Modulation of Immune Response to Group C Meningococcal Conjugate Vaccine Given Intranasally to Mice Together with the LTK63 Mucosal Adjuvant and the Trimethyl Chitosan Delivery System

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Previous work had shown that the immunogenicity of conjugate vaccine against group C meningococci (CRM-MenC) is enhanced when it is delivered intranasally (inl) with mucosal adjuvants, such as mutants of the Escherichia coli enterotoxin (LT), and with delivery systems such as chitosan derivatives. We show, in mice, that the concomitant use of limiting doses of the fully nontoxic LTK63 mutant as a mucosal adjuvant and of the trimethyl derivative of chitosan as a delivery system allows the reduction of each of the components for the induction of antibody and bactericidal responses to CRM-MenC conjugate vaccine delivered inl at titers similar to or higher than those induced by parenteral immunization. These data could affect the design of efficacious mucosal vaccines and their safety.

Vaccination against bacterial meningitis has shown tremendous progress with the introduction of conjugated vaccines consisting of capsular poly- or oligosaccharides chemically conjugated to carrier proteins. These vaccines provide protection to children aged <18 months, who are unable to mount appropriate immune responses to plain polysaccharides, and they induce antigen-specific memory. The conjugated meningococcal vaccine that consists of the diphtheria toxin derivative CRM197 as carrier molecule conjugated with the capsular oligosaccharide of group C Neisseria meningitides (CRM-MenC) [1] has been shown to be efficacious in adolescents and infants in preventing group C meningococcal meningitis [2, 3]. The protection induced by conjugated vaccines against MenC is mediated by bactericidal antibodies and is able to kill the bacteria in the presence of complement [4]. These vaccines are delivered parenterally. Because meningococci colonize the oropharyngeal mucosa, it is likely that mucosal immunization, by inducing effector immune responses at the mucosal sites, may be more effective in conferring protection against meningococcal disease and colonization.

Mutants of the cholera toxin (CT) and of the Escherichia coli enterotoxin (LT) are among the best-characterized mucosal adjuvants. Among these mutants, one, LTK63 (S → K substitution at position 63 of the A subunit), is totally devoid of toxic activity in vitro and in vivo [5]. These mutants behave as powerful mucosal adjuvants in different animal models that have tested viral, bacterial, and parasitic vaccine candidates, and they enhance protective efficacy in appropriate animal models of challenge [6]. Unlike CT, LT mutants such as LTK63 preferentially polarize the cellular immune responses against the coadministered antigens toward a Th0/Th1 functional phenotype [7].

During the past decade, much attention has been devoted to the use of chitosan for the mucosal (mostly intranasal [inl]) delivery of vaccines in experimental animal models and in humans [8–10]. Chitosan is a cationic polysaccharide that originates from the deacetylation of chitin, the most abundant polymer in nature after cellulose and is found in the exoskeletons of crustaceans. Chitosan is considered to be a mucoadhesive polymer. Its mucosal adjuvanticity is possibly mediated by decreasing mucociliary clearance and by opening tight junctions at the level of the mucosal epithelial layer [11]. Similar effects are also exerted by protonated chitosan, such as the N,N,N-trimethyl chitosan chloride (TMC), which offers also a better solubility at neutral pH values than the native chitosan molecule [12]. We evaluated the relative contribution of the chitosan derivative TMC and of the LTK63 mutant to enhance the immunogenicity and protective efficacy of the CRM-MenC conjugate vaccine given inl to mice.

Materials and methods. The CRM-MenC conjugate vaccine and the nontoxic LT mutant LTK63 were prepared as described in detail elsewhere [1, 5]. Chitosan was supplied by Primex. The degree of deacetylation was 94.5%, and the viscosity was 12 mPas, as determined by the supplier. TMC was synthesized by 1-step synthesis, as described elsewhere [13]. The degree of quaternization was determined by 1H-NMR spec-
Female BALB/c mice aged 8–10 weeks (Charles River) were used throughout the study. Groups of 5 mice were immunized 3 times on days 0, 21, and 35 either intranasally (inl), subcutaneously (sc) with 400 μL given at multiple sites. Each immunization dose contained 1–2.5 μg of the MenC saccharide and 2–5 μg of the carrier protein CRM197, given together with aluminium hydroxide (alum) (500 μg), for sc immunizations, or with different doses of the LTK63 mutant and/or of TMC, for inl immunizations. In each experiment, mice immunized sc or inl always received the same amount of CRM-MenC vaccine. All formulations were prepared in PBS (pH 7.4) just before use by mixing the CRM-MenC conjugate vaccine with alum for sc immunizations or with a powder suspension of TMC with or without the LTK63 mucosal adjuvant. In some experiments, variable amounts of TMC (10, 20, or 50 μg) were given inl with or without fixed amounts of LTK63 (1 μg). In other experiments, variable amounts of the LTK63 mucosal adjuvant (0.05, 0.1, or 1 μg) were administered inl with or without fixed amounts of TMC (10 μg).

Serum samples were taken on days 0, 20 (post-1), 34 (post-2), and 45 (post-3), when mice were killed, and nasal washes were obtained and spleens removed. Nasal washes were performed by repeated flushing and aspiration of 1 mL of PBS (pH 7.4) that contained 0.1% bovine serum albumin and 1 mmol/L phenylmethylsulfonyl fluoride (Sigma Chemical).

Titration of serum and mucosal anti-MenC-, anti-CRM-, and anti-LTK63–specific IgG and IgA antibodies was done by ELISA on individual serum samples, as detailed elsewhere [5, 14, 15]. Antibody titers were statistically compared with a 2-tailed Student’s t test. Serum bactericidal activity against N. meningitidis group C strain C11 was titered on pooled serum samples, according to standard procedures described elsewhere [14, 16], using baby rabbit serum (CedarLane) as a source of complement.

Results. The highest serum anti–MenC IgG antibody titers were obtained in groups of mice that had been immunized inl with the CRM-MenC vaccine, together with both the LTK63 mutant and chitosan or TMC (figure 1A, groups 7–9). The antibody titers were comparable (P > .05) to those found in mice immunized with the same dose of vaccine given sc (group 1), except for the response after the first immunization, which induced detectable antibodies only in mice immunized sc. Increasing the dose of TMC from 10 to 50 μg induced a significant enhancement of the serum anti-MenC antibody response (P < .01 for TMC 10 μg [group 4] vs. TMC 50 μg [group 6]) to levels comparable to those observed in mice immunized inl with the vaccine with 1 μg of the LTK63 mutant alone (group 3). Significant enhancement of the anti-MenC antibody response by the addition of 1 μg of LTK63 mutant to the vaccine formulations was evident in groups of mice that received the lowest dose of TMC (10 μg) (P < .01 for group 7 vs. group 4) (figure 1A). Only mice immunized inl with the CRM-MenC vaccine plus adjuvants, but not those immunized sc, had detectable serum IgA antibodies against MenC, irrespective of the adjuvant/TMC dose used in the formulation (data not shown).

Finally, the concomitant use of both the LTK63 mucosal adjuvant and TMC induced bactericidal titers (1:16,000) that were much higher than those induced by LTK63 alone (1:4000), by TMC alone (from 1:1000 to 1:4000), and by vaccine administered sc (1:4000) (figure 1B).

We then asked whether the concomitant use of these adjuvant/delivery systems would have a beneficial effect in allowing the reduction of the doses of LTK63 to achieve a significant mucosal adjuvanticity. The strong adjuvanticity of LTK63 at 1 μg/dose (group 6) dramatically decreased when the mutant was used at dosages of 0.1 or 0.05 μg (groups 4 and 5). The concomitant use of TMC together with the LTK63 mutant fully restored serum anti-MenC antibody responses (groups 7–9) (figure 2A). The enhancing effect of TMC was also evident on the immunogenicity of LTK63 itself (data not shown).

Bactericidal antibody responses were negligible in mice immunized with limiting doses of the LTK63 mutant (figure 2B, groups 4 and 5); however, when the LTK63 was coadministered with TMC (groups 7–9), bactericidal antibody titers increased at levels comparable to or higher than those found in mice that had received the CRM-MenC conjugate vaccine sc (group 1).

Mice immunized inl with the CRM-MenC vaccine plus LTK63 with or without TMC, but not those immunized sc, had detectable IgA antibodies against MenC in the nasal washes. Titers were, however, rather low, and we did not observe any preeminent effect of the coadministration of the LTK63 mutant and TMC on their titers. The sc immunization in the presence of alum, but not inl immunization with the LTK63 mutant, induced the production of anti–MenC IgE antibodies (data not shown).

Discussion. Previous work [15] and our results show that the immunogenicity of inl-delivered vaccines can be enhanced when chitosan and chitosan derivatives, such as TMC, are coadministered with the nontoxic LT mutant LTK63. This has been done by using the CRM-MenC conjugate vaccine and by evaluating the ability of the mucosal immunization to elicit bactericidal antibodies, which are known to mediate protective immunity against meningococci in a serogroup-specific manner [4]. We have already extensively shown that the LTK63 mutant is totally devoid of any residual enzymatic (and, thereby, toxic) activity in vitro and in vivo [5, 17] and that it retains a strong mucosal adjuvant activity for a wide variety of bacterial, viral, and parasitic antigens that are considered to be potential vaccine candidates [6, 17], including conjugate vaccines against Haemophilus influenzae type b [18], meningococci [14, 18], and pneumococci [19]. Some concerns have been raised about po-
Figure 1. Effect of increasing doses of the trimethyl derivative of chitosan (TMC) with and without the LTK63 mutant on the induction of serum anti–group C meningococci (MenC) IgG antibodies (A) and serum bactericidal antibodies (B) after intranasal (inl) immunization with the CRM-MenC conjugate vaccine. Groups of 5 BALB/c mice were immunized either subcutaneously (sc) with aluminium hydroxide (alum)–adjuvanted CRM-MenC vaccine (group 1) or inl with the same vaccine formulated with increasing doses of TMC (10, 20, or 50 μg) with or without a fixed dose of the LTK63 mutant (1 μg) (groups 4–9). Control inl groups received the vaccine without any adjuvant (group 2) or with 1 μg of LTK63 alone (group 3). Immunizations were performed on days 0, 21, and 35, as detailed in Materials and Methods. All groups always received the same amount of CRM-MenC conjugate vaccine, either sc or inl, that consisted of 2.5 μg of the MenC oligosaccharide and 5 μg of CRM197. A, Serum anti–MenC IgG antibody titers measured by ELISA after 1 (post-1), 2 (post-2), and 3 (post-3) immunizations. Each column represents the mean titer ± 1 SD of each group at each time point. B, Serum bactericidal antibody titers tested on pooled samples obtained after 3 immunizations. +, Present; −, absent.

tential risks of the localization of LT and CT mutants in the olfactory bulbs after inl immunization [20] and the induction of inflammatory responses in the surrounding neurological areas. Data obtained by us in animal studies have shown that inl immunization with these mutants does not induce any detectable inflammatory response at the level of the nasal mucosa nor at the level of the olfactory bulb or meninges [17]. Never-
Figure 2. Effect of increasing doses of the LTK63 mutant with and without the trimethyl derivative of chitosan (TMC) on the induction of serum anti–group C meningococci (MenC) IgG antibodies (A) and serum bactericidal antibodies (B) after intranasal (inl) immunization with the conjugate (CRM)–MenC vaccine. Groups of 5 BALB/c mice were immunized either subcutaneously (sc) with aluminium hydroxide (alum)–adjuvanted CRM-MenC vaccine (group 1) or inl with the same vaccine formulated with increasing doses of the LTK63 mutant (0.05, 0.1, or 1 μg) with or without a fixed dose of TMC (10 μg) (groups 4–9). Control inl groups received the vaccine without any adjuvant (group 2) or with 10 μg of TMC alone (group 3). Immunizations were performed on days 0, 21, and 35, as detailed in Materials and Methods. All groups always received the same amount of CRM-MenC vaccine, either sc or inl, consisting of 1 μg of the MenC oligosaccharide and 2 μg of CRM197. A, Serum anti–MenC IgG antibody titers measured by ELISA after 1 (post-1), 2 (post-2), and 3 (post-3) immunizations. Each column represents the mean titer ± 1 SD of each group at each time point. B, Serum bactericidal antibody titers tested on pooled samples obtained after 3 immunizations. +, Present; −, absent.

The use of full doses of TMC would limit the amounts of the LTK63 mutant necessary for the induction of strong and protective antibody responses against MenC. Indeed, at very low doses of the LTK63 adjuvant (i.e., 0.1 or 0.05 μg), high bactericidal antibody titers were induced if the CRM-MenC conjugate vaccine was coadministered together with TMC, but not in its absence. This was also true for the enhancement of the immune response to the CRM carrier and to LTK63 itself (data not shown). These data clearly show that the intrinsic mucosal adjuvanticity of these molecules can be efficiently enhanced by formulation together with appropriate bioadhesive materials, as was the case with nanoparticles reported elsewhere [14]. It is thus expected that the safety profile of these inl-delivered vaccines would be further enhanced by...
the concomitant use of the nontoxic LT mutant and TMC. In addition, the use of the LTK63 mutant favored a Th1-type immune response (data not shown) that otherwise would have been driven toward a Th2 functional phenotype by the TMC used alone inl or by alum given sc.

Our data show that protective immune responses to meningococcal conjugate vaccines can be improved by mucosal immunization using the association of 2 appropriate mucosal adjuvants/delivery systems. In particular, the quality of this protective immune response can be modulated depending on the appropriate dosing of the mucosal adjuvants.

Acknowledgments

We thank Brunella Brunelli and Laura Santini for expert help with bactericidal assays, Marco Tortoli and Giacomo Matteucci for continuous support with animal use, and Paolo Ruggiero for his critical reading of the manuscript.

References