experiments, we elected subsequently to use LTK63 at a dose of 10 μg per mouse, LTwt and LTR72 at 1 μg per mouse, and NadA at 10 μg per mouse for each immunization. Antigen-specific IgG titers were determined by ELISA in serum collected 2 weeks after two booster immunizations. We found that all three proteins induced weak or undetectable antibody responses when administered intranasally to mice in the absence of adjuvant. In contrast, both NadA and NhhA induced strong humoral immunity in the presence of a mucosal adjuvant, with mean IgG titers of greater than $\log_{10}$ 5 (Fig. 1). The enhancing effect of the adjuvants was striking; mice immunized with NhhA or NadA and adjuvant had mean antibody levels which were 3 to 4 $\log_{10}$ units higher than those in mice immunized with NhhA or

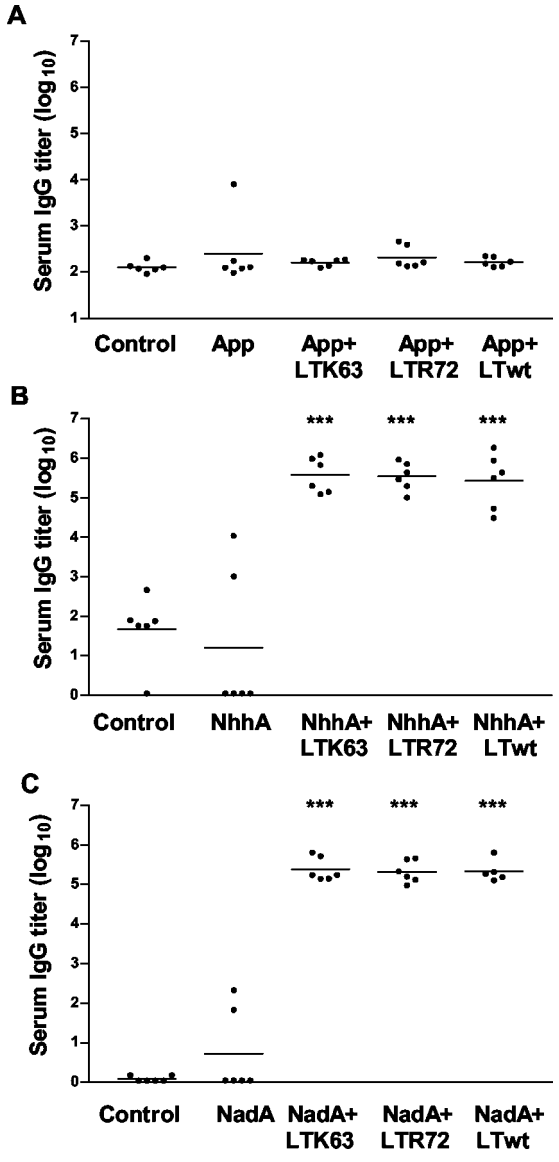

FIG. 1. NhhA and NadA, but not App, induce potent serum IgG responses after intranasal immunization with LT or LT mutants. Mice were immunized intranasally with meningococcal protein App (A), NhhA (B), or NadA (C) together with LTwt (1 μg), LTR72 (1 μg), or LTK63 (10 μg). Serum was collected 2 weeks after the second booster immunization, and specific antibody responses were quantified by ELISA. Antibody titers are expressed as the reciprocal of the serum dilution that gave an OD_{492} of 0.3 above that of preimmune serum. Symbols represent values for five or six individual mice per group; bars represent mean values. Results are representative of two experiments from two independent studies carried out in different laboratories. ***, $P < 0.001$ versus NhhA or NadA alone (ANOVA).

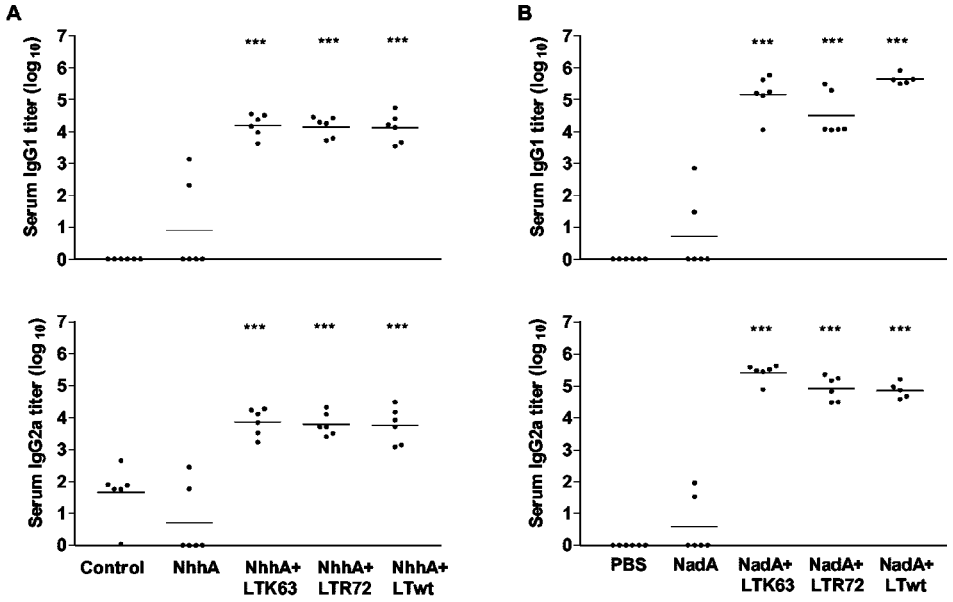

FIG. 2. Intranasal immunization with NhhA (A) and NadA (B) in the presence of mucosal adjuvants induces IgG1 and IgG2a antibodies. Mice were immunized and data are presented as described in the legend to Fig. 1. Symbols represent values for five or six individual mice per group; bars represent mean values. ***, $P < 0.001$ versus NhhA or NadA alone (ANOVA).

NadA alone. There was no significant difference between antibody titers induced with the different LT molecules. However, LTK63 was used at a 10-fold-higher dose (10 μg/mouse) than LTR72 or LTwt; anti-NhhA IgG titers were approximately 1 $\log_{10}$ unit lower when LTK63 used at the equivalent dose (1 μg/mouse; data not shown). Antigen-specific IgG titers remained at background levels in mice immunized with App, even in the presence of a mucosal adjuvant.

Analysis of IgG subclass responses. Analysis of the IgG subclass of the antigen-specific antibody in serum samples revealed that both IgG1 and IgG2a were induced following immunization with NhhA or NadA and mucosal adjuvants. Although we used only twofold-more NadA than NhhA with the same doses of adjuvants, IgG1 and IgG2a titers were approximately 10-fold higher in the mice immunized with NadA (Fig. 2). In NhhA-immunized animals, IgG1 titers were 3 log units above background levels, compared with a difference of 2 log units above background for IgG2a. There was no significant difference between the effects of the different mucosal adjuvants. In contrast, IgG2a titers were marginally higher than IgG1 titers in mice immunized with NadA and either LTR72 or LTK63, but not LTwt, where IgG1 was still the dominant subclass. We were unable to detect either antigen-specific IgG1 or IgG2a antibodies in mice immunized with App in the presence or absence of mucosal adjuvants (data not shown).

Antigen-specific IgA in the respiratory tract. Nasal lavage and lung homogenate samples were used to assess the production of antigen-specific IgA. We could not detect any IgA in mice immunized with App with or without mucosal adjuvants (data not shown) or with NadA and NhhA administered intranasally without adjuvant (Fig. 3). In contrast, significant levels of IgA were detected in nasal lavage (Fig. 3A and B) and lungs (Fig. 3C) of mice intranasally immunized with NhhA or NadA and LTwt. LTR72 was also found to significantly increase IgA titers in nasal and lung lavage samples from mice immunized with NadA. In general, the strongest response was induced with LTwt as the adjuvant and the weakest was induced with LTK63. Statistical analysis revealed no significant difference between LTwt and LTR72 in mice immunized with NadA, but LTK63 was significantly less effective than LTwt in inducing local IgA ($P < 0.01$).

Induction of bactericidal antibodies. Bactericidal antibodies are an important correlate of protection against meningococcal infection. We could not detect bactericidal activity in serum samples from mice immunized with App. Despite impressive serum antibody titers, we also failed to detect bactericidal activity in serum or lung lavage samples from mice immunized with NhhA and mucosal adjuvants (data not shown). The sera from NadA-immunized animals by contrast was strongly bactericidal (Fig. 4). The use of mucosal adjuvants was essential in order to generate bactericidal antibody following intranasal delivery, as bactericidal activity could not be detected in mice immunized intranasally with NadA alone. The strongest bactericidal activity was detected in sera from mice immunized with NadA and LTR72. The bactericidal antibody titers were similar to those generated by parenteral immunization with NadA in Freund's adjuvant (7 and data not shown). We also detected bactericidal activity in lung homogenates from mice immunized with NadA, with the strongest response observed in mice immunized with NadA in the presence of LTK63 (Fig. 5). Mucosal delivery and the use of mucosal adjuvant were essential in order to generate bactericidal antibodies in the respiratory tract; bactericidal activity could not be detected in lungs of mice immunized intranasally with NadA alone (Fig. 5).

Induction of Th1 and Th2 responses. We examined the ability of proteins from serogroup B meningococci to induce cellular immune responses following intranasal delivery with adjuvants by assessing antigen-specific cytokine production by

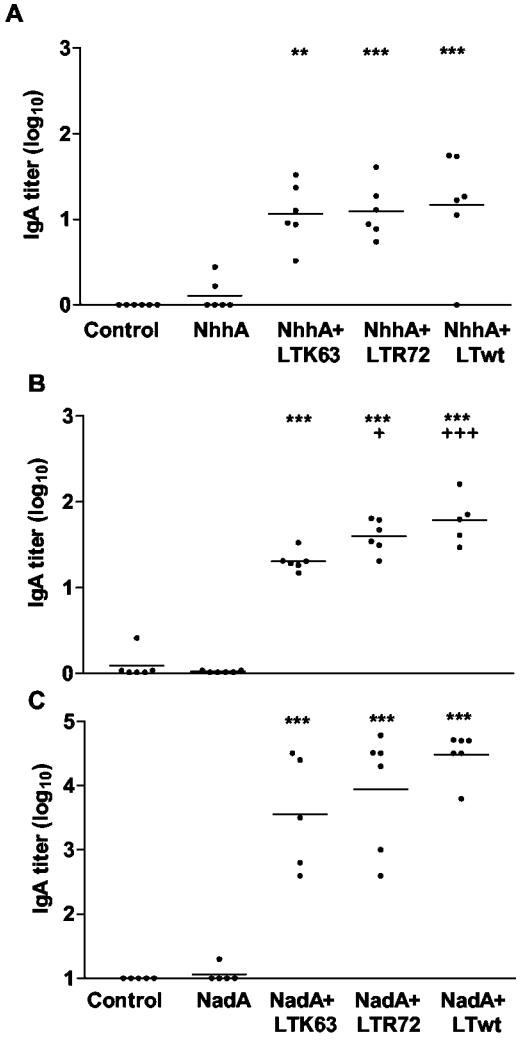

FIG. 3. IgA production following immunization with NhhA or NadA and mucosal adjuvants. Mice were immunized intranasally with meningococcal protein NhhA (A) or NadA (B and C) alone or together with LTwt, LTR72, or LTK63. IgA endpoint titers were determined by ELISA on nasal lavage (A and B) and lung homogenate (C) samples recovered 2 weeks after the third immunization. Symbols represent values for five or six individual mice per group; bars represent mean values. ** and ***, $P < 0.01$ and < 0.001, respectively, versus NhhA or NadA alone (ANOVA); + and +++, $P < 0.05$ and < 0.001, respectively, versus NadA with LTK63 (Tukey-Kramer test).

spleen and lymph node cells from immunized mice. Spleen cells recovered 2 weeks after the third immunization were restimulated in vitro with the relevant meningococcal antigen. Stimulation with PMA and anti-CD3 or with culture medium acted as positive and negative controls, respectively. Cytokine concentrations were determined in the supernatants 3 days after stimulation. In contrast to its failure to induce antibody responses, immunization with App did induce cellular immune responses. Antigen-specific IFN-γ and low levels of IL-5 were detected following intranasal immunization with App in the absence of adjuvant, and responses were enhanced following coadministration with LT mutants, especially LTR72 (Fig. 6). NhhA also induced significant levels of antigen-specific IFN-γ and IL-5 following immunization in the presence of LTR72 or

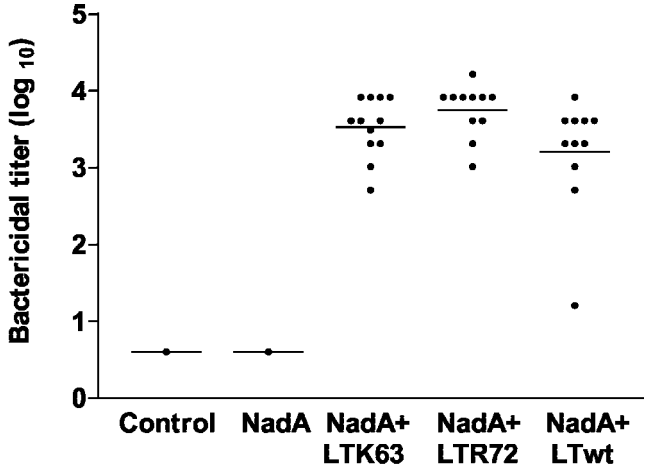

FIG. 4. NadA induces potent bactericidal antibodies after intranasal immunization with LT or LT mutants. Mice were immunized intranasally with PBS, NadA alone, or NadA plus LTwt, LTR72, or LTK63. Bactericidal antibody titers are expressed as the reciprocal of the serum dilution yielding at least 50% killing of the test meningococcal strain. Sera from naïve animals and from animals immunized with NadA alone were tested in pools. Symbols represent values for 12 individual mice per group from two independent experiments; bars represent mean values.

LTK63 (Fig. 6). Similar responses were detected with NhhA formulated with 10 μg of LTK63 or 1 μg of LTR72, but lower concentrations of IFN-γ were produced by antigen-stimulated spleen cells from mice immunized with NhhA and 1 μg of LTK63. In contrast, intranasal immunization with NhhA alone failed to generate significant cellular immune responses.

Th1- and Th2-type responses were also detected following intranasal immunization with NadA in the presence of mucosal adjuvants. Antigen-specific proliferation and cytokine production were at background levels in mice immunized intranasally with NadA alone. In contrast, potent proliferative responses were detected in spleen cells from mice immunized with NadA

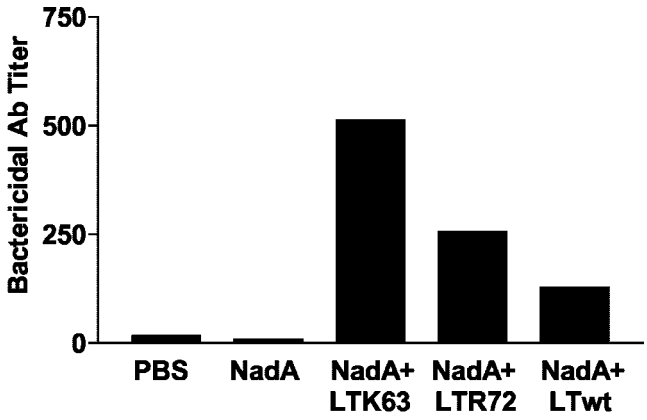

FIG. 5. Bactericidal antibodies in lungs of mice immunized intranasally with NadA and LT or LT mutants. Mice were immunized intranasally with PBS, NadA alone, or NadA plus LTwt, LTR72, or LTK63. Bactericidal antibody titers are expressed as the reciprocal of the lung homogenate dilution yielding at least 50% killing of the test meningococcal strain. Samples were tested in pools and are mean values for triplicate assays.

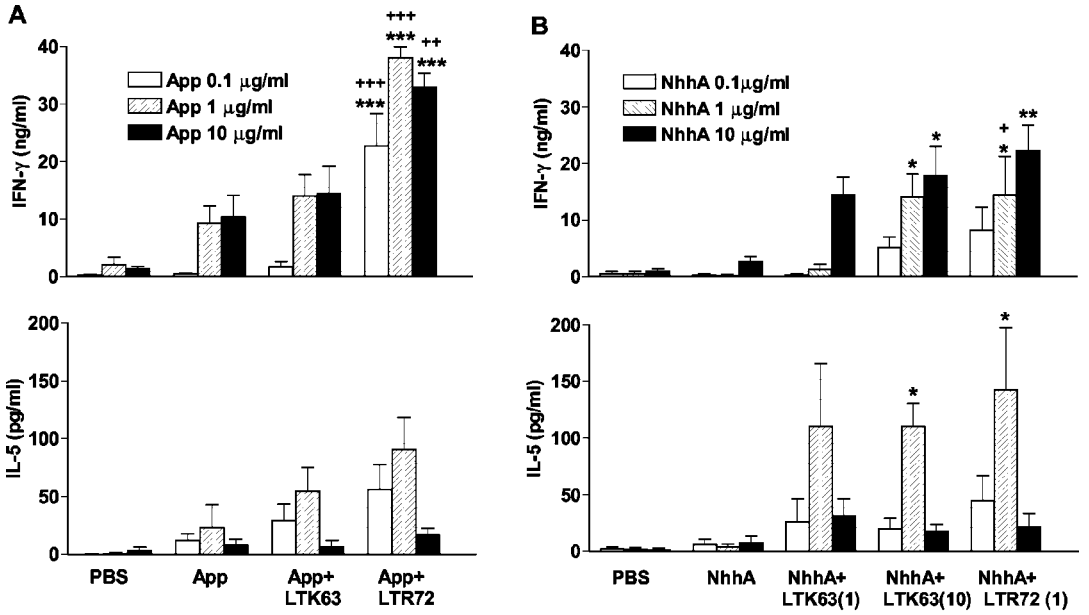

FIG. 6. Induction of systemic Th1- and Th2-type responses in mice immunized with App or NhhA and LTwt, LTR72, or LTK63. Mice were immunized three times (0, 3, and 6 weeks) intranasally with App or NhhA (5 μg) alone or with LTK63 (1 or 10 μg), LTR72 (1 μg), or PBS only as a control. Two weeks after the last immunization spleen cells were stimulated in vitro with antigen (App or NhhA) (1 to 100 μg/ml), and cytokine concentrations in supernatants were determined 3 days later. Cytokine concentrations are expressed as means (+standard deviations) for five mice per group, tested individually in triplicate and are representative of two experiments. *, **, and ***, $P < 0.05$, < 0.01, and < 0.001, respectively, versus App or NhhA alone; + and +++, $P < 0.05$ and < 0.001, respectively, versus App or NhhA with LTK63 (1-μg dose) (Tukey-Kramer test).

in the presence of LTK63 or LTR72, with weaker responses generated with LTwt as the adjuvant (Fig. 7). Furthermore, significant levels of IFN-γ and IL-5 and lower levels of IL-10 (Fig. 7) and IL-4 (data not shown) were detected in antigen-stimulated spleen or draining lymph node (data not shown) cells from mice immunized intranasally with NadA and LTK63, LTR72, or LTwt, but not with NadA alone. The strongest responses were detected in mice immunized with LTR72 as the adjuvant. Spleen cells from all mice produced cytokines in response to PMA and anti-CD3 (data not shown).

DISCUSSION

This study demonstrates that intranasal immunization of mice with NadA, a conserved, putative adhesin found in serogroup B strains of *N. meningitidi*s, is capable of generating local and systemic cellular and humoral immunity when coadministered with mucosal adjuvants. Moreover, the antibodies induced are bactericidal, a correlate of protection against serogroup B meningococci.

Childhood immunization with conjugate polysaccharide vaccines has led to a dramatic reduction in cases of bacterial meningitis caused by *H. influenzae* and serogroup C meningococci (4, 27). However, many children and young adults are still vulnerable to potentially fatal infection with serogroup B strains of *N. meningitidis*. Since the polysaccharide capsule cannot be used to vaccinate against serogroup B meningococci, research has concentrated on identifying candidate protein antigens. However, the rate of antigenic variation among surface-exposed moieties of *N. meningitidis* has been a major obstacle to the development of protein vaccines. Although protective antigens have been identified, they may have limited efficacy against heterologous challenge (34).

In an effort to design a novel vaccine against serogroup B meningococci with broader protective efficacy, we have taken advantage of a systematic screen of the *N. meningitidis* B genome (23), which identified surface-exposed, immunogenic proteins, many of which are conserved among serogroup B strains. These proteins were found to induce bactericidal antibodies in mice following parenteral delivery with complete Freund's adjuvant. In this study we focused on three antigens, App, NhhA, and NadA, all of which are either novel or putative adhesins, and examined their potential as components of a mucosal vaccine against serogroup B meningococci. Inhibition of binding by antibodies targeted against adhesins could have an important inhibitory effect on the initial attachment of bacteria to the nasopharyngeal epithelium, the first step in infection by *N. meningitidis*. It has been shown that immunization with the FimH adhesin from uropathogenic *E. coli* led to greater than 99% reduction in colonization of the bladder mucosa in a murine cystitis model (12).

Although most licensed vaccines are administered parenterally, mucosal vaccines have a number of advantages, including avoidance of the need for injections, which improves patient compliance and eliminates problems associated with the disposal or reuse of contaminated needles. Mucosal delivery also has the advantage of stimulating local, as well as systemic, immune responses, thus preventing bacterial colonization in naïve individuals and preventing dissemination of bacteria that breach the mucosal barrier. However, most antigens are poorly immunogenic when delivered at mucosal sites and typically require the use of mucosal adjuvants. We have employed LTwt

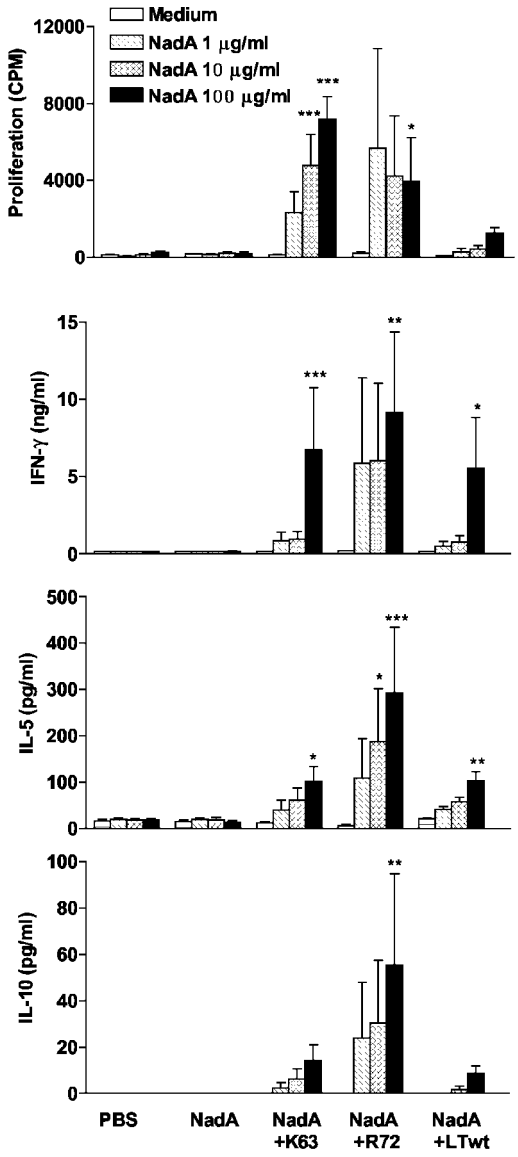

FIG. 7. Induction of Th1- and Th2-type responses in mice immunized with NadA and LT or LT mutants. Mice were immunized three times (0, 3, and 6 weeks) intranasally with PBS, NadA (10 μg) alone, or NadA with LTK63 (10 μg), LTR72 (1 μg), or LTwt (1 μg). Two weeks after the last immunization spleen cells from immunized mice were stimulated in vitro with antigen (NadA) (1 to100 μg/ml), and cytokine concentrations in supernatants were determined 3 days later. Cytokine concentrations are expressed as means (+standard deviations) for five mice per group, tested individually in triplicate, and are representative of two experiments from two independent studies. *, **, and ***, $P < 0.05$, < 0.01, and < 0.001, respectively, versus NadA alone (ANOVA).

and two mutant derivatives, LTR72, with significantly reduced toxicity, and LTK63, which is completely nontoxic. Both mutants have been shown to enhance immune responses to a range of foreign antigens and in preclinical studies were found to have no adverse effects in vivo (1, 21, 24). LTK63 has been successfully tested in human volunteers with a nasally delivered influenza subunit vaccine (21). However, potential toxicity problems associated with intranasal administration of AB-type toxins as mucosal adjuvants have been raised. Van Ginkel and coworkers (36) have reported GM1 ganglioside-dependent binding of radiolabeled cholera toxin (CT) and the B subunit of CT (CT-B) to olfactory nerves and epithelium and retrograde transport to the olfactory bulb. Furthermore, intranasal vaccination with radiolabeled tetanus toxoid together with CT resulted in uptake of tetanus toxoid into the olfactory nerve and epithelium but not the olfactory bulb. Recent reports have indicated that a commercial, intranasally administered influenza vaccine containing LTwt as the adjuvant significantly increased the risk of developing Bells palsy among vacinees, and consequently the vaccine was withdrawn from clinical use (16). Preclinical studies indicated that radiolabeled LT intranasally administered can be detected in the olfactory bulb of BALB/c mice but not in other strains of mice or in rats, rabbits, or baboons (39). It has also been reported by Francotte et al. (http://www.niaid.nih.gov/dmid/enteric/intranasal.htm) that intranasal administration of CT, LTwt, and the LT mutants LTR192G and LTS63K resulted in overt inflammation of the meninges and severe lesions in the olfactory bulb of BALB/c mice, whereas LTB or the nonbinding mutant LTR192G/G33D did not undergo transport to the olfactory bulb. However, it has also been reported that clinical-grade preparations of the LT mutants LTK63 and LTR72 did not induce inflammation, detected by histological changes, in the olfactory bulbs, brain, or meninges of outbred mice or rabbits (21).

In this study we found that serogroup B meningococcus antigens induced weak or undetectable immune responses when delivered nasally without an adjuvant. In contrast, LT and the LT mutants augmented cellular or humoral immune responses to these antigens when delivered to mice by the nasal route. However, the antigen considerably influenced the nature and magnitude of the immune responses induced following nasal immunization. Two of the three antigens, NadA and NhhA, stimulated serum IgG titers of greater than $\log_{10}$ 5 when delivered nasally with LTwt, LTR72, or LTK63, with no significant differences between the effects of the adjuvants. In contrast, App failed to induce significant IgG production, even in the presence of adjuvant. We have no evidence to suggest that App is homologous to self-antigens, and indeed it is recognized by human convalescent-phase sera and sera from asymptomatic carriers of *N. meningitidis*, suggesting that it is immunogenic in humans (10). App has been shown to elicit antibodies in mice and rabbits following parenteral administration with Freund's adjuvant (10, 31). Therefore, it is possible that it was degraded following immunization by the nasal route or that it may not have refolded properly following purification from *E. coli*. Although we could not detect specific IgG by ELISA, App-specific antibodies were detected in sera from App-immunized mice by Western blotting (data not shown). Furthermore, cellular immune responses, IFN-γ production in particular, were detected in mice immunized intranasally with App and LT mutants, and this is consistent with the previously reported T-cell-stimulating activity of App in humans (10). Alternatively, App may have immunomodulatory effects on specific elements of the immune response, and this possibility is under investigation.

All three meningococcus B antigens stimulated strong systemic cellular responses when delivered intranasally in the presence of a mucosal adjuvant. Antigen-specific cytokine pro-

duction was almost undetectable in mice immunized with meningococcus B proteins alone. In contrast, significant concentrations of IFN-γ and IL-5 were detected in antigen-stimulated spleen cells from mice immunized with App, NhhA, and NadA in the presence of LT mutants, suggesting that both Th1 and Th2 cells were induced in vivo. However, enhancement of IFN-γ was more striking in mice immunized with App and LTR72, whereas IL-4, IL-5, and IL-10 were also enhanced by LTR72 and LTK63 in mice immunized with NadA. Coadministration with adjuvants was clearly necessary for strong induction of cellular immunity, and the mutant toxin LTR72 was the most effective mucosal adjuvant. This finding provides further evidence that the ADP-ribosyltransferase activity of AB-type toxins plays an important role in their adjuvant effect for T-cell as well as antibody responses. This is also consistent with the suggestion that excess enzyme activity may result in reduced immune responses, possibly due to toxicity or apoptotic activity in vivo (22, 29). Nevertheless, the present study also reveals that LTK63, which is devoid of enzyme activity and potential toxicity in vivo, can also augment cellular and humoral responses, albeit at a higher dose.

The mixed Th1/Th2 responses were consistent with the detection of both IgG1 and IgG2a antibodies in the sera of mice immunized with NhhA or NadA formulated with mucosal adjuvants. Although serum IgG1 titers were higher than IgG2a titers in mice immunized with NhhA, IgG2a titers were higher in mice immunized with NadA in the presence of either of the LT mutants. While there is no definitive evidence of a role for either Th1 or Th2 cells in immunity to serogroup B meningococci, it has been suggested that Th2 responses are stronger among older children than infants and may be associated with improved protection against infection (25).

The induction of IgA is potentially an important advantage of mucosal over parenteral vaccines as a vigorous local IgA response could effectively stop an infection at the point of initial contact between the bacterium and host at the mucosal surface. Antigen-specific IgA was detected in the lungs and nasal washes of mice immunized with either NhhA or NadA, but not with App. These responses were totally dependent on coimmunization with a mucosal adjuvant.

Serum bactericidal activity is widely regarded as the gold standard predictive correlate of protection against *N. meningitidis* infection (2). In our study, only NadA administered with LT adjuvants was capable of stimulating bactericidal antibody in serum, with LTR72 promoting the highest titers. Furthermore, bactericidal antibody titers were comparable to those generated following parenteral immunization with NadA and Freund's adjuvant (7). We also detected bactericidal activity in lung homogenates from mice immunized intranasally with NadA and LTwt or LT mutants. The bactericidal assay employed does not allow discrimination between serum IgG antibodies that may have transuded into the lungs and locally produced IgA. Therefore, we have no way of knowing whether the bactericidal activity of the lung samples is related to IgA induction. There was an inverse relationship between the bactericidal activity and IgA titers. In contrast, the highest bactericidal activity was observed with lung samples from mice immunized with NadA and LTK63, a combination that induced the highest IgG2a antibody response. The relationship between antibody subclass and protection against *N. meningitidis* infection is not completely clear. Some studies have found no correlation between subclass and serum bactericidal activity (8, 14). Others have shown a positive correlation between IgG1 and IgG3 antibodies and serum bactericidal activity in humans (17, 38). Interestingly, these subclasses are most effective at complement binding and activation (3). Our data show that NadA formulated with LT adjuvants induces high levels of IgG1, IgG2a, and bactericidal antibody. However, NhhA also induced strong IgG1 and IgG2a responses but no bactericidal activity. Therefore, it is possible that other features of the antibodies, including specificity or avidity, may determine their bactericidal activity. It is clear, however, that the capacity of NadA to induce strong cellular immune responses and systemic and local bactericidal antibodies, together with its known conservation among disease-associated strains, makes it an excellent candidate for inclusion in a vaccine against serogroup B meningococci.

ACKNOWLEDGMENTS

This work was supported by a grant from the European Commission within the 5th Framework Programme: Mucosal Immunization and Vaccine Development (MUCIMM), contract no. QLK2CT 1999 00228. K.H.G.M. and E.C.L. are supported by Science Foundation Ireland (grant no. 00/PI.I/B045). C.H. is supported by a NEOVAC grant from the European Commission, contract no. QLK2-CT-1999-00429.

REFERENCES

1. **Baudner, B. C., O. Balland, M. M. Giuliani, P. Von Hoegen, R. Rappuoli, D. Betbeder, and G. Del Giudice.** 2002. Enhancement of protective efficacy following intranasal immunization with vaccine plus a nontoxic LTK63 mutant delivered with nanoparticles. Infect. Immun. **70:**4785–4790.
2. **Borrow, R., N. Andrews, D. Goldblatt, and E. Miller.** 2001. Serological basis for use of meningococcal serogroup C conjugate vaccines in the United Kingdom: reevaluation of correlates of protection. Infect. Immun. **69:**1568–1573.
3. **Burton, D. R., L. Gregory, and R. Jefferis.** 1986. Aspects of the molecular structure of IgG subclasses. Monogr. Allergy **19:**7–35.
4. **Cadoz, M.** 1998. Potential and limitations of polysaccharide vaccines in infancy. Vaccine **16:**1391–1395.
5. **Cartwright, K., N. Noah, and H. Peltola.** 2001. Meningococcal disease in Europe: epidemiology, mortality, and prevention with conjugate vaccines. Report of a European advisory board meeting Vienna, Austria, 6–8 October, 2000. Vaccine **19:**4347–4356.
6. **Coligan, J. E., B. M. Dunn, H. L. Ploegh, D. W. Speiches, and P. T. Wingfield (ed.).** 1997. Current protocols in protein science, p. 6.0.1–6.7.14, 9.4.1–9.4.16. John Wiley & Sons, Inc., New York, N.Y.
7. **Comanducci, M., S. Bambini, B. Brunelli, J. Adu-Bobie, B. Arico, B. Capecchi, M. M. Giuliani., and V. Masignani.** 2002. NadA, a novel vaccine candidate of *Neisseria meningitidis.* J. Exp. Med. **195:**1445–1454.
8. **de Kleijn, E. D., L. van Eijndhoven, C. L. Vermont, B. Kuipers, H. van Dijken, H. Rümke, R. de Groot, L. van Alphen, and G. van den Dobbelsteen.** 2001. Serum bactericidal activity and isotype distribution of antibodies in toddlers and school children after vaccination with RIVM hexavalent PorA vesicle vaccine. Vaccine **20:**352–358.
9. **Giuliani, M. M., G. Del Giudice, V. Giannelli, G. Dougan, G. Douce, R. Rappuoli, and M. Pizza.** 1998. Mucosal adjuvanticity and immunogenicity of LTR72, a novel mutant of *Escherichia coli* heat-labile enterotoxin with partial knockout of ADP-ribosyltransferase activity. J. Exp. Med. **187:**1123–1132.
10. **Hadi, H. A., K. G. Wooldridge, K. R. Robinson, and D. A. A. Ala'Aldeen.** 2001. Identification and characterization of App: an immunogenic autotransporter protein of *Neisseria meningitidis.* Mol. Microbiol. **41:**611–623.
11. **Hendrixon, D. R., M. L. de la Morena, C. Stathopoulos, and J. W. St. Geme III.** 1997. Structural determinants of processing and secretion of the *Haemophilus influenzae* Hap protein. Mol. Microbiol. **26:**505–518.
12. **Langermann, S., S. Palaszynski, M. Barnhart, A. G. Pinkner, J. Burlein, P. Barren, S. Koenig, S. Leath, C. H. Jones, and S. J. Hultgren.** 1997. Prevention of mucosal *Escherichia coli* infection by FimH-based systemic vaccination. Science **276:**607–611.
13. **Lepow, M. L., and P. A. Hughes.** 2003. Meningococcal immunology. Immunol. Allergy Clin. North Am. **23:**769–786.
14. **Lissolo, L., G. Maitre-Wilmotte, P. Dumas, M. Mignon, B. Danve, and M. J.**

Quentin-Millet. 1995. Evaluation of transferrin-binding protein 2 within the transferrin-binding protein complex as a potential antigen for future meningococcal vaccines. Infect. Immun. **63:**84–90.
15. **Litt D. J., S. Savino, M. Comanducci, M. Pizza, C. A. Ison, M. Levin, R. Rappuoli and J. S. Kroll.** 2002. Novel putative surface antigens from *N. meningitidis* strain MC58 which are consistently recognised by antibodies from patients convalescing from meningococcal disease, p. 133. *In* D. A. Caugant and E. Wedeger (ed), Abstracts of the 13th International Pathogenic Neisseria Conference, Oslo, Norway.
16. **Mutsch, M., W. Zhou, P. Rhodes, M. Bopp, R. T. Chen, T. Linder, C. Spyr, and R. Steffen.** 2004. Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland. N. Engl. J. Med. **26:**896–903.
17. **Naess, L. M., T. Aarvak, A. Aase, F. Oftung, E. A. Hoiby, R. Sandin, and T. E. Michaelsen.** 1999. Human IgG subclass responses in relation to serum bactericidal and opsonic activities after immunization with three doses of the Norwegian serogroup B meningococcal outer membrane vesicle vaccine. Vaccine **17:**754–764.
18. **Peak, I. R. A., Y. Srikhanta, M. Dieckelmann, E. R. Moxon, and M. P. Jennings.** 2000. Identification and characterisation of a novel conserved outer membrane protein from *Neisseria meningitidis.* FEMS Immunol. Med. Microbiol. **28:**329–334.
19. **Peeters, C. C., I. J. Claassen, M. Schuller, G. F. Kersten, E. M. van der Voort, and J. T. Poolman.** 1999. Immunogenicity of various presentation forms of PorA outer membrane protein of *Neisseria meningitidis* in mice. Vaccine **17:**2702–2712.
20. **Peltola, H.** 1998. Meningococcal vaccines. Current status and future possibilities. Drugs **55:**347–366.
21. **Peppoloni, S., P. Ruggiero, M. Contorni, M. Morandi, M. Pizza, R. Rappuoli, A. Podda, and G. Del Giudice.** 2003. Mutants of the *Escherichia coli* heat-labile enterotoxin as safe and strong adjuvants for intranasal delivery of vaccines. Expert Rev. Vaccines **2:**285–293.
22. **Pizza, M., M. R. Fontana, M. M. Giuliani, M. Domenighini C. Magagnoli, V. Giannelli, D. Nucci, W. Hol, R. Manetti, and R. Rappuoli.** 1994. A genetically detoxified derivative of heat-labile *Escherichia coli* enterotoxin induces neutralizing antibodies against the A subunit. J. Exp. Med. **180:**2147–2153.
23. **Pizza, M., V. Scarlato, V. Masignani, M. M. Giuliani, B. Arico, M. Comanducci, G. T. Jennings, L. Baldi, E. Bartolini, B. Capecchi, et al.** 2000. Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing. Science **287:**1816–1820.
24. **Pizza, M., M. M. Giuliani, M. R. Fontana, E. Monaci, G. Douce, G. Dougan, K. H. G. Mills, R. Rappuoli, and G. Del Giudice.** 2001. Mucosal vaccines: non toxic derivatives of LT and CT as mucosal adjuvants. Vaccine **19:**2534–2541.
25. **Pollard, A. J., R. Galassini, E. M. Rouppe van der Voort, M. Hibberd, R. Booy, P. Langford, S. Nadel, C. Ison, J. S. Kroll, J. Poolman, and M. Levin.** 1999. Cellular immune responses to *Neisseria meningitidis* in children. Infect. Immun. **67:**2452–2463.
26. **Poolman, J. T.** 1995. Development of a meningococcal vaccine. Infect. Agents Dis. 413–428.
27. **Ramsay, M. E., N. Andrews, E. B. Kaczmarski, and E. Miller.** 2001. Efficacy of meningococcal serogroup C conjugate vaccine in teenagers and toddlers in England. Lancet **357:**195–196.
28. **Rosenstein, N. E., B. A. Perkins, D. S. Stephens, L. Lefkowitz, M. L. Carter, R. Danila, P. Cieslak, K. A. Shutt, T. Popovic, A. Schuchat, et al.** 1999. The changing epidemiology of meningococcal disease in the United States, 1992–1996. J. Infect. Dis. **180:**1894–1901.
29. **Ryan, E. J., E. McNeela, M. Pizza, R. Rappuoli, L. O'Neill, and K. H. G. Mills.** 2000. Modulation of innate and acquired immune responses by *Escherichia coli* heat labile toxin: distinct pro- and anti-inflammatory effects of the non-toxic AB complex and the enzyme activity. J. Immunol. **165:**5750–5759.
30. **Scarselli, M., R. Rappuoli, and V. Scarlato.** 2001. A common conserved amino acid motif module shared by bacterial and intercellular adhesins: bacterial adherence mimicking cell cell recognition? Microbiology **147:**250–252.
31. **Serruto, D., J. Adu-Bobie, M. Scarselli, D. Veggi, M. Pizza, R. Rappuoli, and B. Arico.** 2003. Neisseria meningitidis App, a new adhesin with autocatalytic serine protease activity. Mol. Microbiol. **48:**323–334.
32. **Soriano-Gabarro, M., J. M. Stuart, and N. E. Rosenstein.** 2002. Vaccines for the prevention of meningococcal disease in children. Semin. Pediatr. Infect. Dis. **13:**182–189.
33. **St. Geme, J. W., III, D. Cutter, and S. J. Barenkamp.** 1996. Characterization of the genetic locus encoding *Haemophilus influenzae* type b surface fibrils. J. Bacteriol. **178:**6281–6287.
34. **Tappero, J. W., R. Lagos, A. M. Ballesteros, B. Plikaytis, D. Williams, J. Dykes, L. L. Gheesling, G. M. Carlone, E. A. Hoiby, J. Holst, H. Nokleby, E. Rosenqvist, G. Sierra, C. Campa, F. Sotolongo, J. Vega, J. Garcia, P. Herrera, J. T. Poolman, and B. A. Perkins.** 1999. Immunogenicity of 2 serogroup B outer-membrane protein meningococcal vaccines: a randomized controlled trial in Chile. JAMA **281:**1520–1527.
35. **Tettelin, H., N. J. Saunders, J. Heidelberg, A. C. Jeffries, K. E. Nelson, J. A. Eisen, K. A. Ketchum, D. W. Hood, J. F. Peden, R. J. Dodson, et al.** 2000. Complete genome sequence of *Neisseria meningitidis* serogroup B strain MC58. Science **287:**1809–1815.
36. **Van Ginkel, F. W., R. J. Jackson, Y. Yuki, and J. R. McGhee.** 2000. Cutting edge: the mucosal adjuvant cholera toxin redirects vaccine proteins into olfactory tissues. J. Immunol. **165:**4778–4782.
37. **Van Ulsen, P., L. van Alphen, C. T. P. Hopman, A. van der Ende, and J. Tommassen.** 2001. In vivo expression of *Neisseria meningitidis* proteins homologous to the *Haemophilus influenzae* Hap and Hia autotransporters. FEMS Immunol. Med. Microbiol. **32:**53–64.
38. **Vermont, C. L., H. H. van Dijken, C. J. van Limpt, R. de Groot, L. van Alphen, and G. P. van Den Dobbelsteen.** 2002. Antibody avidity and immunoglobulin G isotype distribution following immunization with a monovalent meningococcal B outer membrane vesicle vaccine. Infect. Immun. **70:**584–590.
39. **Zurbriggen, R., I. C. Metcalfe, R. Gluck, J. F. Viret, and C. Moser.** 2003. Nonclinical safety evaluation of Escherichia coli heat-labile toxin mucosal adjuvant as a component of a nasal influenza vaccine. Expert Rev. Vaccines **2:**295–304.

Editor: S. H. E. Kaufmann