Nasopharyngeal Colonization by *Neisseria lactamica* and Induction of Protective Immunity against *Neisseria meningitidis*

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**Background.** Natural immunity to *Neisseria meningitidis* may result from nasopharyngeal carriage of closely related commensals, such as *Neisseria lactamica*.

**Methods.** We enrolled 61 students with no current carriage of *Neisseria* species and inoculated them intranasally with 10,000 colony-forming units of *Neisseria lactamica* or sham control. Colonization was monitored in oropharyngeal samples over 6 months. We measured specific mucosal and systemic antibody responses to *N. lactamica* and serum bactericidal antibody (SBA) and opsonophagocytic antibodies to a panel of *N. meningitidis* serogroup B strains. We also inoculated an additional cohort following vaccination with *N. lactamica* outer-membrane vesicles (OMV) produced from the same strain.

**Results.** Twenty-six (63.4%) of 41 inoculated individuals became colonized with *N. lactamica*; 85% remained colonized at 12 weeks. Noncarriers were resistant to rechallenge, and carriers who terminated carriage were relatively resistant to rechallenge. No carriers acquired *N. meningitidis* carriage over 24 weeks, compared with 3 control subjects (15%). Carriers developed serum IgG and salivary IgA antibodies to the inoculated *N. lactamica* strain by 4 weeks; noncarriers and control subjects did not. Cross-reactive serum bactericidal antibody responses to *N. meningitidis* were negligible in carriers, but they developed broad opsonophagocytic antimeningococcal antibodies. OMV vaccinees developed systemic and mucosal anti-*N. lactamica* antibodies and were relatively resistant to *N. lactamica* carriage but not to natural acquisition of *N. meningitidis*.

**Conclusions.** Carriers of *N. lactamica* develop mucosal and systemic humoral immunity to *N. lactamica* together with cross-reacting systemic opsonophagocytic but not bactericidal antibodies to *N. meningitidis*. Possession of humoral immunity to *N. lactamica* inhibits acquisition of *N. lactamica* but not of *N. meningitidis*. Some individuals are intrinsically resistant to *N. lactamica* carriage, independent of humoral immunity.
N. meningitidis. It is preferentially carried by young children, whereas carriage of N. meningitidis is rare [7, 8]. As childhood progresses, N. lactamica carriage becomes less frequent, and N. meningitidis carriage becomes more frequent [9]. In concert with this prevalence of SBA and IgG antibody, titers to N. meningitidis increase [10].

Elucidating the mechanism of natural protection will aid development of vaccines against N. meningitidis, particularly serogroup B strains, for which no broadly effective vaccines are available. To provide definitive evidence of the role of N. lactamica in natural immunity to N. meningitidis, we inoculated young adults with live N. lactamica, assessed subsequent carriage of Neisseria species, and measured their immune responses.

MATERIALS AND METHODS

Participants and Study Design

We screened volunteers for pharyngeal carriage of Neisseria species. Healthy individuals aged 18–45 years, who were negative for carriage after 2 throat swabs taken 2 weeks apart, were enrolled in the study. Those excluded from enrollment were smokers, pregnant women, those with a history of meningitis, immunocompromized persons or those with allergies or antibiotic or steroid exposure within the previous 3 months, meningococcal vaccination within the previous 5 years, and contacts of immunocompromised persons. Our study was approved by the UK National Research Ethics Service and was monitored by an independent safety committee.

Volunteers were randomized to receive either N. lactamica strain Y92 - 1009 [11] (sequence type 3493, clonal complex 613) or phosphate-buffered saline (PBS) under investigator-blind conditions. Vials of 2 × 10^9 bacteria, stored in Frantz medium containing 30% (v/v) glycerol, were prepared in cGMP pharmaceutical manufacturing facilities at the Centre for Emergency Preparedness and Response, Porton Down. The vials were stored at −80°C and then thawed and diluted in PBS before inoculation. We conducted inoculation with the volunteer supine. We administered into each nostril 500 μL of PBS containing either 10^4 N. lactamica per mL or PBS containing no bacteria. We chose this dose because we could prepare it with reproducible precision, and in pilot experiments, it induced colonization in at least 50% of volunteers (data not shown). Before inoculation and over 24 weeks, volunteers provided oropharyngeal samples for Neisseria screening. They also provided blood and saliva samples for immunological analysis. Figure 1 illustrates our study design. Six months after we completed our study, we invited volunteers to enroll for reswabbing, and those who tested negative were invited to be rechallenged with N. lactamica at the same dose.

To observe the role of N. lactamica humoral immunity in carriage of Neisseria, we recruited study participants of a recently completed phase 1 clinical trial of intramuscular vaccination safety and immunogenicity. This trial used outer-membrane vesicles (OMV) of the same strain of N. lactamica Y92 1009 (EudraCT number 2005-002191-15) [11]. We observed that the vaccine in this trial elicited high levels of systemic antibody (IgG) against N. lactamica and modest cross-protective immunity against a panel of 6 strains of serogroup B N. meningitidis [11]. At study termination, 4 weeks after a fourth dose of OMV, volunteers were enrolled and screened for Neisseria carriage; 13 of 17 were Neisseria negative and received live challenge. Neisseria carriage and immune responses were measured over 4 weeks. (Figure 1).

Oropharyngeal Sampling for Neisseria Species and Microbiological Procedures

We obtained oropharyngeal samples from all volunteers by both posterior pharyngeal swabbing and gargling. In pilot work, this combination had a 96% sensitivity for detection of Neisseria species (Supplementary Table 1). Posterior pharyngeal swab specimens were plated directly onto GC-selective agar (See http://www.bd.com/ds/technicalCenter/inserts/GC_Lect_Agar.pdf). Gargle samples comprise 5 mL of 0.9% (w/v) sodium chloride. Volunteers gargled for 20 s and 5 mL of Mucolyse (Pro Lab Diagnostics) was added to the sample, which we centrifuged at 5,000 g for 5 min. We then used a 200 µL pellet to inoculate GC-selective agar.

N. lactamica colonies were identified using X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactoside, Sigma), resulting in blue-green precipitate formation [12]. Negative colonies were Gram stained, tested for oxidase reaction, and characterized using the API NH system (Biomerieux).

We confirmed that recovered isolates were the inoculated strain based on an Rsrl restriction sitem which is absent from the pyruvate dehydrogenase E1 gene (pdhC) of N. lactamica Y92-1009 but present in the other 450 N. lactamica strains in the MLST database [13]. A 910-bp segment of pdhC was amplified by colony polymerase chain reaction (PCR; primers 5′-tcgcgatgattccgttcta-3′ and 5′-actaactaacaagtctgcg-3′) and digested with Rsrl to yield either an intact 910-bp band from strain Y92-1009 or bands at 618 and 292 bp from spontaneously acquired strains. We used a gel-purified fragment of pCR4Blunt as a positive control substrate for the restriction digest.

Serological Assays

ORACOL (Malvern Medical Supplies) devices were used for saliva collection, extracted by 5-min centrifugation in transport buffer at 2,000 g. Aliquots were stored at −80°C. Blood samples were centrifuged for serum separation and stored at −20°C.

Enzyme-Linked Immunosor bent Assay

We performed anti-N. lactamica IgG OMV enzyme-linked immunosorbent assay ELISA, as described elsewhere [11].
N. lactamica OMV-specific IgA was determined as for IgG, except that PBS containing .1% (v/v) Brij35 was used as the wash buffer and goat anti-human IgA phosphatase conjugate (Jackson Immunoresearch) was used for detection. Total IgA was also determined for each saliva sample in a similar assay using plates coated with anti-human IgA.

N. lactamica OMV-specific IgA and total IgA in saliva were determined and expressed as a ratio of specific IgA units per microgram of total IgA, with fold increases calculated from these values.

SBA Assay

The assay was performed against 6 representative serogroup B N. meningitidis strains (44/76-SL, M01-240101, M01-240013, M01-240149, M01-240355, M01-240185), as described elsewhere [11]. Human serum (25%) was used as an exogenous complement source, and SBA titers were expressed as the reciprocal of the final dilution, giving ≥50% killing at 60 min, compared with the control (inactive complement and/or no test serum). Titers <2 were assigned a value of 1 for analytical purposes.

Opsonophagocytosis Assay

We performed opsonophagocytosis Assay (OPA) as described elsewhere [11]. Each sample was analyzed in duplicate. A significant increase in OPA level between time points for an individual was an increase that exceeded the variability of data from all duplicates in each assay, with a probability of >95%, calculated using a z test.
among 210 healthy volunteers screened for nasopharyngeal carriage of N. lactamica, 138 were noncarriers at enrollment (Figure 1). Sixty-one of these noncarriers were willing and able to enter the challenge study. Of these, 41 were challenged intranasally with 10^4 cfu of N. lactamica and 26 (63.4%; 95% confidence interval [CI], 49.5%–77.9%) became colonized. In the latter group, carriage of N. lactamica remained stable over 12 weeks, and 17 remained colonized at 24 weeks (Table 2). In all cases of N. lactamica carriage, the isolate was typed by PCR as the inoculum strain. All 15 individuals who were challenged with but not colonized by N. lactamica remained culture negative for N. lactamica. Among 20 control subjects who received sham preparation, none carried N. lactamica over the study course, but 3 volunteers (15%) acquired N. meningitidis. In contrast, none of the 41 who received N. lactamica live challenge acquired N. meningitidis (P = .032) over 24 weeks. Transient carriage of commensal Neisseria species was detected in 3 volunteers (2 challenged and 1 control subject). One was characterized as Neisseria cinerea. Two were not identified further.

In samples taken before challenge, concentrations of salivary IgA and serum IgG against N. lactamica were no different between control and challenged groups or between challenged volunteers who became colonized or not (analysis of variance IgA: P = .615 IgG: P = .752). Median specific total salivary IgA values were 6.9 for controls, 5.7 for noncolonized, and 7.8 for colonized. Median total serum IgG titer values were 424 for control subjects, 573 for noncolonized, and 559 for colonized.

**Induction of Immunity**

Following inoculation with live N. lactamica, volunteers who were colonized generated increased specific salivary IgA to N. lactamica over 12 weeks (Figure 2), compared with those who were challenged but not colonized (P < .001) and control subjects (P = .05). Likewise, there was an increase in serum IgG to N. lactamica in colonized compared with noncolonized individuals, (P = .005) and control subjects (P = .005) (Figure 2). Among control subjects, 3 individuals had a >2-fold increase in either IgA or IgG titer (data not shown), suggesting possible contact with Neisseria species, despite negative throat swab culture results. In individuals who spontaneously lost carriage of N. lactamica, mucosal IgA and serum IgG responses were not significantly different than those observed in individuals who remained colonized, comparing median fold increase at the time of colonization loss, with final fold increase in those colonized (IgA: P = .845, IgG: P = .823) or maximum fold-increase values between the groups (IgA: P = .759, IgG: P = .61; data not shown).

Twelve weeks after inoculation, a > 4 fold rise in SBA against one or more of 5 standard N. meningitidis strains was observed in 69% of colonized individuals, compared to 53% of noncolonized and 55% controls (Figure 3). These differences were not significant when comparing each group (P = .513) or live-challenged versus control subjects (P = .577). We observed no consistent pattern of SBA response to the 6 representative N. meningitidis strains tested. Overall, the mean geometric SBA titers in all volunteers were low grade (Supplementary Table 2). We did not include in this analysis control subjects who became colonized with N. meningitidis.

In contrast, we observed significant OPA responses against ≥1 strains of N. meningitidis (colonized, 65%; noncolonized, 53%; control subjects, 28%) (Figure 3). Across the 3 groups, these differences were significant (P = .048), with colonized individuals exhibiting a significant increase in OPA against this panel of meningococci, compared with control subjects (P = .030). We observed no significant increase in those not colonized.

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**Table 1. Study design for the initial challenge study, rechallenge, and challenge of volunteers previously vaccinated with a Neisseria lactamica outer-membrane vesicle (OMV) vaccine (OMV Challenge)**

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<tr>
<td>Blood sampling</td>
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<tr>
<td>Blood sampling</td>
<td>+ + + + + + + + + + +</td>
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**Statistical Analysis**

Statistical analysis was performed using GraphPad Prism 5 software. Categorical variables are presented as percentages with contingency table analysis including χ2 test and Fisher’s exact tests. This analysis was performed for colonization data, SBA, and OPA results.

Serological data were analyzed using 1-way analysis of variance, except where stated. For serological analysis among smaller groups (ie, OMV and baseline naive individuals), a Mann-Whitney test was used. Log-transformed data were used to construct areas under the curve for comparison of immune responses over the 12-week study period.

**RESULTS**

**Colonization by Neisseria lactamica**

Among 210 healthy volunteers screened for nasopharyngeal carriage of Neisseria species, 138 were noncarriers at enrollment (Figure 1). Sixty-one of these noncarriers were willing and able to enter the challenge study. Of these, 41 were challenged intranasally with 10^4 cfu of N. lactamica and 26 (63.4%; 95% confidence interval [CI], 49.5%–77.9%) became colonized. In the latter group, carriage of N. lactamica remained stable over 12 weeks, and 17 remained colonized at 24 weeks (Table 2). In all cases of N. lactamica carriage, the isolate was typed by PCR as the inoculum strain. All 15 individuals who were challenged with but not colonized by N. lactamica remained culture negative for N. lactamica. Among 20 control subjects who received sham preparation, none carried N. lactamica over the study course, but 3 volunteers (15%) acquired N. meningitidis. In contrast, none of the 41 who received N. lactamica live challenge...
Table 2. Colonization: positive swabs for all *Neisseria* species recorded over the 6-month study period, following live challenge with *N. lactamica*. Control subjects (*n* = 20), noncolonized (*n* = 15), and colonized (*n* = 26). Volunteers with consistently negative swabs are not shown.

<table>
<thead>
<tr>
<th>Study Volunteer</th>
<th>1 wk</th>
<th>2wks</th>
<th>4wks</th>
<th>7wks</th>
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Challenged with but not subsequently colonised by *N.lactamica* (*n* = 15)

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<th>2wks</th>
<th>4wks</th>
<th>7wks</th>
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Controls (*n* = 20)

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<th>4wks</th>
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- Culture positive for *Neisseria lactamica* and PCR positive Y92 1009
- Culture positive for *Neisseria meningitidis*
- Culture positive for commensal *Neisseria*
- Culture Negative for *Neisseria*

Rechallenge of Volunteers with *N. lactamica*

Six months after we completed our study, 11 of 26 volunteers who had initially carried *N. lactamica* were no longer colonized. Following a second challenge with $10^4$ cfu *N. lactamica*, 2 of 11 volunteers became colonized. This was a reduction in carriage rate from 100% to 18% ($P = .015$) (Figure 1). Likewise, 13 of...
the 15 persons who were originally challenged but not colonized were rechallenged with *N. lactamica*, but none became colonized. Of these, 6 were then rechallenged with a higher dose of $10^5$ cfu, and 3 (50%) became colonized.

The median specific to total salivary IgA was 6.5 in naive individuals (all subjects before either inoculation with control PBS or live challenge with *N. lactamica*). Before rechallenge, the median IgA was 9.2 in the previously colonized group ($P = .028$) and 5.8 in the noncolonized group (not significant). Respective values for serum IgG titers were 522 in naive individuals, 1087 in previously colonized ($P = .002$) individuals, and 519 in noncolonized (not significant). We saw no evidence of a booster response 4 weeks after rechallenge in either group. With regard to induction of cross-reacting SBA against the panel of *N. meningitidis* strains, 5 (46%) of 11 of the previously colonized and 4 (23%) of 13 of the noncolonized individuals had a $\geq 4$-fold increase in SBA titer, which was not statistically significant ($P = .675$).

**N. lactamica** Intranasal Challenge of *N. lactamica* OMV Vaccine Recipients

Amongst 17 individuals who had received 4 doses of an *N. lactamica* OMV vaccine, 3 were carriers of *N. meningitidis* (carrier rate, 17.6%) at the point of enrollment, 4 weeks after their fourth (booster) dose. Thirteen of the volunteers who were non-carriers of *Neisseria* species at enrollment received live challenge with $10^4$ *N. lactamica*. We detected a significantly lower uptake of *N. lactamica* carriage (215.3% of 13; $P = .004$), compared with that observed in naive individuals (Figure 1). Both median values for salivary IgA (OMV group, 9.2; challenge group, 6.5; $P = .024$) and serum IgG (OMV group, 10510; challenge group, 522; $P = .001$) were significantly elevated at baseline in volunteers who had received OMV vaccination. Of the 11 who were not colonized by *N. lactamica* after experimental challenge, 2 (15.3%) were subsequently colonized by *N. meningitidis* (Figure 1) by 4 weeks after challenge.

Following live challenge, 9 (69%) of 13 OMV-vaccinated individuals had a significant SBA response against $\geq 1$ strain of meningococci; the same percentage SBA response was seen following colonization by *N. lactamica* in unvaccinated individuals.

**DISCUSSION**

We have demonstrated that young adults can be colonized with *N. lactamica* and that this induces salivary IgA and serum IgG

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**Figure 2.** Response in specific: total salivary IgA and total serum IgG to *Neisseria lactamica* over the 12-week study period. Controls ($n = 20$), noncolonized ($n = 15$), colonized ($n = 26$). A significant increase was shown in specific:total salivary IgA in the colonized individuals, compared with noncolonized individuals ($P = .001$) and control subjects ($P = .05$). Likewise, there was an increase in total serum IgG titer to *N. lactamica* in colonized individuals, compared with noncolonized individuals ($P = .005$) and control subjects ($P = .006$). Analysis was performed using Kruskal-Wallis of the area under the curve.

**Figure 3.** Twelve-week serological data: comparison of serum bactericidal antibody (SBA; percentage with a $\geq 4$-fold increase against $\geq 1$ *Neisseria meningitidis* strain) and of Opsonophagocytosis (percentage with a significant increase in FI-C against $\geq 1$ *N. meningitidis* strain). Response against a panel of 6 serogroup B *N. meningitidis* strains. Control subjects ($n = 20$), noncolonized ($n = 15$), and colonized ($n = 26$). A $\geq 4$-fold increase in SBA titer was observed in 69% of colonized individuals, compared with 53% of noncolonized individuals and 55% of control subjects. These differences were not significant when comparing each group ($\chi^2 P = .513$). OPA analysis revealed a significant difference between the 3 groups ($\chi^2 P = .048$), with colonized individuals exhibiting a significant increase in opsonophagocytic activity against this panel of meningococci, compared with control subjects (Fisher’s exact test, $P = .030$).
against this organism by 4 weeks. Despite this humoral immunity, carriage of the organism persisted in most for at least 24 weeks, but re-acquisition of the organism was inhibited after carriage had been terminated naturally. Colonization induced cross-reactive opsonophagocytic but not bactericidal antibodies to *N. meningitidis*. Colonization restricted acquisition of carriage of *N. meningitidis*. These findings are consistent with the strong epidemiological evidence that carriage of *N. lactamica* is protective against meningococcal disease, even during the early phase of life, when SBA titers are low [10, 14].

Currently, an SBA titer ≥4 in the presence of human complement is used as a threshold for meningococcal vaccine efficacy [15], but it has been argued that this may exceed the threshold required for natural protection [16]. Natural humoral immunity against meningococcal disease could be mediated by cumulative generation of antibodies that mediate opsonophagocytosis or are below the threshold of detection in the SBA assay. Our data suggest that such immunity does not prevent meningococcal carriage, because OMV vaccination generates similar levels of cross-reactive opsonophagocytic antibody, however, did not protect against *N. meningitidis* carriage. From this we conclude that it may be the physical presence of *N. lactamica* in the nasopharynx, or alternatively non-humoral acquired responses, such as cell mediated immunity, which inhibits acquisition of meningococcal carriage amongst *N. lactamica* carriers.

Mathematical modelling has predicted that *N. lactamica* carriage inhibits *N. meningitidis* carriage for a mean of 4.7 years [17]. Only a few infection studies have reported dual colonization of both *Neisseria* species, and all have described very low levels of meningococcal infection in the presence of elevated *N. lactamica* carriage [18, 19]. This effect could result from direct inhibition by competition for nutrients or adhesion [20].

The young adults studied here had, as expected, relatively low mucosal and systemic, specific anti-*N. lactamica* antibodies at baseline. Although no initial difference in antibody levels was detected among those who did or did not become colonized, the very high concentrations of antibodies generated by OMV vaccination did retard *N. lactamica* (but not *N. meningitidis*) colonization. This effect on colonization by cognate strains reflects the serogroup-specific effect of meningococcal conjugate vaccines on meningococcal carriage [21]. We found that induction of IgA and IgG occurred only following infection by the organism, implying that this requires the organism to be sampled intimately over a prolonged period in the mucosa. Likewise, McCool et al [22, 23] inoculated human volunteers with *Streptococcus pneumoniae* and detected induction of systemic anti-pneumococcal antibody but only in those who were successfully colonized. The clear generation of mucosal and systemic immunity responding to colonization in our current study contradicts previous assumptions that *N. lactamica* adapts to maintain immunological ignorance in the host [24]. However, lack of a booster effect in those undergoing repeat inoculation after shedding *N. lactamica* colonization confirms that *N. lactamica* does not strongly prime mucosal T or B cell memory [24].

One striking observation of our current study is that a proportion of individuals were resistant to repeated experimental carriage. We could explain this by differences in mucosal or systemic antibody. Furthermore, only in 50% carriage in this group could be induced by inoculation with a 10-fold higher dose. This implies that carriage varies intrinsically by individuals’ susceptibility to these microorganisms. Rake [25] serially sampled the nasopharynx of laboratory workers continually exposed to meningococci and identified individuals who were highly susceptible, transiently susceptible, and non-susceptible to infection by *N. meningitidis*. *N. meningitidis* attaches to nasopharyngeal epithelium via multiple receptor-adhesin interactions [26], including between opacity-associated adhesion (Opa) proteins of the organism and human carcino-embryonic antigen cell adhesion molecule (CEACAM) proteins. Host variation in receptor specificity might underlie colonization resistance. Indeed, we know that haplotypes of CEACAM-encoding genes are associated with susceptibility to meningococcal disease [27].

Limitations of our study include sampling frequency and detection methods. A recent review [28] supports our method of swabbing the posterior pharyngeal wall with direct plating as the most sensitive technique. However, we could have supplemented our method with direct PCR detection of organisms [29]. Increased sampling frequency may have revealed higher rates of dual carriage with *N. meningitidis*. Our control population included some individuals who exhibited ≥4-fold increases in SBA titer over our study period. This probably is a simple consequence of sampling young healthy adults in a university hospital community and may reflect undetected carriage of *N. meningitidis* during our study.

The pattern of opsonophagocytic and bactericidal responses in colonized and control volunteers was similar, but only the opsonophagocytic response was significant. We contend this is a difference due to biology rather than to experimental method, because it has been shown that the pattern of immunoglobulin isotypes generated after antigen challenge can determine opsonophagocytic versus bactericidal activity [30]. Also, it is already established that humans who receive non-polysaccharide meningococcal vaccines can exhibit opsonophagocytic responses when bactericidal responses are absent [31].

In conclusion, carriage of *N. lactamica* results in a specific mucosal and serum antibody response, plus the generation of cross-reacting antibodies against a broad range of serogroup B *N. meningitidis* strains, but these antibodies promote opsonophagocytosis rather than serum bactericidal activity. These
findings support the hypothesis that carriage of N. lactamica contributes to protection against meningococcal carriage and disease, but this is unlikely to be due to induction of cross-reacting humoral immunity.

**Supplementary Material**

Supplementary materials are available at Clinical Infectious Diseases online (http://www.oxfordjournals.org/our_journals/cid/).

Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Acknowledgments**

We thank the volunteers who participated in the study: Linda Godwin, and Margaret Lee, who contributed technical expertise; the Clinical Research Facility at Sheffield Royal Hallamshire Hospital for hosting the Challenge study; Professors Roger Finch and Richard Moxon for reviews of safety issues; Meningitis UK and all their donors for financial support of this study; and Christoph Tang and Joel Ernst, for their critical review of our manuscript.

_Potential conflicts of interest._ All authors: no conflicts.

**References**


