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Influence of age and carriage status on salivary IgA to *Neisseria meningitidis*

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SUMMARY

Asymptomatic carriage of *Neisseria meningitidis* is common (5–35% of individuals) while the incidence of invasive meningococcal disease is fairly low (<1–5 per 100 000 per annum in Europe). Naturally acquired protective immunity may account for this difference. In this study, we investigated the relationship between anti-meningococcal salivary IgA and age and carriage. We showed that salivary IgA to a range of meningococcal antigens increased successively with age with some specificity for commonly circulating serosubtypes. In a group of 258 students 37 (14%) of whom were carriers of *N. meningitidis* serogroup B, higher levels of specific IgA were associated with carriage. Stratified analysis revealed a positive relationship between smoking and specific anti-*N. meningitidis* IgA independent of current carriage, weighted odds ratio (OR) 4·1 (95% CI 1·1–18) and OR 3·8 (95% CI 0·96–16) for reference strains B:1:P1.14 and B:4:P1.5,4 respectively. These data implicate IgA as a factor in host defence from meningococcal invasion, although the precise mechanisms remain uncertain.

INTRODUCTION

Despite an effective meningococcal serogroup C conjugate (MenC) vaccination programme, *Neisseria meningitis* (largely serogroup B disease) is still the commonest cause of bacterial meningitis in the United Kingdom, and is a frequent cause of septicaemia and shock in children and young adults (Health Protection Agency, see http://www.hpa.org.uk/). Asymptomatic meningococcal carriage is common, occurring in 5–10% of the general population [1–3], a figure which may be as high as 35% in late teenage years [4, 5]. The incidence of invasive meningococcal disease, in contrast, is relatively low at <1–5 per 100 000 per annum in Europe.

The disparity between the frequency of carriage and disease may be partly accounted for by the low virulence of most carriage strains but also suggests a role for naturally acquired protective immunity [6, 7]. The process by which natural immunity arises is poorly understood but it is thought to occur through single or multiple colonization events at the mucosal surface by *N. meningitidis*, related organisms such as *N. lactamica* and antigenically cross-reacting flora.

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Although there is considerable evidence that circulating complement-fixing IgG antibody, high in bactericidal activity, is one of the mainstays of protection against invasive disease, mucosal clearance through the elaboration of secretory IgA is also considered to be important in limiting colonization by *N. meningitidis* and preventing early invasion. We have previously shown that T-cell-mediated immunological memory to *N. meningitidis* antigens is present at the mucosal level and that the magnitude of these responses is strongly associated with increasing age [8]. In this study, we investigated whether secretory IgA is implicated in the mucosal immune response to *N. meningitidis* serogroup B (MenB) by determining the relationship between anti-MenB salivary IgA, age and current asymptomatic carriage.

MATERIALS AND METHODS

Meningococcal antigens

MenB strain H44/76 (B15:P1.7,16;L3,7,9), isogenic strains expressing different PorA types TR4 (B15:P1.7–8,4), TR10 (B15:P1.5–2,10) and a spontaneous PorA-negative mutant (B15:P1.–.–) were provided by the National Vaccine Institute, The Netherlands [9, 10]. Outer membrane vesicles (OMVs) were prepared by deoxycholate detergent extraction, as previously described [8].

Subjects and samples

The following subjects were sampled in compliance with the relevant guidelines and institutional practices as part of other immunological studies:

Adults (*18–45 years*). Fifteen consecutive healthy adult volunteers (United Bristol Healthcare Trust Local Research Ethics Committee E4388).

Children (*7 years*). Fifteen healthy children selected at random from 100 consecutive subjects attending the Avon Longitudinal Study of Parents and Children (ALSPAC; for details see http://www.alspac.bris.ac.uk), (Ethics Committee number: E4233).

Infants (*2–3 months*) *and toddlers* (*2–3 years*). Thirty healthy subjects selected at random from two other immunological studies (South Sheffield LREC 00/0089).

College students (*carriage study*). A total of 258 volunteers recruited from two colleges in Hereford, England (Herefordshire District LREC.454).

Data was collected on age, sex and smoking. Meningococcal carriage status of the volunteers was determined by selective culture of mouth and tonsil swabs, as previously described [5]. Strains isolated were sent to the Health Protection Agency Meningococcal Reference Unit, Manchester, for *N. meningitidis* confirmation and serological phenotype characterization. Bacterial lysates of the carriage strains were made by heating bacterial suspensions for 1 h at 60 °C, and using as the coating antigen for ELISAs at 4 μg/ml, as described below. Salivary IgA concentrations specific for the strain(s) being carried at the time of sampling were measured for each MenB carrier in the study ($n=37$), in addition to two other reference MenB strains: a *N. meningitidis* B:1:P1.14 (18/37 subjects carrying a strain with the P1.14 variable region) and a *N. meningitidis* B:4:P1.5,4 (1/37 subjects carrying a strain with the P1.5 and P1.4 variable regions). Twenty non-carriers from the study were also chosen at random and tested for salivary IgA against the two reference strains.

Saliva samples were obtained using oral testing kits (Malvern Medical Developments Ltd, Worcester, UK) [11] by rubbing the sponge over the gums at the base of the teeth for ~1 min. Sterile forceps were used to remove the sponge from the swab and a 5-ml syringe used to extract the saliva directly into a cryo vial for storage at −80 °C until use.

ELISA to measure salivary IgA levels specific for *N. meningitidis* OMVs

Using a protocol based on the method of Borrow et al. [11], Maxisorp ELISA plates (Nunc, Roskilde, Denmark) were coated with 4 μg/ml OMV in carbonate buffer (pH 9·6), consisting of 15 mM Na_2CO_3 (BDH Chemicals, Poole, UK) and 35 mM $NaHCO_3$ (BDH Chemicals), overnight at 4 °C. After blocking with PBS, 0·1% Tween-20 (Sigma, St. Louis, MO, USA) and 1% BSA (Sigma), test samples were serially diluted and incubated for 2 h at room temperature. As there is no standard available for *N. meningitidis*-specific IgA, a pool of serum from a volunteer with high serum IgA levels was used on every plate to provide a consistent reference. Plates were washed and incubated for 1 h at room temperature with horseradish peroxidase-conjugated rabbit anti-human IgA (Dako, Glostrup, Denmark) to detect antibody. The assay was left to develop with *o*-phenylenediamine dihydrochloride (OPD) (Sigma) and stopped after 30 min with 1 M H_2SO_4. Absorbance readings at 490 nm were recorded. Arbitrary values were assigned to samples using weighted probit regression analysis

[12] to compare with the internal serum standard used.

Western blotting with saliva as an antibody source

For Western blotting [13], OMVs in NuPAGE LDS sample buffer (Invitrogen, CA, USA), were boiled briefly and loaded onto a NuPAGE 10% Bis–Tris pre-cast gel (Invitrogen). This was run at 180 V for 1 h. Transfer was carried out after soaking in transfer buffer (2·91 g Trizma base, 1·47 g glycine, 0·188 g SDS and 100 ml methanol in 400 ml distilled water) using a Trans-blot semi-dry transfer cell (Bio-Rad, Hercules, CA, USA) for 15 min at 15 V. The gel was then stained using GelCode Blue stain reagent (Pierce, Rockford, IL, USA). Nitrocellulose was blocked overnight at 4 °C with PBS and 5% BSA. Saliva samples diluted in PBS, 0·1% Tween-20 and 1% BSA were incubated with the blot for 2 h at room temperature. After washing, alkaline phosphatase-conjugated goat anti-human IgA (Dako) was added and incubated for 1 h at room temperature. The blot was developed with Nitro Blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP), and the reaction stopped with water.

Statistical analysis

Analysis was carried out using Prism version 3.0 (GraphPad Software, San Diego, CA, USA) and Epi-Info, version 6.0 (CDC, Atlanta, GA, USA). All data-points are represented individually and medians are shown. The Kruskal–Wallis H test was used to examine differences in IgA levels between age groups. Friedman's non-parametric ANOVA was used to compare IgA levels in different meningococcal strains within both age groups and carriage groups, and comparisons between carriers and non-carriers were made using the Mann–Whitney U test. The relationship between IgA levels and smoking was assessed by weighted stratified Mantel–Haenszel analysis.

RESULTS

Salivary IgA levels to meningococcal OMVs increased with age

To investigate the relationship between mucosal IgA acquisition and age, salivary IgA specific for three MenB OMVs, differing solely in their serosubtype, was measured by ELISA across four age groups of subjects (Fig. 1). Salivary IgA to both the TR4 (B15:P1.7–8,4) and the PorA-negative (B15:P1.–.–) OMVs increased successively with age group (Fig. 1). A similar increase in IgA levels was observed with the TR10 (B15:P1.5–2,10) OMV, although the differences were much less marked and the titres were highest in children not adults. Within each age cohort, significantly different concentrations of salivary IgA were seen in all three OMVs (infants $P=0{\cdot}0002$, toddlers $P<0{\cdot}0001$, children $P=0{\cdot}0023$, adults $P<0{\cdot}0001$) with levels to the TR10 OMV consistently lower than to both the TR4 and the PorA-negative OMV (Fig. 1).

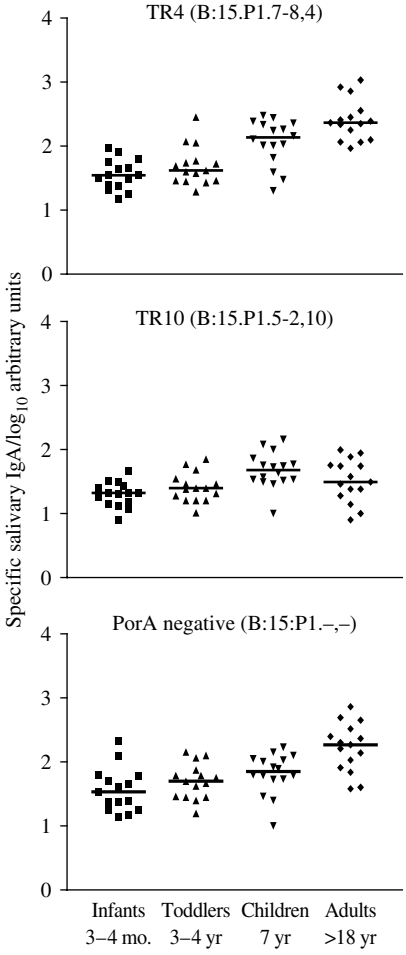

Fig. 1. Relationship between age and anti-meningococcal IgA detected by ELISA. IgA concentrations towards strain TR4 (B15:P1.7–8,4) and the PorA-negative mutant are generally higher than to the TR10 (B15:P1.5–2,10) strain. Each group represents 15 individuals. Group medians are shown by horizontal lines.

Saliva contains IgA to meningococcal antigens

To investigate the specificity of the anti-MenB IgA detected by ELISA, Western blotting against MenB

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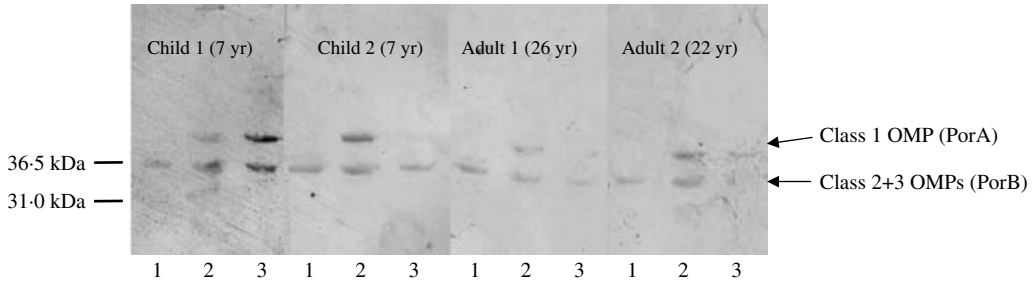

Fig. 2. Specificity of anti-meningococcal IgA detected by Western blot. Lane 1, PorA-negative OMV; lane 2, strain TR4; lane 3, TR10. The outer membrane proteins indicated are based on molecular weights: PorA (46 ± 1 kDa), PorB (41/38 ± 1 kDa), Rmp (33 ± 1 kDa) and Opa/Opc (28 ± 1 kDa) [31].

OMVs using saliva as an antibody source was carried out. Multiple bands were observed, the most prominent being consistent with the outer membrane proteins PorA and PorB (Fig. 2). Comparisons of several adults and children revealed some variation in band intensity but the pattern of reactivity appeared fairly consistent and cross-reactivity was not restricted at younger ages.

Anti-meningococcal IgA levels in saliva are higher in carriers and smokers

To explore the relationship between current colonization by *N. meningitidis* and anti-meningococcal IgA levels in saliva we made use of a large carriage study of students attending two colleges in the same town in the United Kingdom [5]. In the study, 90/258 individuals (35%) were found to be carriers of *N. meningitidis* of which 37 (41%) were carrying MenB strains (14 had more than one strain). The mean ages and male to female ratios in the carriage and non-carriage groups were similar (18·9 *vs.* 19·5 years and 22:14 *vs.* 8:13 respectively).

Comparison of anti-meningococcal salivary IgA against two reference MenB strains (B:1:P1.14 and B:4:P1.5,4) in 37 carriers and 20 randomly selected non-carriers showed that carriage was associated with an increase in MenB-specific IgA (median values of 115·6 and 113·0 compared with 34·3 and 44·7 respectively) (Fig. 3). Amongst carriers, no significant strain-specific difference in salivary IgA levels was found (median value of 142·0 against the carriage strain compared to 115·6 and 113·0 for the reference MenB strains respectively, $P = 0{\cdot}47$) (Fig. 3).

In these students, carriage rates were higher in smokers than non-smokers (49/90 *vs.* 51/168, $P < 0{\cdot}001$) [5]. Although the numbers in each group are fairly small, stratified analysis revealed a relationship between smoking and specific anti-*N. meningitidis* IgA independent of current carriage, weighted odds ratio (OR) 4·1 (95% CI 1·1–18) and OR 3·8 (95% CI 0·96–16) for the reference strains B:1:P1.14 and B:4:P1.5,4 respectively (Fig. 4). The ages and sex ratios of smoking groups were similar to those of the carriage groups.

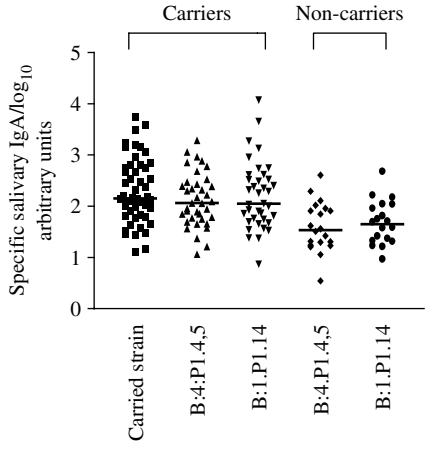

Fig. 3. The influence of meningococcal carriage on anti-meningococcal IgA. There is no significant difference between the levels of IgA towards the strain carried at the time of sampling and two alternative strains, B:1:P1.14 and B:4:P1.5,4 ($P = 0{\cdot}4692$). Salivary IgA levels to the two alternative strains are considerably lower in non carriers ($P = 0{\cdot}009$ and $P = 0{\cdot}0017$ respectively). Medians are shown as horizontal bars.

DISCUSSION

Carriage of *N. lactamica* in young children [14] and of *N. meningitidis* in late teenage and early adulthood [6] are linked to the development of 'natural' immunity, providing protection against meningococcal disease in most adults [15]. Initial colonization by these bacteria occurs in the nasopharynx where IgA is the most

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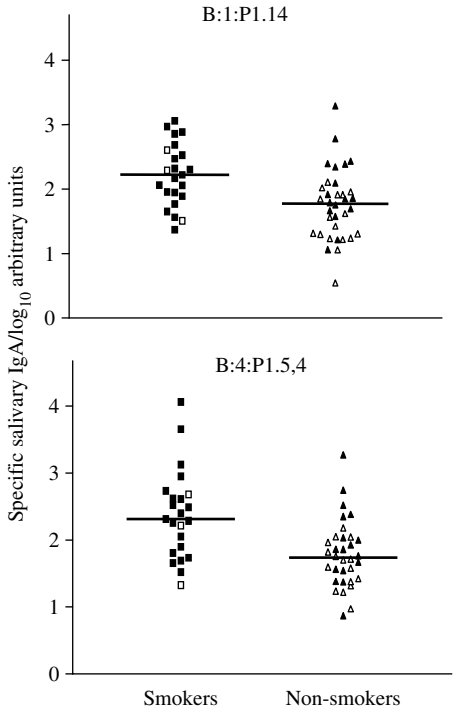

Fig. 4. Relationship between carriage, smoking and anti-meningococcal IgA. Specific salivary IgA levels in smokers ($n=23$) and non smokers ($n=34$) against meningococcal strains B:1:P1.14 and B:4:P1.5,4. Carriers are depicted by solid symbols (▲, ■), and non-carriers by open symbols (△, □). Medians are shown by horizontal bars.

abundant antibody, approximately 10 times the concentration of IgG [16]. IgA has a major role in protection against upper respiratory tract infection by viruses [17, 18] and is also thought to protect against invasion by bacterial colonizers such as *N. meningitidis*, *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Moraxella catarrhalis* [19–22]. In this study, we showed age-related increases in levels of salivary IgA to *N. meningitidis* in line with age-related increases in the prevalence of carriage [7]. Since carriage is transient in most individuals [4, 23], these findings suggest acquisition of immunological memory at the mucosal level. The tonsil is a common site of colonization by *N. meningitidis* [24] and is an important inductive site for mucosal immunity. We have previously demonstrated mucosal T-cell-mediated immune memory which increases with age [8]. The presence of salivary IgA suggests that this cellular immunity is associated with long-lived plasma cells resident in the mucosa. Although the principle tissue source of mucosal IgA is unknown, antibody-secreting cells (ASC) specific for antigens of the mucosal pathogen *S. pneumoniae* have been demonstrated in tonsils of otherwise healthy children [25], and we have found *N. meningitidis*-specific ASCs in the tonsil (R. E. Horton, unpublished observations).

PorA is an immunodominant surface antigen of *N. meningitidis* [26] which gives rise to the serosubtype designation of meningococcal strains. We have found that the prevalence of MenB serosubtypes in the UK population at the time of sampling was reflected in the concentrations of serosubtype-specific IgA detected. High salivary IgA levels against the P1.7,4 serosubtype (detected in 38·3% of carriers in England and Wales, 2000–2001) were demonstrated, whereas the P1.5,10 serosubtype (detected in 1·18% of carriers) gave rise to lower IgA concentrations. The observed influence of PorA on IgA levels is in line with previous studies of carriage and systemic immunity [27, 28] and our immunoblotting experiments have confirmed the presence of salivary IgA to this meningococcal surface protein. It is noteworthy, however, that salivary IgA was also detected against the PorA-negative OMVs, suggesting immunity to other antigens including outer membrane proteins such as PorB.

To investigate the link between current carriage and salivary IgA levels, we studied higher-education college students, a population with a high *N. meningitidis* colonization rate. Robinson et al. have previously reported the association of *N. meningitidis* carriage in UK university students with increasing concentrations of salivary IgA to a reference MenB strain, H44/76 [27, 28]. We showed that carriers have higher levels of anti-meningococcal salivary IgA against both the reference and carriage strains, suggesting either considerable cross-reactivity and/or multiple previous carriage events. Remarkably, the anti-*N. meningitidis* IgA levels appear to be increased in smokers. We, and others, have previously shown that smoking is associated with increased *N. meningitidis* carriage rates [29, 30], but not increased incidence of invasive disease. We have found higher anti-meningococcal IgA levels in the saliva of smokers than in non-smokers irrespective of current carriage. It is widely accepted that conventional sampling considerably underestimates carriage of *N. meningitidis* [5, 24] and, therefore, under-ascertainment may partially explain this observation. Nonetheless, it is likely that our finding of a relationship between smoking and specific IgA levels reflects increased current as well as previous asymptomatic carriage. Whether smoking also increases the intensity of carriage, or the frequency of carriage events leads to a prolonged

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period of carriage or augments the immune response to *N. meningitidis* has yet to be ascertained.

In conclusion, we have shown that anti-*N. meningitidis* IgA concentrations in saliva increased both with age and current carriage. Smokers also showed increased IgA levels which may reflect the higher rates or intensity of *N. meningitidis* carriage. It is widely assumed that IgA has an important role in mucosal defence against *N. meningitidis* which is supported by our data. Investigations into the precise mechanisms of this IgA-mediated defence are now required.

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REFERENCES

1. **Greenfield S, Sheehe PR, Feldman HA.** Meningococcal carriage in a population of 'normal' families. J Infect Dis 1971; **123**: 67–73.
2. **Caugant DA, Hoiby EA, Magnus P, et al.** Asymptomatic carriage of Neisseria meningitidis in a randomly sampled population. J Clin Microbiol 1994; **32**: 323–330.
3. **Cartwright KA, Stuart JM, Jones DM, Noah ND.** The Stonehouse survey: nasopharyngeal carriage of meningococci and *Neisseria lactamica*. Epidemiol Infect 1987; **99**: 591–601.
4. **Neal KR, Nguyen-Van-Tam J, Jeffrey N, et al.** Changing carriage rate of *Neisseria meningitidis* among university students during the first week of term: cross sectional study. Br Med J 2000; **320**: 846–849.
5. **Orr HJ, Gray SJ, Macdonald M, Stuart JM.** Saliva and meningococcal transmission. Emerg Infect Dis 2003; **9**: 1314–1315.
6. **Goldschneider I, Gotschlich EC, Artenstein MS.** Human immunity to the meningococcus. II. Development of natural immunity. J Exp Med 1969; **129**: 1327–1348.
7. **Coen PG, Cartwright K, Stuart J.** Mathematical modelling of infection and disease due to Neisseria meningitidis and Neisseria lactamica. Int J Epidemiol 2000; **29**: 180–188.
8. **Davenport V, Guthrie T, Findlow J.** Evidence for naturally acquired T cell-mediated mucosal immunity to Neisseria meningitidis. J Immunol 2003; **171**: 4263–4270.
9. **Peeters CC, Rümke HC, Sundermann LC, et al.** Phase I clinical trial with a hexavalent PorA containing meningococcal outer membrane vesicle vaccine. Vaccine 1996; **14**: 1009–1015.
10. **van der Ley P, van der Biezen J, Poolman JT.** Construction of Neisseria meningitidis strains carrying multiple chromosomal copies of the porA gene for use in the production of a multivalent outer membrane vesicle vaccine. Vaccine 1995; **13**: 401–407.
11. **Borrow R, Fox AJ, Cartwright K.** Salivary antibodies following parenteral immunization of infants with a meningococcal serogroup A and C conjugated vaccine. Epidemiol Infect 1999; **123**: 201–208.
12. **Bailey M, Williams NA, Wilson AD.** PROBIT: weighted probit regression analysis for estimation of biological activity. J Immunol Methods 1992; **153**: 261–262.
13. **Wedege E, Kuipers B, Bolstad K.** Antibody specificities and effect of meningococcal carriage in icelandic teenagers receiving the Norwegian serogroup B outer membrane vesicle vaccine. Infect Immun 2003; **71**: 3775–3781.
14. **Gold R, Goldschneider I, Lepow ML.** Carriage of Neisseria meningitidis and Neisseria lactamica in infants and children. J Infect Dis 1978; **137**: 112–121.
15. **Pollard AJ, Frasch C.** Development of natural immunity to Neisseria meningitidis. Vaccine 2001; **19**: 1327–1346.
16. **Kerr M.** The strucrue and function of human IgA. Biochem J 1991; **271**: 285–296.
17. **Weltzin R, Monath TP.** Intranasal antibody prophylaxis for protection against viral disease. Clin Microbiol Rev 1999; **12**: 383–393.
18. **Petrescu A.** Secretory antibodies in respiratory virus infections. Virologie 1984; **35**: 133–138.
19. **Kauppi M, Eskola J, Kayhty H.** Anti-capsular polysaccharide antibody concentrations in saliva after immunization with Haemophilus influenzae type b conjugate vaccines. Pediatr Infect Dis J 1995; **14**: 286–294.
20. **Kauppi M, Saarinen L, Kayhty H.** Anti-capsular polysaccharide antibodies reduce nasopharyngeal colonization by Haemophilus influenzae type b in infant rats. J Infect Dis 1993; **167**: 365–371.
21. **Stutzmann Meier P, Heiniger N, Troller R.** Salivary antibodies directed against outer membrane proteins of Moraxella catarrhalis in healthy adults. Infect Immun 2003; **71**: 6793–6798.
22. **Zhang Q, Choo S, Everard J.** Mucosal immune responses to meningococcal group C conjugate and group

A and C polysaccharide vaccines in adolescents. Infect Immun 2000; **68**: 2692–2697.
23. **Ala'Aldeen DA, Neal KR, Ait-Tahar K.** Dynamics of meningococcal long-term carriage among university students and their implications for mass vaccination. J Clin Microbiol 2000; **38**: 2311–2316.
24. **Greiner O, Berger C, Day PJ.** Rates of detection of Neisseria meningitidis in tonsils differ in relation to local incidence ofinvasive disease. J Clin Microbiol 2002; **40**: 3917–3921.
25. **Zhang Q, Choo S, Finn A.** Immune responses to novel pneumococcal proteins pneumolysin, PspA, PsaA, and CbpA in adenoidal B cells from children. Infect Immun 2002; **70**: 5363–5369.
26. **Wiertz EJ, Delvig A, Donders EM.** T-cell responses to outer membrane proteins of Neisseria meningitidis: comparative study of the Opa, Opc, and PorA proteins. Infect Immun 1996; **64**: 298–304.
27. **Jones GR, Christodoulides M, Brooks JL.** Dynamics of carriage of Neisseria meningitidis in a group of military recruits: subtype stability and specificity of the immune response following colonization. J Infect Dis 1998; **178**: 451–459.
28. **Robinson K, Neal KR, Howard C.** Characterization of humoral and cellular immune responses elicited by meningococcal carriage. Infect Immun 2002; **70**: 1301–1309.
29. **Blackwell CC, Weir DM, James VS.** Secretor status, smoking and carriage of *Neisseria meningitidis*. Epidemiol Infect 1990; **104**: 203–209.
30. **Stuart JM, Cartwright KA, Robinson PM.** Effect of smoking on meningococcal carriage. Lancet 1989; **2**: 723–725.
31. **Tsai CM, Frasch CE, Mocca LF.** Five structural classes of major outer membrane proteins in Neisseria meningitidis. J Bacteriol 1981; **146**: 69–78.