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Induction of Protective Serum Meningococcal Bactericidal and Diphtheria-Neutralizing Antibodies and Mucosal Immunoglobulin A in Volunteers by Nasal Insufflations of the *Neisseria meningitidis* Serogroup C Polysaccharide-CRM197 Conjugate Vaccine Mixed with Chitosan

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Thirty-six healthy volunteers received either a single intramuscular injection of *Neisseria meningitidis* serogroup C polysaccharide (MCP)-CRM197 conjugate vaccine in alum or two nasal insufflations 28 days apart of the same vaccine powder, without alum, mixed with chitosan. Nasal immunization was well tolerated, with fewer symptoms reported than after intramuscular injection. The geometric mean concentrations of MCP-specific immunoglobulin G (IgG) after one nasal immunization were 3.25 μg/ml in naïve subjects and 14.4 μg/ml in subjects previously immunized parenterally, compared with 4.30 μg/ml in naïve subjects immunized intramuscularly. The geometric mean titer of serum bactericidal antibody (SBA) rose 24-fold after two nasal immunizations in naïve subjects and was comparable to parenteral immunization (1,080 versus 1,625). All subjects achieved SBA titers associated with protection after two nasal immunizations: even those with titers of <8 at entry. A single nasal immunization boosted the SBA titer to ≥128 in 96% of previously immunized subjects, and two immunizations achieved this level in 92% of naive subjects. MCP-specific IgG levels were ~70% IgG2 and ~20% IgG1 after nasal or intramuscular immunization. Increases in CRM197-specific IgG and diphtheria toxin-neutralizing activity were observed after nasal or intramuscular immunization, with balanced IgG1/IgG2 and higher IgG4. Significant MCP-specific secretory IgA was detected in nasal wash only after nasal immunization and predominantly on the immunized side. Simple nasal insufflation of existing MCP-CRM197 conjugate vaccines in chitosan offers an inexpensive but effective needle-free prime and boost against serogroup C *N. meningitidis* and diphtheria.

Neisseria meningitidis poses a significant global challenge with over 250,000 cases of serogroup A disease in sub-Saharan Africa annually (35) and outbreaks of group C throughout the developing world or when peoples are mixed together such as in the Saudi Arabian hajj. In developed countries, serogroup B predominates, followed by serogroup C in outbreaks (35). Polysaccharide antigens are inherently T helper cell independent and benefit from increased immunogenicity and memory by conjugation with protein carriers such as tetanus toxoid or CRM197. Short-term efficacy rates of conjugate vaccines are around 90% after three immunizations in the United Kingdom infant schedule or a single immunization in toddlers (6). Their introduction has reduced rates of pediatric *N. meningitidis* serogroup C infection in the United Kingdom significantly (6). Conjugate vaccines are extremely expensive to produce, and the requirement for multiple immunizations in young infants adds to the cost. Reports of breakthrough infection a year after the United Kingdom infant schedule (36) have prompted a discussion on the need for booster immunizations, probably in the second year of life but potentially in adolescence or even adulthood. While parenteral serogroup A and C polysaccharide vaccines have brought benefit in some African countries such as Egypt (28) and Benin (16), the problem of cold chain integrity and transmission of blood-borne viruses by parenteral immunization, together with high costs of conjugate vaccines, remains a major challenge to deployment throughout areas where *N. meningitidis* is highly endemic such as sub-Saharan Africa.

In human and animal models of immunization, the nasal route induces a systemic and disseminated mucosal immune response, making it attractive to deliver vaccines against mucosally transmitted invasive bacteria such as *N. meningitidis*. Various human studies have reported nasal delivery of nonreplicating antigens from *N. meningitidis* (1, 15, 20), *Shigella* (13), diphtheria/tetanus toxoids (2), cholera toxin B subunit (8, 31), *Pseudomonas* (23), and *Bordetella pertussis* (9, 10). Most studies have used developmental vaccines and/or complex or expensive delivery devices that have regulatory and cost implications likely to delay deployment to the areas with the highest disease burden. The cationic polysaccharide chitosan is primarily a mucoadhesive agent, as well as a mucosal adjuvant, which enhanced systemic and mucosal immune responses in

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animal models of intranasal immunization with CRM197 (24) and influenza antigens (5). Chitosan enhances transepithelial transport of antigen through an effect on tight junctions and by decreasing mucociliary clearance (4). We employed chitosan as we found it to be effective in a previous phase 1 clinical trial of a CRM197 diphtheria vaccine (25). As the nasal administration of alum is associated with adverse reactions (2), the Chiron "Menjugate-C" vaccine was selected as the alum adjuvant is in the diluent and not the polysaccharide-CRM197 powder.

This study sought to address whether the nasal delivery of a currently available and licensed meningococcal vaccine via the simplest possible delivery system could induce levels of immunity even roughly equivalent to the same vaccine given intramuscularly with alum adjuvant. As nasal immunization is particularly attractive for adolescent or adult booster vaccines, we included subjects previously immunized with Menjugate-C as part of the United Kingdom national immunization strategy in addition to meningococcal vaccine-naïve subjects. We also sought to determine whether CRM197, present as an immunological carrier, could induce useful immune responses against diphtheria toxin, as we had seen excellent responses when unconjugated CRM197 was given nasally (25). As we reported that priming and subsequent boosting restricted to the same nostril led to secretory immunoglobulin A (SIgA) responses restricted to the immunized side (25), we also allocated subjects to receive either two right-sided immunizations (ipsilateral) or a right-side immunization followed by a left-side ("contralateral") immunization to further explore the compartmentalization of the nasal immune system.

We report here the remarkably efficient and reliable induction of levels of meningococcal and diphtheria immunity associated with protection against disease, by simple syringe insufflation of Menjugate-C in chitosan.

MATERIALS AND METHODS

Subjects, immunization protocol, vaccine, and sample collection. The study was approved by the UK Medicines Control Agency (DDX reference MF8000/11871, subsequently converted to Clinical Trials Approval) and Wandsworth Local Research Ethics Committee. All subjects provided written informed consent. Exclusion criteria included contraindications for meningococcal conjugate vaccination present in the manufacturer's data sheet; having received a meningococcal vaccine within the preceding 12 months; known hypersensitivity to any component of the vaccines; severe or multiple allergies, including to drugs or pharmaceutical agents; history of nasal surgery; concomitant drug treatment or cardiac or respiratory disease; known impairment of immune function or receiving immunosuppressive therapy; acute infections; and women who were pregnant or capable of becoming pregnant who did not agree to have pregnancy testing and take effective contraception. As chitosan is produced from chitin, a substance derived from the exoskeleton of crustaceans, subjects with hypersensitivity to shellfish were routinely excluded from this phase 1 study, even though chitosan derivatives are widely used as excipients in pharmaceutical preparations and dietary supplements without reports of allergy induction.

Subjects who reported never having received a meningococcal vaccine previously were randomly assigned to receive either two intranasal immunizations 28 days apart—ipsilateral (both to the right nostril, group 1) or contralateral (right nostril for the first and left nostril for the second immunization, group 3)—or a single intramuscular immunization (group 4). Subjects who reported having received a meningococcal vaccine previously received two ipsilateral intranasal immunizations 28 days apart (group 2).

The vaccine used was Menjugate-C (Chiron Vaccines) supplied by Farillon UK and composed of 10 μg of meningococcal C oligosaccharide conjugated to between 12.5 and 25 μg of CRM197 per dose. Two batches of vaccines were used in the study, with the first 12 subjects receiving one batch (10 intranasal, 2 intramuscular) and the remaining subjects receiving the second batch. All subjects received the same dose of Menjugate-C by either nasal or intramuscular immunization. However, the intramuscular vaccine contained alum adjuvant, whereas the intranasal vaccine was free of alum. For the intramuscular immunization, the vaccine was resuspended in the alum-containing diluent provided and injected into the right deltoid. For intranasal immunization, the vaccine powder was removed by scraping from the vials and placed in a 5-ml Combitips Plus syringe (Eppendorf, Hamburg, Germany). The Combitips Plus syringe is a sterile polypropylene device widely used to dispense sterile liquids, with the advantages of a solid displacement plunger that expels powder without residual airspace and a very narrow opening to the tip that generates a fine aerosol. Seven milligrams of a GMP-certified powder formulation of chitosan glutamate G213 (FMC BioPolymer AS, Drammen, Norway) was added, and the plunger was replaced partially. The syringe was vortexed in the vertical position using a bench-top vortex to mix the powders and pack them loosely into the tip. Nasal vaccines were prepared and used within a day or two and kept at 4°C on desiccant before use. For nasal immunization, the tip of the syringe was placed into the nostril as far as it would go without causing discomfort, with the subject sitting in a semireclined position. The plunger was quickly depressed to expel the contents as an aerosol, and the subject was instructed to press on the nostril for a few minutes after the syringe was removed. Subjects remained under supervision for 30 min before leaving and were provided with a semistructured diary card to record any symptoms in the 28 days after each immunization. Symptoms recorded on the diary cards were logged as adverse events, and severity was ascribed as follows: mild, awareness of sign or symptom, but easily tolerated; moderate, discomfort enough to cause interference with usual activity; severe, incapacitating, with inability to do usual activity; and life-threatening, immediate risk of death from the event as it occurred. Adverse events (AE) were also classified as related to immunization as follows: not related, the AE is completely independent of vaccine administration; remote, the temporal association between the AE and the vaccine or the nature of the AE is such that the vaccine is not likely to have had any reasonable association with the observed illness/event (cause-and-effect relationship improbable but not impossible); possible, the AE follows a reasonable temporal sequence from vaccination but could have been produced by either the subject's clinical state or by vaccine administration; probable, the AE follows a reasonable temporal sequence from vaccination and is likely or very likely to be due to the study vaccine rather than another cause; definite, the AE has a direct cause-and-effect relationship to the study vaccine.

Nasal lavage was performed by inserting a Foley urinary catheter into the nostril, inflating the balloon gently to seal, and instilling normal saline, which was then aspirated after 5 min. Each nostril was sampled independently in all subjects on days −7, 14, and 28 and also on days 42 and 56 in those receiving intranasal vaccine. Samples were held on ice before immediately freezing at −80°C to prevent proteolysis of SIgA. Blood was drawn and serum separated for all subjects on days 0, 14, and 28 and also on days 42 and 56 in those receiving intranasal vaccine.

Enzyme-linked immunosorbent assay for serum and nasal wash IgG, IgG subclasses, and IgA. The enzyme-linked immunosorbent assay method used in this study has been described previously (17). Briefly, the concentration of coating antigen was 5 μg/ml in carbonate/bicarbonate buffer (pH 9.6; Sigma) for antibodies against meningococcal C polysaccharide (MCP) (NIBSC code 98/730) and also 5 μg/ml for antibodies against CRM197 (gift of Chiron Vaccines, Siena, Italy). The plates (96-well MaxiSorp; Nunc) with coating antigen were incubated at 4°C overnight. All serum samples were diluted to 1/100 and 1/300 with 0.05% phosphate-buffered saline–Tween 20, respectively. Nasal samples were diluted 1/2 or from neat. On each plate, one standard curve was set up to assign results as mass units. Negative and positive controls were also set up on each plate to monitor the reproducibility of the assays. The specific IgG or IgA antibody was detected by 1/10,000-diluted peroxidase-conjugated mouse anti-human IgG γ-chain-specific antibody (Sigma) or anti-human IgA α-chain-specific antibody (Sigma). The specific IgG subclasses were detected by peroxidase-conjugated sheep anti-human antibodies (Binding Site). The dilutions were used for each subclass as follows: 1/1,000 for IgG1, 1/500 for IgG2 and IgG3, and 1/250 for IgG4. The plates were developed with 3,3′,5,5-tetramethylbenzidine (Sigma) and read at 650 nm using a Vmax kinetic microplate reader (Molecular Devices, Crawley, United Kingdom). If a reading was above or below the relevant standard curve, further measurements were taken with higher or lower dilutions.

An anti-meningococcal serogroup A/C reference serum pool, CDC 1992 (NIBSC code 99/706), a commercial serum with a known specific antibody concentration, was used to obtain standard curves for measuring IgG and IgA antibodies against MCP. Two serum pools, one from five sera with a high concentration of IgG antibody against CRM197 (CRM1) and the other from seven subjects with high IgA concentrations (IgAss), were used as standards for

TABLE 1. Reactogenicity of nasal and intramuscular immunizations

Symptom	No. of subjects with symptom or % of group[a]							
	1st immunization				2nd immunization			
	i.m.	Nasal				Nasal			
		Naive		Ipsilateral preimmunized	% of whole group	Naive		Ipsilateral preimmunized	% of whole group
		Contralateral	Ipsilateral			Contralateral	Ipsilateral		
Injection site pain	4				66				
Mild	4				66				
Nasal discharge		3	4	6	48	0	1	4	19
Mild		2	2	6	19	0	1	4	19
Moderate		1	2	0	11	0	0	0	0
Nasal discomfort		0	3	3	22	1	2	4	26
Mild		0	1	2	11	0	1	3	15
Moderate		0	2	1	11	1	1	1	11
Nasal congestion		2	0	5	26	1	1	3	19
Mild		2	0	5	26	1	1	2	15
Moderate		0	0	0	0	0	0	1	4
Headache		2	4	2	30	1	1	4	22
Mild		0	3	1	15	0	1	1	7
Moderate		1	1	1	11	1	0	3	15
Severe		1	0	0	4	0	0	0	0
Sneezing		2	1	1	15	0	1	1	7
Mild		1	0	1	7	0	1	1	7
Moderate		1	1	0	7	0	0	0	0
Sore throat		0	1	0	4	1	1	0	7
Mild		0	1	0	4	0	1	0	4
Moderate		0	0	0	0	1	0	0	4
Itchy nose		1	1	0	7	0	1	0	4
Mild		0	1	0	4	0	1	0	4
Moderate		1	0	0	4	0	0	0	0
Watery eyes		1	0	0	4	2	0	0	7
Mild		0	0	0	0	1	0	0	4
Moderate		1	0	0	4	1	0	0	4
Eye pain		0	1	1	7	0	0	0	0
Mild		0	1	1	7	0	0	0	0
Severe		0	0	0	0	0	0	0	0

[a] Number of subjects recording symptoms on the diary card during 28 days after each immunization other than those designated as "unrelated" or "remote" relationship to immunization. Subjects may have had more than one occurrence of any symptom during this time but are counted only once. Row percentages are calculated for the 27 subjects in the nasal group and 6 subjects in the intramuscular (i.m.) groups. Severity grades were omitted if no subjects reported symptoms of that grade.

the measurement of IgG and IgA antibodies against CRM197. The commercial serum with a known concentration of specific IgG antibody, but without IgG subclass concentrations, from NIBSC (code 99/706) was also used as a standard for measurement of specific IgG1, IgG2, IgG3, and IgG4 antibodies against MCP. The concentrations of the above three standards with unknown concentration were assigned as μg/ml using an equal-potency method. This method and its principle have been described before in detail (17) and employs SPS01 (a calibration material for a specific protein assay, which is a normal human serum pool with known concentrations of IgG, IgG subclasses, and IgA antibodies, provided by the Immunology Quality Services, Sheffield, United Kingdom). By this method, the sum of specific subclass concentrations in the anti-meningococcal serogroup A/C reference serum pool was 31.51 μg/ml, which is very close to the concentration assigned by the company itself (32 μg/ml). CRM1 and IgAss contain 25 μg/ml anti-CRM197 IgG and 7.7 μg/ml anti-CRM197 IgA antibodies, respectively.

Neutralizing antibody assay. Serum samples were tested for specific anti-diphtheria toxin-neutralizing antibodies using a method similar to Miyamura et al. (26) as described previously (24). Briefly, serial twofold or threefold dilutions of serum or standard antitoxin for diphtheria (equine antisera from CBER, FDA, Bethesda, Md.; 6 IU/ml, diluted 1/200 prior to assay) in M199 medium were added to the wells of 96-well tissue culture plates. Plates were incubated at 37°C for 3 h with diphtheria toxin (Chiron Corporation). Following incubation, 10^4 Vero cells in M199 medium, supplemented with 10% fetal calf serum (FCS), were added to the plates. The neutralizing effect of antibodies versus diphtheria toxin was evaluated by analysis of the growth of Vero cells after 3 days of incubation at 37°C. Supernatants from each well were removed, and viable cells, which remained adherent to the plates, were fixed and stained with crystal violet. Following solubilization of the dye, cell density was detected by measurement of the absorbance at 540 nm. The levels of neutralizing antibodies in serum samples were expressed as IU/ml with reference to absorbance values obtained for the standard antitoxin. A level of greater than 0.01 IU/ml was taken as protective.

Serum meningococcal bactericidal antibody assay. Serum samples were tested for serum meningococcal bactericidal antibody (SBA) at the Vaccine Evaluation Department, Manchester Royal Infirmary, Manchester, United Kingdom, using

TABLE 2. Timing of onset of symptoms

Symptom and immunization	No. with symptom at day of onset[a]:							
	1st immunization				2nd immunization			
	0	1–2	3–14	15–28	0	1–2	3–14	15–28
Nasal								
Nasal discharge	10	2	2	1	5	0	0	3
Nasal discomfort	3	2	1	0	6	0	1	1
Nasal congestion	6	1	2	1	5	0	1	2
Headache	5	1	5	3	4	1	3	2
Sneezing	3	1	1	0	2	0	1	2
Sore throat	1	0	0	1	1	0	1	0
Itchy nose	1	1	1	0	1	0	1	0
Watery eyes	1	0	0	0	2	0	0	1
Eye pain	1	0	1	0	0	0	1	0
Intramuscular								
Headache	0	1	1	1				
Injection site pain	1	3	0	0				

[a] All symptoms recorded on the diary card during 28 days after each immunization are shown whether designated as related to immunization or not. Some subjects may have more than one episode of each type, and all episodes are counted. Symptoms lasting longer than 1 day are counted only on the day of onset.

baby rabbit sera as a source of complement in standardized protocols reported previously (11).

Statistical analysis. GraphPad Prism software was used for statistical analysis. Geometric mean concentrations (GMC) or titers (GMT) were calculated. Paired Student's *t* test was used to compare log-transformed data for within-group differences, and a two-tailed *P* value of <0.05 was taken as significant. Primary immunogenicity endpoints were antigen-specific serum IgG and IgA levels and SBA at 28 days after first (nasal and intramuscular groups) and second (day 56, nasal groups only) immunizations, compared with the preimmunization (day 0) level. Secondary endpoints were as follows: SIgA responses in nasal wash on days 28 and 56 compared to day 0 and day 56 MCP SIgA in left nostril compared with right nostril. Other secondary endpoints were diphtheria toxin-neutralizing activity (DNTA) on days 0 and 56 for subjects receiving intranasal immunization and days 0 and 28 for those receiving intramuscular vaccine. Repeated-measures one-way analysis of variance was also used to detect a trend in the antigen-specific SIgA response in nasal wash of each group for each nostril independently, using total antigen-specific SIgA values and total antigen-specific IgA/total SIgA ratio to correct for dilution by nasal lavage. Bonferroni's multiple-comparison test was used to detect differences in serum IgG subclass levels. No between-group comparisons were made, as this study was not powered to detect significant differences in unpaired data.

RESULTS

Subjects and route of immunization. Thirty-six healthy adult volunteers (10 male and 26 female; median age, 27 years, 25/75th centiles, 23.5/30 years; range, 20 to 43 years) were recruited. All subjects reported parenteral diphtheria vaccination more than 5 years previously. Nineteen subjects (12 male and 7 female; median age, 29 years) who reported never having received a meningococcal vaccine previously were randomly assigned to receive two ipsilateral intranasal immunizations ($n = 7$) or contralateral nasal immunizations ($n = 6$) or a single intramuscular immunization ($n = 6$). Seventeen healthy adult volunteers (2 male and 15 female; median age, 24 years) who reported having received a meningococcal vaccine (possibly a conjugated or unconjugated-polysaccharide) more than 1 year previously received two ipsilateral intranasal immunizations.

Safety and tolerability of the nasal vaccine. Intranasal immunization using the Combitips Plus syringe was well tolerated, with only transient and mild-to-moderate symptoms. No subjects withdrew from the study or refused to have the second intranasal immunization. Table 1 reports the frequency and severity of the most common symptoms reported by subjects, except those classified as only "remotely" related to immunization or unrelated. Table 2 reports the day of onset of all symptoms recorded by the subjects whether classified as related to immunization or not. On average, around one-third of subjects reported nasal symptoms such as congestion, discharge, or discomfort after the first immunization, and around a quarter reported such symptoms after the second, which compares favorably with approximately two-thirds reporting injection site pain after intramuscular immunization. Nasal symptoms did not occur within 20 min of delivery, and there were no instances recorded of loss of the vaccine due to sneezing or nasal discharge. There was no obvious trend to an exacerbation of nasal symptoms when ipsilateral immunization was used or in subjects who had previously received the parenteral vaccine.

Serum meningococcal C polysaccharide-specific IgG responses. As expected, intramuscular immunization with Menjugate-C induced a significant (13.4-fold) increase in the GMC of MCP-specific IgG in subjects naïve to meningococcal vaccine (Table 3). In subjects naïve to meningococcal vaccine receiving nasal immunization, there were a 3.3-fold increase in GMC from day 0 to day 28 after the first nasal immunization and a further 3.3-fold rise in GMC by day 56 (28 days after the second nasal immunization): a 10.7-fold rise overall. In contrast, in those subjects reporting previous parenteral immunization with meningococcal vaccine (over a year previously), while there was a 6.5-fold increase in GMC from day 0 to day 28 after the first nasal immunization, there was only a 1.3-fold increase after the second nasal immunization, which was not significant. In this group, two nasal immunizations induced an 8.6-fold increase in GMC overall.

Some escape of the insufflated powder from the nostril was noted on 6 of the 60 nasal immunizations: three occurrences on the first dose and three on the second, and the tip became blocked as it was introduced for the first dose on one occasion, requiring it to be cleared before the vaccine could be successfully delivered. These apparent delivery "failures" did not appear to affect the immune response of the individuals, as shown in Table 4, which compares individual serum MCP IgG responses to the GMC for the group.

Serum meningococcal C polysaccharide-specific IgG subclass responses. We studied IgG subclass profile on day 28 after a single intramuscular immunization and on day 56 after two nasal immunizations (Table 5). For one subject, a day 56 sample was not available and so the day 42 sample was used. After two nasal immunizations, we found MCP-specific IgG1/IgG2/IgG3/IgG4 ratios of 20:71:3:6 for the nasal group, with a similar profile in both naïve and preimmunized subjects and after intramuscular immunization.

Serum meningococcal C polysaccharide-specific IgA responses. Parenteral immunization with Menjugate induced a 9.1-fold increase in the GMC of MCP-specific IgA in subjects naïve to meningococcal vaccine (Table 3). Nasal immunization induced a pattern of serum MCP IgA responses similar to

TABLE 3. Serum MCP-specific IgG and IgA antibody GMC and SBA GMT

Group (no.) and day	GMC in μg/ml (95% CI)[a]		SBA GMT (95% CI)[b]
	IgG	IgA	
Intramuscular naïve (group 4)[c]			
0 (6)	0.32 (0.11–0.91)	0.74 (0.29–1.86)	4.49 (0.78–25.8)
14 (6)	4.53 (0.82–25)	6.99 (1.47–33.2)	
28 (6)	4.30 (0.89–21)**	6.75 (1.69–27.0)*	1,625 (122–21,652)**
Nasal naïve (groups 1 and 3)			
0 (13)	0.99 (0.39–2.51)	1.09 (0.71–1.65)	44.1 (6.68–290)
14 (13)	3.12 (0.80–12.2)	2.23 (1.27–3.94)	
28 (13)	3.25 (0.87–12.2)**	2.03 (1.34–3.08)**	57.5 (5.55–596)†
42 (12)	12.7 (4.18–38.4)	3.00 (1.75–5.12)	
56 (13)	10.6 (3.75–30.0)**	3.22 (1.76–5.89)**	1,080 (332–3,509)***/**
Nasal preimmunized (group 2)			
0 (17)	2.22 (1.07–4.57)	1.28 (0.85–1.93)	301 (72.2–1,257)
14 (17)	14.7 (8.64–25.1)	2.94 (1.81–4.78)	
28 (17)	14.4 (8.57–24.1)***	2.30 (1.38–3.82)**	1,308 (590–2,900)**
42 (16)	16.0 (9.78–26.1)	2.83 (1.66–4.82)	
56 (16)	19.1 (12.8–28.7)†	2.98 (1.72–5.14)*	2,139 (1,055–4,336)**/0.07
Nasal nonimmune[c]			
0 (9)			2.94 (1.84–4.70)
28 (9)			10.9 (1.48–80.1)†
56 (8)			362 (109–1,202)***/***

[a] *P* values for differences between days 0 and 28 and days 28 and 56: †, not significant ($P > 0.05$); *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.
[b] *P* values for differences between days 0 and 28 and days 0 and 56/28 and 56. Symbols are the same as those for footnote *a*.
[c] Subjects with SBA of ≤8 at entry in groups 1, 2, and 3.

serum IgG, in that naïve subjects had low but significant increases after both the first and second immunizations, whereas previously immunized subjects had a greater response to the first immunization (Table 3).

Serum meningococcal C bactericidal activity. SBA titers of ≥8 have been associated with protection in other studies (3, 11), and ≥128 has been used as an extremely strict surrogate of protection (3). The effect of immunization on SBA is shown in Table 3. As expected, parenteral immunization of subjects naïve to meningococcal vaccine with Menjugate-C induced a significant (362-fold) increase in the SBA GMT, with all subjects achieving levels of ≥8 and 5 of 6 subjects (83%) achieving levels of ≥128.

After one nasal immunization, there was no significant increase in the SBA GMT in subjects naïve to meningococcal vaccine, but after the second nasal immunization, there was a 24-fold rise from day 0 in this group and the SBA GMT was comparable to that of the parenterally immunized group (Table 3). After the first nasal immunization, 7/13 subjects (54%) had SBA of <8, and after two nasal immunizations, all subjects had SBA of ≥8 and 12/13 (92%) had SBA of ≥128.

In subjects reporting previous parenteral immunization with meningococcal vaccine (over a year previously, group 2), there were a 4.3-fold increase in SBA GMT after the first nasal immunization and a further 1.6-fold increase after the second nasal immunization, which did not quite reach statistical significance ($P = 0.07$). This suggests that the previous parenteral immunization effectively primed for an anamnestic response to nasal boosting but that a ceiling was reached after the first nasal immunization in some subjects, as seen with the IgG levels. After both the first and second nasal immunizations, no subjects had an SBA of <8 and only 1 (4%) had an SBA of <128.

To evaluate the reliability of nasal immunization in subjects who appeared to be genuinely nonimmune at entry, we pooled the nasal groups 1, 2, and 3 and stratified the SBA responses according to a preimmunization SBA of ≤8 (Table 3). This is a very strict definition, as SBA titers equal to 8 have been associated with protection (3, 11). Nine subjects had preimmunization levels of SBA that were ≤8. After the first nasal immunization, six subjects (67%) still had levels below 8 while the other two achieved levels of >128. After the second nasal immunization, all subjects had SBA of >8 and only 1 (13%)

TABLE 4. Serum MCP-specific IgG responses in subjects experiencing vaccine delivery problems

Delivery failure[b]	MCP IgG level (μg/ml) on day[a]:		
	0	28	56
Group 1 (naïve ipsilateral)			
Tip blockage on day 0	0.49	0.97	36.09
Escape of powder from nostril on day 0	1.37	50.92	59.36
GMC for group 1	0.42	1.16	4.44
Group 2 (preimmunized)			
Escape of powder from nostril			
Day 0	1.39	5.01	6.27
Days 0 and 28	3.30	24.64	19.02
Day 28	1.30	26.39	39.60
Day 28	13.09	15.53	26.53
GMC for group 2	2.22	14.4	19.1

[a] Specific MCP serum IgG.
[b] Day 28 results are for different subjects.

TABLE 5. Serum MCP-specific and CRM197-specific IgG subclass GMC and percentage of each subclass in total IgG concentration

Parameter for group	MCP				CRM197			
	Intramuscular naive	Nasal		Total nasal[c]	Intramuscular naive	Nasal		Total nasal[c]
		Naive[a]	Preimmunized[b]			Naive[a]	Preimmunized[b]	
No.	6	13	17	30	6	13	17	30
GMC (μg/ml)								
IgG1	4.3	3.3	5.6	4.4	66.4	19.4	30.6	25.1
IgG2	10	14.7	16.5	15.7	21.7	23.3	22.6	22.9
IgG3	0.56	0.48	1.0	0.7	0.4	0.29	0.23	0.25
IgG4	1.1	1.4	1.2	1.3	92.0	43.3	39.1	40.9
% of total IgG[d]								
IgG1	27	17	23	20	37	22	33	28
IgG2	63	74	68	71	12	27	24	26
IgG3	4	2	4	3	0.2	0.3	0.2	0.3
IgG4	7	7	5	6	51	50	42	46
Between-subclass differences[e]								
IgG1 vs IgG2	NS	***	*	***	NS	NS	NS	NS
IgG1 vs IgG3	*	***	***	***	*	***	***	***
IgG1 vs IgG4	NS	NS	***	***	NS	NS	NS	NS
IgG2 vs IgG3	***	***	***	***	NS	***	***	***
IgG2 vs IgG4	**	***	***	***	NS	NS	NS	NS
IgG3 vs IgG4	NS	*	NS	NS	**	***	***	***

[a] Groups 1 and 3.
[b] Group 2.
[c] Groups 1, 2, and 3.
[d] May not total 100% due to rounding.
[e] Bonferroni's multiple-comparison test: NS, not significant ($P > 0.05$); *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

had an SBA of <128. It appears therefore that two nasal immunizations may be required to ensure protective levels are reliably achieved in naïve subjects.

Serum CRM197-specific IgG responses. All subjects reported previous childhood and adolescent immunization with standard diphtheria toxoid vaccines, which would have induced cross-reacting antibodies to CRM197. The serum CRM197 IgG appeared slightly lower on day 0 in the subjects reporting no previous immunization with a meningococcal vaccine (groups 1, 3, and 4), suggesting that the CRM197 present in that vaccine may have boosted antibody levels slightly (Table 6).

Two batches of vaccines were used in the study, with the

TABLE 6. Serum CRM197-specific IgG and IgA antibody GMC and DTNA GMT

Group (no.) and day	GMC in μg/ml (95% CI)[a]		DTNA GMT (95% CI)[b]
	IgG	IgA	
Intramuscular naïve			
0 (6)	1.92 (0.73–5.04)	0.64 (0.46–0.90)	0.24 (0.09–0.61)
14 (6)	69.8 (11.2–436)	1.33 (0.32–5.53)	
28 (6)	87.8 (14.6–527)**	1.70 (0.44–6.61)†	518 (14.6–18363)**
Nasal naïve[c]			
0 (13)	4.48 (2.14–9.40)	0.62 (0.43–0.91)	2.91 (0.17–50.0)
14 (13)	11.6 (5.39–24.7)	0.95 (0.68–1.32)	
28 (13)	10.8 (4.76–24.4)*	0.82 (0.62–1.08)†	
42 (12)	26.3 (12.9–53.7)	0.83 (0.56–1.22)	
56 (13)	23.4 (11.4–48.1)***	0.99 (0.80–1.22)†	181 (10.7–3055)**
Nasal preimmunized[d]			
0 (17)	8.06 (3.68–17.7)	0.63 (0.42–0.97)	32.6 (2.83–377)
14 (17)	19.1 (8.39–43.7)	0.76 (0.44–1.32)	
28 (17)	19.8 (9.08–43.2)**	0.66 (0.36–1.22)†	
42 (16)	32.5 (16.2–65.4)	1.10 (0.81–1.49)	
56 (16)	28.2 (11.3–70.3)**	1.00 (0.69–1.46)†	387 (53.0–2826)*

[a] P values for differences between days 0 and 28 and days 28 and 56: †, not significant ($P > 0.05$); *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.
[b] P values for differences between days 0 and 28 and days 0 and 56. Symbols are the same as those for footnote *a*.
[c] Groups 1 and 3 combined.
[d] Group 2.

potential for the amount of CRM197 to vary between 12.5 and 25 μg per dose due to differences in efficiency of conjugation. However, intramuscular immunization was able to induce a 46-fold increase the CRM197-specific IgG GMC. In subjects who reported not receiving a meningococcal vaccine previously (in the United Kingdom, this is most likely to be CRM197-containing meningococcal conjugate), there were also significant increases in CRM197-specific IgG GMC: 2.4-fold after the first nasal immunization and a further 2.2-fold after the second nasal immunization. In subjects who reported having had a previous meningococcal vaccine, there were significant responses after both first (2.5-fold) and second (1.4-fold) nasal immunizations, with a similar pattern to those seen to MCP.

Serum CRM197-specific IgG subclass responses. In contrast to the results with MCP, after two nasal immunizations, we found CRM197-specific IgG1/IgG2/IgG3/IgG4 ratios of 28:26:0.3:46 for the nasal group, with a similar profile in both naïve and preimmunized subjects and after intramuscular immunization (Table 5).

Serum CRM197-specific IgA responses. In marked contrast to serum CRM197 IgG responses and MCP IgA responses, neither intramuscular nor two intranasal immunizations induced significant serum CRM197 IgA responses (Table 6).

Serum diphtheria toxin-neutralizing activity. A level of serum diphtheria toxin-neutralizing activity (SDTNA) above 0.01 U/ml is associated with protection. Due to constraints on sample volumes, we were only able to measure SDTNA on days 0 and 28 after intramuscular immunization and 0 and 56 after two nasal immunizations. There were a 2,158-fold increase in the GMT SDTNA of subjects after intramuscular immunization and a 62-fold rise after two nasal immunizations (Table 6) in subjects in groups 1 and 3. There was a 12-fold rise in the SDTNA after two nasal immunizations of subjects who had already received previous meningococcal immunization. As none of the subjects had a preimmunization SDTNA of <0.01, an analysis of efficacy of intranasal immunization to achieve levels associated with protection was not possible.

Meningococcal C polysaccharide-specific secretory IgA responses in nasal wash. The most significant responses were seen in subjects that had received parenteral meningococcal vaccine previously and who therefore received ipsilateral nasal immunization (Table 7). In these subjects, a significant 5.2-fold rise in the MCP-specific SIgA GMC from day 0 was seen in the immunized nostril after the second nasal immunization. A 2.4-fold, just significant, increase in MCP-specific SIgA GMC also occurred in the unimmunized nostril. However, there was only a significant linear trend in the MCP-specific SIgA increase on the immunized side ($P < 0.0001$). This was also true for the MCP-specific SIgA/total SIgA ratio ($P = 0.01$), which corrects for dilution during lavage. Furthermore, the MCP-specific SIgA GMC at day 56 was significantly higher in the immunized than the unimmunized nostril ($P = 0.04$), as was the MCP-specific SIgA/total SIgA ratio ($P = 0.03$).

A similar pattern was seen with ipsilateral nasal immunization of subjects who had not previously had parenteral meningococcal vaccine (group 1), who manifested slight increases after each nasal immunization, especially on the immunized side. However because of a scatter in the day zero values, these changes did not reach statistical significance in the small number of subjects studied, except paradoxically in the unimmu-

TABLE 7. Nasal wash MCP-specific and CRM197-specific secretory IgA antibody GMC

Group	GMC in ng/ml (95% CI) for nostril lavaged at day:								
	Unimmunized side				Immunized side				
	0	14	28/42[a]	56	P^b	0	14	28/42[a]	56	P^b
MCP										
Intramuscular naïve	77 (36–164)	223 (71–700)	157 (62–395)		NS	100 (44–223)	285 (114–717)	281 (68–1168)		NS
Intranasal										
Naïve ipsilateral	80 (62–103)	206 (113–375)	143 (109–188)	199 (139–285)	***	184 (86–394)	329 (86–1266)	182 (79–419)	288 (109–761)	NS
Preimmune ipsilateral	86 (48–152)	185 (124–275)	155 (104–231)	204 (94–443)	*	97 (49–191)	136 (78–238)	441 (219–891)	505 (230–1108)	***
Naïve contralateral	102 (71–145)	67 (23–199)	118 (66–213)	139 (61–317)	NS	72 (50–103)	112 (85–149)	158 (75–332)	106 (73–153)	NS
CRM197										
Intramuscular naïve	22 (4–122)	78 (23–259)	72 (27–191)		NS	37 (8–175)	81 (23–283)	72 (51–102)		NS
Intranasal										
Naïve ipsilateral	59 (34–102)	120 (47–308)	94 (41–215)	152 (99–235)	*	83 (44–157)	101 (56–182)	85 (57–127)	117 (59–232)	NS
Preimmune ipsilateral	37 (16–83)	97 (63–150)	70 (41–121)	77 (37–162)	NS	54 (28–105)	90 (54–150)	73 (48–112)	127 (71–226)	*
Naïve contralateral	51 (28–93)	49 (9–264)	55 (19–158)	68 (27–172)	NS	40 (23–70)	70 (37–132)	120 (52–276)	83 (37–185)	*

[a] Day 28 for intramuscular group and day 42 for nasal immunization groups.
[b] P value for differences between days 0 and 28 (intramuscular group) and days 0 and 56 (nasal groups) for each nostril separately: NS, not significant ($P > 0.05$); *, $P < 0.05$; ***, $P < 0.001$.

nized nostril, where by chance the day zero values had less variance.

In contrast, contralateral nasal immunization induced poor responses, and while there were some increases in MCP-specific SIgA on the immunized side on day 42 and on the unimmunized side on day 56, which correlated with the order in which the nostrils were immunized, overall there were no significant increases or linear trends using this sequence of nasal immunization.

In some subjects naïve to previous exposure to a meningococcal vaccine, there was an increase in nasal wash MCP-specific SIgA after intramuscular immunization, which was not restricted to the left or right nostril (Table 7), as might be expected given the parenteral route of immunization. However, the difference in the GMC between day 0 and 28 was not significant, nor was there a linear trend in the values.

We observed the same pattern of responses for each group with regard to kinetics over time and immunized versus unimmunized dominance, regardless of whether the data were expressed as MCP-specific SIgA (Table 7) or corrected for the effect of lavage dilution as the MCP-specific SIgA/total SIgA ratio (data not shown).

CRM197-specific secretory IgA responses in nasal wash. Overall the responses to CRM197 were extremely low in all groups (Table 7). Very slight, and just significant, increases on day 56 compared to day 0 were observed on the right side in those subjects receiving contralateral immunization (group 3) and on the left in naïve subjects receiving ipsilateral immunization (group 1)—which is paradoxical as these are the opposite sides to the immunization and suggest a nonspecific source of antibody. Subjects who had received both parenteral diphtheria and meningococcal vaccines (group 2) had low but significant response on the immunized side. Parenteral immunization did not induce significant responses.

DISCUSSION

Nasal immunization with MCP-CRM197 powder in chitosan was well tolerated, with around a third of subjects reporting mild-to-moderate nasal symptoms, in contrast to two-thirds reporting pain after intramuscular injection. Also reassuring was a decrease in the frequency of symptoms after the second nasal immunization and no trend to increased symptoms in subjects who had previously received a meningococcal vaccine.

The delivery technique for nasal immunization was deliberately simple and inexpensive, and the formulation has potential for optimization. Therefore, while intramuscular immunization with alum adjuvant induced the highest levels of antibody overall, the similar levels of MCP-specific serum IgG induced in naïve volunteers after a single nasal immunization and the higher levels induced by a single nasal immunization in subjects already preimmunized are encouraging for the use of nasal powder delivery for both primary and booster meningococcal vaccines. The MCP IgG GMC responses observed in our study after nasal immunization in vaccine-naïve subjects were similar to those reported by Goldblatt et al. (14) after parenteral immunization of young adults with the same vaccine, either naïve subjects (GMC, 40.6; 95% confidence interval [CI] 29.2 to 56.6) or as a booster of unconjugated AC polysaccharide vaccine (GMC, 41.1; 95% CI, 29.9 to 56.5). The SBA responses we observed after nasal immunization were also similar to those in the previous study of parenteral immunization (14) (SBA GMT of naïve subjects, 734.6; 95% CI, 474.3 to 1,138; and preimmunized subjects, 454.9; 95% CI, 264 to 782). We found two nasal immunizations were required to ensure adequate levels of serum antibody in naive subjects but induced a level of SBA associated with protection in all subjects who had unprotective levels at entry and titers of $\geq$128 in 87%. In contrast, a single intranasal immunization appeared sufficient as a booster, as a second immunization did not result in increased responses, and 96% of subjects achieved SBA of $\geq$128 after just one nasal insufflation. CRM197 is widely used in humans as a licensed polysaccharide antigen carrier and despite possible variation in the amount present in each dose, both nasal and intramuscular immunizations with Menjugate-C induced comparable levels of CRM197 IgG antibodies and markedly increased diphtheria toxin-neutralizing activity. This observation is worthy of more detailed study, as it offers prospects for economic savings if conjugate vaccines can usefully boost diphtheria immunity.

Whether the failure of preimmunized subjects to further increase MCP-specific serum IgG was due to local secretory IgA or transudated IgG blocking antigen uptake or to the timing of the second dose will require further study. Partial loss of vaccine powder from the nostril and blockage of the tip did not affect immune responses, suggesting scope for optimization of formulation and reduction of antigen. Lying the subject down may prevent escape of vaccine, and further reductions in dose may be achieved by additional mucosal adjuvants such as enterotoxogenic *Escherichia coli* heat-labile toxin mutants, which enhance immune responses to nasal immunization with MCP in chitosan in mice (7, 12). Further studies of acceptability and immunogenicity of nasal powder insufflation in babies and toddlers are indicated, as are studies of whether the efficient bioadhesive and ciliary paralytic actions of chitosan (33) can overcome problems of chronic rhinitis and nasal discharge observed in infants in resource-poor areas.

Protein antigens preferentially induce IgG1 and IgG3, as well as IgG4 on repeated boosting; whereas polysaccharides induce more IgG2, due to respective T-helper-cell-dependent or -independent priming. We observed a predominantly IgG2 response to MCP, with less IgG1 and almost no IgG3 or IgG4 whether the antigen was delivered nasally or parenterally. This pattern has been observed in studies of parenteral immunization with MCP (18, 19). In contrast, CRM197-specific IgG subclasses were mixed, with equal levels of IgG1 and IgG2 and high levels of IgG4 in many subjects, probably indicating repeated boosting. Again this pattern was independent of route of delivery and has been seen with other protein antigens delivered nasally (1, 27) and with parenteral diphtheria vaccines (29). It would appear therefore that nasal or parenteral immunization has less influence on the IgG subclass profile than the protein or polysaccharide nature of the antigen. Recent observations that breakthrough infections may occur some years after primary immunization with MCP-conjugate vaccines (36) have raised questions over their ability to induce long-term protection. Further studies of the memory phenotype and mucosal homing markers present on antigen-specific B cells and duration of antibody responses after nasal and parenteral MCP-conjugate vaccines will therefore be of interest.

In a rodent model of antigen uptake by nasal mucosa-associated lymphoid tissue (22, 32), it was found that particulate antigens were taken up by M cells, presented to B and T cells within nasal mucosa-associated lymphoid tissue which drains to posterior cervical lymph nodes, and induced both local and systemic immunity. In contrast, soluble antigen was taken directly to superficial cervical lymph nodes and induced tolerance rather than immunity. An objective of our study was to investigate the observation from our previous study (25) that nasal immunization induces a significant SIgA response almost exclusively in the nostril that was immunized—an example of in situ maturation of plasmablasts and tight compartmentalization within the "common mucosal immune system," also seen after tonsillar injection (30). In this study, the highest MCP-specific SIgA nasal wash levels were observed in subjects who had been previously immunized parenterally and nasally immunized ipsilaterally. In these the SIgA response was largely restricted to the immunized nostril, suggesting that the SIgA secreted in the anterior portion of the nares is induced by locally resident plasmablasts and not from migratory cells in a "common mucosal immune system." The compartmentalization was not as pronounced as in a previous study (25) in which powder was insufflated into the lower part of the nostril, rather than injected higher up. By washing out only the lower part of the nasal cavity, we may only have detected localized responses to powder deposited low down, to the apparent disadvantage of the syringe insufflation method, which may also be more effective at driving powder through the opening in the septum between the nostrils.

Previous parenteral diphtheria toxoid immunization appeared to prime only for a serum IgG and not a nasal SIgA response to CRM197 whatever the route of boosting. In contrast, MCP-specific IgA could be induced in both nasal wash and serum. Although mucosal carriage of serogroup C *Neisseria meningitidis* is low in the United Kingdom (35), the responses to MCP may reflect previous mucosal priming by colonization of the respiratory tract with cross-reacting *Neisseria meningitidis* antigens. A similar pattern of responses has been described previously after parenteral immunization with tetanus toxoid, which induced only a serum IgG response, compared with a serum IgA response to parenteral immunization with polysaccharide antigens from *Haemophilus influenzae*, *N. meningitidis*, and *Streptococcus pneumoniae* (34). It would appear that mucosal priming is superior to induce mucosal responses on boosting. The ability of mixed mucosal-systemic prime-boost strategies to induce a balanced mucosal and systemic immune response is obviously attractive for vaccine against a mucosally acquired invasive organism such as *N. meningitidis.* Further study of the phenotype of circulating B cells secreting antibody to these antigens after mucosal-prime/parenteral boost, could be useful, as it has been shown previously that oral poliomyelitis vaccine can prime for the induction of B cells bearing mucosal addressins ($\alpha 4\beta 7$ integrin) in response to subsequent parenteral boost (21).

In conclusion, this phase 1 study indicates that an available, alum-free, polysaccharide conjugate vaccine can reliably induce levels of immunity associated with protection against both meningococcal C disease and diphtheria when delivered nasally as powder, comparable with the same vaccine delivered intramuscularly. The amount of polysaccharide antigen used was only 0.1% (wt/wt) of the delivered powder, allowing multiple polysaccharide or other antigens to be combined in one delivery. The potential exists for powder prefilled into a small disposable syringe, with an autodeactivating plunger for single use, sealed in packaging designed to keep the powder dry. Industrial-scale methods to dispense powders are in development, together with dry formulations that may extend the lifetime of powders outside the cold chain. By saving on the cost of liquid diluent and removing needles and the need to assemble or reconstitute a liquid vaccine, such a system could offer a cheap but reliable method to deliver vaccines nasally, while removing the risks and expense associated with needles or jet-injecting devices, and opens the possibility of an affordable multivalent, needle-free delivery system applicable to resource-poor settings.

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