Combinations of Protein Polysaccharide Conjugate Vaccines for Intranasal Immunization

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The ability of 2 mutants of heat-labile Escherichia coli enterotoxin (LTK63 and LTR72) to enhance the immunogenicity of 2 protein polysaccharide conjugate vaccines, Neisseria meningitidis group C (MenC) and Haemophilus influenzae type B (Hib), both of which are conjugated to the nontoxic mutant of diphtheria toxin (CRM197), after intranasal (inl) immunization in mice was evaluated. In addition, the question of whether combining both vaccines in a single formulation with heat-labile E. coli enterotoxin mutants reduced the response to either vaccine was investigated. The results showed that potent serum antibody responses against MenC and Hib could be elicited by inl immunization in combination with the mucosal adjuvants. Moreover, IgA mucosal responses were induced only in animals immunized through the inl route. Finally, the coadministration of 2 conjugate vaccines simultaneously did not adversely affect the responses against either. These studies support the rationale for developing mucosal vaccines, based on combining protein polysaccharide conjugates with heat-labile E. coli enterotoxin mutants, for infants and young children.

Vaccination continues to be the optimal approach for the prevention and control of infectious diseases. However, as new vaccines are developed, more and more immunizations become necessary, resulting in significant problems for vaccine compliance. Hence, combining several vaccines into a single formulation is an approach that has been widely used for many years [1]. However, when 2 existing vaccines are mixed, there is a concern that 1 or both might have reduced potency because of “antigenic competition,” which has been reported elsewhere [2].

Mucosal delivery of vaccines represents an attractive approach to overcome the problem of the large number of injections administered to young children and, if successful, could significantly improve compliance. In addition, since most pathogens initially infect at mucosal surfaces, inducing mucosal immunity at the site of infection would likely contribute to optimal protective immunity. Of the various mucosal delivery options available, the intranasal (inl) route appears to be the most practical because it offers easy access with relatively simple devices that already have been mass produced. In addition, on many occasions, inl immunization has been shown to induce more-potent immune responses with less antigen than alternative routes. Nevertheless, most vaccines, especially those based on recombinant proteins, are unable to induce potent responses after inl immunization unless strong adjuvants and/or delivery systems are used. Cholera toxin (CT) and heat-labile Escherichia coli enterotoxin (LT) are powerful mucosal adjuvants when coadministered with soluble antigens [3]. However, because of their toxicity, their use in humans is not possible. Therefore, mutants of LT were constructed by site-directed mutagenesis. Two of these, LTK63 and LTR72, were selected for further evaluation; they were shown to be nontoxic (LTK63) or to have only residual toxicity (LTR72) in vitro and in vivo and to retain adjuvant activity [4].

Inl immunization with LTK63 and LTR72 mucosal adjuvants induces strong humoral and cellular responses against a variety of antigens [4]. In addition, in some animal models, protective immunity has been induced against a challenge with various pathogens (e.g., Helicobacter pylori, Bordetella pertussis, influenza virus, and pneumococcus) [5, 6]. In general, purified polysaccharides (PSs) are poor immunogens and require conjugation to a carrier protein to improve their immunogenicity [7]. The Journal of Infectious Diseases 2002; 186:1358–61 © 2002 by the Infectious Diseases Society of America. All rights reserved. 0022-1899/2002/18609-0022$15.00
Figure 1. Geometric mean serum IgG antibody titers against *Neisseria meningitidis* group C (MenC). Groups of 10 female BALB/c mice were immunized with 10 μg of MenC or *Haemophilus influenzae* (Hib) alone or with the 2 vaccines combined. The adjuvanticity of heat-labile *Escherichia coli* enterotoxin mutants LTK63 and LTR72 (1 and 10 μg for both) was compared with that of cholera toxin (CT; 1 μg) by the intranasal route and with an aluminum hydroxide vaccine (alum; 10 μg, administered intramuscularly [im]). **A**, Geometric mean antibody titers to MenC determined by ELISA. Error bars, SD within ±1 SE. **B**, Complement-mediated bactericidal activity (BCA) titers against MenC were measured in pooled serum samples obtained from each group. Titers were determined by calculating the serum dilution, showing a 50% reduction in the no. of cfu after 1 h of incubation. **C**, Mucosal IgA responses to MenC were measured by bioluminescent assay in nasal washes obtained 2 weeks after the third immunization. Titers represent the log dilution values linearly extrapolated from log relative units data, at the cutoff value of >2 SD above the mean background.

In the present study, we examined inl immunization as an alternative route of vaccine delivery and assessed the immunogenicity of *Neisseria meningitidis* group C (MenC) and *Haemophilus influenzae* type B (Hib) in combination with LT mutants. A second objective was to investigate whether the antibody responses to the 2 vaccines would be impaired when the 2 PS vaccines were conjugated to the same carrier protein and were delivered simultaneously.

**Materials and Methods**

**Vaccines.** MenC capsular oligosaccharide conjugate and Hib conjugate vaccines were produced by using selective end-reducing group activation of sized oligosaccharide, followed by subsequent coupling to the protein carrier CRM197 through a hydrocarbon spacer (Chiron Siena), as described elsewhere [8]. The vaccines were diluted in PBS and combined with LTK63 and LTR72, obtained as described elsewhere [10], aluminum hydroxide (Superfos Biosector), or CT (Sigma).

**Animals and immunizations.** Two identical studies were performed simultaneously. Groups of 10 female BALB/c mice (Charles River), 6–10 weeks old, were immunized inl with 10 μg of MenC or Hib alone, MenC and Hib combined with CT (1 μg), or MenC and Hib combined with the LT mutants (1 μg and 10 μg for both mutants). For comparison, an additional group of mice was immunized intramuscularly (im) with 10 μg of MenC or Hib adsorbed to aluminum hydroxide. The vaccines were prepared on the same day of immunization, and mice were immunized on days 0, 21, and 35. Fifty microliters of each vaccine was injected into the thigh or instilled into alternate nostrils in unanesthetized mice. Blood samples were taken on day 49, along with nasal wash samples. To evaluate whether the immunogenicity of each PS conjugate vaccine was impaired when the 2 vaccines were used in combination, a third, identical, study was performed concurrently, in which the 2 vaccines were administered simultaneously to the same groups of mice at the same doses and with the same regimen described above.

**Immunoassays.** The antibody responses against MenC were measured by an ELISA, using a modification of a procedure described elsewhere [11]. In brief, ELISA plates were coated with adipic dihydrazide–derived MenC overnight at 4°C. The specific antibodies were developed with goat anti–mouse IgG–horseradish peroxidase conjugate. The MenC PS IgG antibody titers for the test samples and the internal control were expressed as the reciprocal of the serum dilution, giving an optical density of 1.0. Each serum sample was assayed in duplicate, and the average value was used to calculate the geometric mean and the SD within ±1 SE. The antibody responses against Hib polyribosylribitol phosphate (PRP) were determined similarly to the MenC ELISA, except that the plates were coated with bovine serum albumin–conjugated PRP. Titers were expressed as the optical density measured at 450 nm for serum diluted 1:50.

Nasal washes were assayed for anti–MenC IgA using a bioluminescent assay, as described elsewhere [12], with adipic dihydrazide–derived MenC as the coating antigen. A goat anti–mouse IgA-biotin conjugate was added as a first antibody, and Strepta-
Results

Responses to MenC after inl immunization alone and in combination with Hib. The geometric mean serum IgG antibody titers against MenC are shown in figure 1A. The serum antibody responses elicited by both LT mutants were significantly greater than those obtained with the antigen alone. LTR72 exhibited a higher adjuvanticity than did LTK63 at lower doses. Of note, the antibody responses induced by inl immunization with both LT mutants were comparable to those achieved with wild-type CT or those induced by im immunization with the aluminum hydroxide vaccine. Significantly, the addition of a second PS vaccine conjugated to CRM197 did not adversely affect the antibody responses to either vaccine. The levels of bactericidal antibodies induced by inl immunization with LT mutants closely correlate with the ELISA serum IgG responses and also were comparable to the responses induced by CT or im immunization with the aluminum hydroxide vaccine (figure 1B).

Nasal wash samples obtained after inl immunization with MenC combined with both LT mutants showed higher IgA titers than those obtained by inl immunization with MenC in the absence of adjuvants (figure 1C). As expected, im immunization elicited very low IgA titers.

Responses to Hib after inl immunization alone and in combination with MenC. The geometric mean serum IgG antibody titers against PRP are shown in figure 2. Similar to the data reported above for MenC, the antibody responses induced by both LT mutants were greater than those achieved with the antigen alone. LTR72 again showed better adjuvanticity. Comparable titers were induced in mice immunized inl with LT mutants and with the aluminum hydroxide vaccine by im immunization. In addition, there was no evidence of competition after combined inl immunization with the 2 PS conjugate vaccines, and the responses induced against Hib with MenC and Hib in combination were comparable to the responses induced by immunization with Hib alone.

Discussion

We have demonstrated that potent serum antibody responses against MenC and Hib can be induced by inl immunization with conjugate vaccines in combination with the mucosal adjuvants LTK63 and LTR72. Moreover, for the MenC vaccine, the antibodies induced by inl immunization had potent bactericidal activity, which is known to correlate with protective immunity [13]. In addition, IgA responses in the nasal cavity were induced only in animals immunized through the inl route. Inducing secretory immunity is important, because the upper respiratory tract is the portal of entry for several pathogens, including MenC and Hib.

On the basis of the antibody titers obtained with conjugate vaccines given alone and in combination and the bactericidal activity measured against MenC, the combination of 2 vaccines coadministered with the LT mutants did not negatively influence the antibody responses against MenC or Hib. Therefore, these results suggest that inl immunization is an effective route of vaccination for protein PS conjugate vaccines in combination with LT mutants. Of interest, the same dose of LT mutants was sufficient to significantly enhance the immunogenicity of both conjugate vaccines administered simultaneously. This result is particularly important, because it would reduce the amount of adjuvant needed and the risks associated with potential toxicity. Of importance, we have previously shown that preexisting immunity against the LTK63 mutant does not affect the ability of the mutant to act as an adjuvant for a second antigen [14]. Furthermore, the potency of mucosally-delivered vaccines may be further improved by formulating the vaccines in bioadhesive delivery systems [15]. In conclusion, our findings show that combining protein PS conjugate vaccines with LT mutants for inl immunization is an attractive approach for the development of mucosal vaccines for pediatric use.
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References