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Evidence for Naturally Acquired T Cell-Mediated Mucosal Immunity to Neisseria meningitidis¹,²

Victoria Davenport,³* Terry Guthrie,* Jamie Findlow,† Ray Borrow,† Neil A. Williams,* and Robert S. Heyderman*

Naturally acquired protective immunity against Neisseria meningitidis is thought to partially explain the disparity between the high levels of carriage in the human nasopharynx and the rare incidence of disease. To investigate this immunity to Neisseria meningitidis at the mucosal level, in vitro cellular responses to outer membrane vesicle preparations derived from this pathogen were examined using mononuclear cells from the palatine tonsils of adults and children. Characterization of these responses was achieved by depletion of CD45RA⁺, CD45RO⁺, and CD19⁺ populations and outer membrane vesicles derived from isogenic mutants expressing different serosubtypes of the major outer membrane protein, porin A (PorA), no PorA and membrane preparations from a mutant with no LPS (LpxA⁻). The magnitude of cellular proliferative responses against the outer membrane vesicles were strongly associated with age and were largely T cell mediated, involving both CD45RO⁺ and CD45RA⁺ T cell phenotypes. Responses were not dependent on LPS but consisted of both PorA cross-specific and non-PorA-dependent responses. Cellular immunity against Neisseria meningitidis was found to be frequently associated with systemic IgG Abs but was not associated with serum bactericidal Abs. For the first time our results demonstrate an age-associated acquisition of mucosal T effector/memory cell responses to Neisseria meningitidis. This mucosal cellular immunity can be present in the absence of serum bactericidal Abs, a classical marker of protective immunity.


Meningococcal disease in the form of septicaemia and meningitis causes significant morbidity and mortality among infants and young adults in industrialized countries and is associated with major epidemics in sub-Saharan Africa (1–3). Although Neisseria meningitidis is carried in the nasopharynx of between 5 and 40% of individuals (4–6), the development of meningococcal disease is rare, particularly in adults over 25 years (6, 7).

The disparity between carriage and disease rates is partly accounted for by the low virulence of many carrier strains but also suggests a role for naturally acquired protective immunity. Although poorly understood, natural immunization is thought to occur through prolonged or intermittent colonization at the mucosal surface by N. meningitidis or related organisms such as Neisseria lactamica (5–8). Mucosal as well as systemic immune mechanisms have been implicated in this process (6, 9), with both serum and salivary Abs demonstrated following meningococcal carriage (6, 10–12). Complement-fixing IgG Abs that are high in bactericidal activity (serum bactericidal Abs; SBA⁴) are thought to be important mediators of protective immunity against N. meningitidis (13).

N. meningitidis serogroup B (Men-B) remains the only major serogroup for which there is currently no effective vaccine (14, 15) and has been associated with increased disease rates in the U.K., Norway, Chile, Cuba, Brazil, and recently New Zealand (16, 17). Unlike vaccines against the other serogroups of N. meningitidis (A, C, Y, W135) that utilize capsular polysaccharide conjugated to a protein carrier (18), Men-B polysaccharide vaccines are poorly immunogenic, which is thought to be due to homology with polysialic acid in fetal neural tissue (19). The Men-B vaccines that have been most extensively investigated in human clinical trials are based on multiple subcapsular surface Ags in which porin A (PorA), a class 1 outer membrane protein (OMP), appears to be a dominant immunogen (20–24). These include two partially purified Men-B OMP vaccines, developed by the Finlay Institute in Cuba and the National Institute of Public Health in Norway, that are presented as proteoliposomes vesicles adsorbed onto aluminium hydroxide (21, 22, 25), and an outer membrane vesicle (OMV) hexavalent vaccine developed at the Netherlands Institute of Public Health and the Environment (Rijksinstituut voor Volksgezondheid & Milieu), derived from two recombinant strains each expressing three different PorA proteins (23). Although these vaccines have been shown to engender a 50–80% protective efficacy, this has frequently been found to be strain specific, variable in infants under 18 mo of age, and of limited longevity (20).

The initial immune response following natural colonization occurs at the mucosal surface of the nasopharynx, which may act as a specialized microcompartment (26). T cells at these sites are

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¹Department of Pathology and Microbiology, School of Medical Sciences, University of Bristol, Bristol, United Kingdom; and ²Vaccine Evaluation Department, Medical Microbiology Partnership, Manchester Royal Infirmary, Manchester, United Kingdom

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³Address correspondence and reprint requests to Dr. Victoria Davenport, Department of Pathology and Microbiology, School of Medical Sciences, University of Bristol, University Walk, Bristol BS8 1TD, U.K. E-mail address: c.victoria.davenport@bristol.ac.uk

⁴Abbreviations used in this paper: SBA, serum bactericidal Ab; KLH, keyhole limpet hemocyanin; OMV, outer membrane vesicle; LpxA⁻, LPS-deficient mutant OMV; Men-B, N. meningitidis serogroup B; MNC, mononuclear cell; OMP, outer membrane protein; PT, palatine tonsil; PorA, porin A; QR, quantum red; Tri, Tricolor; TT, tetanus toxoid.

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cruicial to the regulation of Ab class-switching, affinity maturation, and immunological memory (27–30). Depending on the nature of the T cell help, contact with Ag at a mucosal surface can either lead to a predominantly proinflammatory Th1-type response, an Ab-associated Th2 response (29), or, in the absence of danger signal, tolerance (31, 32). It is unclear whether meningococcal carriage results in immunological priming or immune unresponsiveness, as with microbial colonizers at other mucosal sites and to what extent mucosal cellular immunity accounts for the disparity between the levels of SBA and protective immunity observed in vaccine trials (22, 33). In this study, we aimed to ascertain whether T cell-mediated immunological memory to N. meningitidis is located at the mucosal level, to characterize the nature of this response, and to determine its relationship to circulating humoral immunity.

Materials and Methods

Patients and clinical materials

Palatine tonsils (PT) and sera were obtained from 15 adults (ages 17 years 2 mo to 36 years 5 mo; 8 females) and 15 children (ages 3 years 5 mo to 14 years 6 mo; 12 females) with no history of atopy or meningococcal disease, who were undergoing tonsillectomy for airway obstruction or recurrent tonsillitis. The collection of the PT and the research described complies with relevant guidelines and institutional practices (University of Bristol Hospital Trust Local Research Ethics Committee E4388). Samples of PT were either used fresh for the isolation of mononuclear cells (MNC) or fixed in formal saline and embedded in paraffin before immunocytochemistry.

Control and meningococcal Ags

Men-B strain H44/76 (B:15:P1.7,16:L3,7,9), isogenic strains expressing different PorA types TR52 (B:15:P1.5,2), TR4 (B15:P1.7-8,4), TR10 (B:15:P1.5-2,10), and a spontaneous PorA-negative mutant (B:15:P1.1,−,−) were kind gifts from the RIVM (The Netherlands) (34, 35). Meningococcal OMV were prepared by deoxycholate detergent extraction (34, 35) and were used at final concentrations of 0.1, 0.5, and 1.0 μg/ml protein/ml as indicated. Final concentrations of 10–20 μg/ml keyhole limpet hemocyanin (KLH) Ag (Calbiochem CN Biosciences U.K., Beeston, U.K.), 100 ng/ml tetanus toxoid vaccine (TT; Aventis Pasteur MSD, Maidenhead, U.K.), and 30,000 endotoxin U/ml (8.3 μg/ml) Escherichia coli 011:B4 LPS (Sigma-Aldrich, Gillingham, U.K.) were used as controls. All Ags were diluted in RPMI 1640 containing 100 U/ml penicillin, 0.1 mg/ml streptomycin (Life Technologies, Paisley, U.K.), 4 mM L-glutamine (Roche, Basel, Switzerland) and passed twice through a French press (BG Electronics, Farnborough, Hampshire, U.K.) at 15,000 psi. Whole cells were pelleted by 5-min cold centrifugation (25,000 g) and washed in PBS.

Preparation of LPS-deficient mutant OMV cell membranes (LpxA−)

A LpxA− mutant strain of Men-B was a kind gift from Drs. P. van der Ley and L. van Alphen (RIVM, The Netherlands) (37). Inner and outer cell membranes were harvested by 5-min cold centrifugation (25,000 × g) and washed in PBS. Pellets were resuspended in 50 mM Tris-HCl buffer (pH 8.0) containing 50 μg/ml RNase (Roche, Basel, Switzerland) and passed twice through a French press (BG Electronics, Farnborough, Hampshire, U.K.) at 15,000 psi. Whole cells were pelleted by 5-min cold centrifugation (25,000 × g) and membranes were separated from the supernatant on a discontinuous sucrose gradient (15% on 55% w/v cushion) in 3 mM EDTA buffer (pH 8.0) by centrifugation for 2 h at 150,000 × g. The membrane fraction was then collected from the interface and the sucrose concentration was lowered to 30% w/v with EDTA buffer followed by separation on a second gradient of 35% w/v sucrose on top of 50% w/v at 70,000 × g for 18 h. The upper three bands were harvested. Membrane pellets were obtained by centrifugation for 2 h at 150,000 × g. Membranes were washed in PBS and stored at −20°C.

Isolation of MNC from PT

MNC were separated using a method modified from Quiding-Jarbrink et al. (39). PT were dissected into 2-mm3 pieces using a scalpel, then dispersed through industrial stainless steel mesh (Potter and Son, Bristol, U.K.) in 10 ml of HBSS supplemented with 10 mM HEPES (Life Technologies). Samples were washed in HBSS and separated by centrifugation on Histopaque 1077 (Sigma-Aldrich) for 30 min at 400 × g.

Cellular proliferation assay

Tonsilar T cell proliferative responses were determined using a method adapted from Plebanski et al. (40) and Williams et al. (41). PT MNC at 1.5 million, 1 million, and 0.6 million/ml were cultured for 24–30 h at 37°C in 24-well flat-bottom plates (Nunc, Roskilde, Denmark) with Ag, mitogen, or medium alone in final volumes of 2 ml/well. Media and plates were cultured at 5%CO2 for up to 9 days. On days 3–9 of culture, triplicate 100-μl samples were removed and plated directly into 96-well round-bottom plates (Costar, Appleton Woods, Birmingham, U.K.). The cells were then pulsed with 0.4 μCi [3H]thymidine (Amersham Pharmacia Biotech, Little Chalfont, U.K.) for 24 h and frozen at −20°C. Plates were harvested and cellular tritiated thymidine incorporation ([3H]Tdr) was quantified using a 1540 microbeta liquid scintillation counter (Wallac, Crown Hill, Milton Keynes, U.K.), giving results in corrected cpm.

Cellular depletion by MACs sorting

Cell depletion experiments (CD19+ B cells, CD45RO+ T cells, CD45RA+ T/B/NK cells) were performed by negative selection using MACs microbeads and magnetic sorting according to the manufacturer’s instructions (Miltenyi Biotec, Bailey, Surrey, U.K.). In brief, 2 × 10^7 filtered cells were incubated in 1600 μl of cold buffer (calf serum, magnesium-free PBS supplemented with 2 mM EDTA and 0.5% human AB serum) with 400 μl of microbeads for 15 min. Washed cells were separated on a LS+ column (Miltenyi Biotec) with a 25-μg needle placed within a MACS magnetic separator. The depleted cells were washed in cold buffer, pelleted, and separated on a second LS+ column. The purity of the depletion was determined by FACS.

Abs used for flow cytometric analysis

The following mAbs conjugated with quantum red (QR), PE, FITC, or Tricolor (Tri) were used: CD19-Tri (Caltag Medystems, Towcester, U.K.), CD14-Tri (Caltag Medystems), HLA-DP, DQ, DR-FITC (BD PharMingen, San Diego, CA), CD25-FITC (BD PharMingen), CD4-QR (Sigma-Aldrich), CD8-QRST (Sigma-Aldrich), CD3-Tri (Caltag Medystems), CD45RO-PE (BD PharMingen), and CD45RA-FITC (Sigma-Aldrich). Isotype-matched controls IgG1-FITC (BD PharMingen), IgG2a-PE (BD PharMingen), and IgG2a-Tri (Caltag Medystems) were included.

Flow cytometric analysis of PT MNC preparations

Freshly isolated (10^7) PT MNC were stained with the appropriate mouse mAbs in FACS buffer (HBSS supplemented with 10 mM HEPES and 5% mouse serum) for 15 min at 4°C in the dark. Stained samples were washed and fixed in FACS lysing solution (BD PharMingen) for 10 min at room temperature. Cells were analyzed on a FACScan (BD Pharmacia) to collect 10,000 events. MNC were gated on forward scatter and side scatter. Data was subsequently analyzed using WinMDI version 2.8 software (J. Trotter, The Scripps Research Institute, La Jolla, CA).

Immunocytochemistry of PT

The phenotypes of the cellular components of the PT and their distribution were determined in 4-μm Formalin-fixed paraffin-embedded sections. Staining mouse mAbs to CD20, CD8, and HLA-DR was adapted from Trotter, The Scripps Research Institute, La Jolla, CA). Endogenous peroxidases were quenched by 10-min incubation in 0.3% hydrogen peroxide (v/v) and nonspecific Fc binding was prevented by incubation in 10% milk protein for 5 min. The sections were then incubated with the mAbs for 1 h at room temperature. Labeling was detected with a DAKO EnVision kit according to the manufacturer’s instructions and counterstained in hematoxylin. Ag retrieval was required for optimal staining of sections with all of the mAbs. This consisted of heating samples for 20 min by microwave using either citric acid retrieval (10.5 g citric acid/5 L, buffered with 2 M NaOH to pH 6.0) for CD20, CD8, and HLA-DR, or DQ, DR, or high pH Ag retrieval (microwave buffer, pH 9.0; DAKO) for CD4.

Serum bactericidal Ab

Sera were transported frozen to the Meningococcal Reference Unit and tested for the presence of SBA against the strains H44/76, TR4, TR10, and...
TR52 by standard assay (Centers for Disease Control and Prevention protocol version 092096) (42). Human plasma was used as the source of complement and heat-inactivated patient serum samples as the source of Ab. Results were expressed as the reciprocal of the minimum sample titer that caused 50% or greater bacterial lysis. SBA against meningococci at titers of 4 or more have been correlated with immunity (11) and were taken as positive. Those below 4 were negative and designated a titer of 2 for analytical purposes.

**OMV ELISA**

Immunol 2 HB (Thermo Labsystems, Franklin, MA) ELISA plates were coated with OMV preparations at 1 μg/ml in bicarbonate buffer consisting of 15 mM Na2CO3 (Sigma-Aldrich), and 35 mM NaHCO3 (Sigma) at pH 9.6, overnight at 4°C. Two-fold serum dilutions in 10 mM PBS, 0.05% Tween 20 (Sigma-Aldrich), and 5% newborn bovine serum (ICN Pharmaceuticals, Basingstoke, U.K.) were added to the OMV-coated plates and incubated overnight at 4°C. Plates were washed and Ab was detected using an alkaline phosphatase-conjugated anti-human IgG (BioSource International, Camarillo, CA) and incubated for 2.5 h at room temperature. The assay was developed using p-nitrophenol phosphate substrate (Sigma-Aldrich) and stopped after 2 h with 3 M NaOH (Sigma-Aldrich). Arbitrary values were assigned from an internal serum standard using a four-parameter logistic log model.

**SDS-PAGE and immunoblotting**

OMV components were separated by SDS-PAGE as described by Laemmli (43). OMV samples were reduced and separated on a 12% (w/v) polyacrylamide gel (25 mA for 60 min). Proteins were transferred from gel to nitrocellulose membrane (Bio-Rad, Richmond, CA) using semidy transfer (15 V for 15 min). Gels were silver stained for LPS as previously described (44). Membranes were stained for proteins using human sera as the source of primary Ab (1/1000). Ab binding was detected with goat anti-human IgG conjugated to alkaline phosphatase (1/1000; Southern Biotechnology Associates, Birmingham, AL) and Fast Red (Sigma-Aldrich).

**Statistical analysis**

All proliferation experiments were performed in triplicate to give arithmetic means, which were used for all subsequent analysis. Means demonstrated SEs of <5%. Nonparametric tests were subsequently performed using SPSS version 11.0 for Windows (Lead Technologies, Chicago, IL) and results were presented as medians. A Bonferroni correction was applied to allow for multiple testing of the same data set. Friedman’s nonparametric ANOVA was performed to examine differences between the tonsillar MNC response to Ags within subject groups. To control for the potential mitogenic effects of LPS, OMV responses were compared with those of LPS-treated controls. Comparisons of data from adults and children were performed using the Mann-Whitney U test. Two-tailed Spearman’s rank correlations were performed to determine the association between peak proliferative responses and age of PT donor, and peak proliferative responses and SBA titer.

**Results**

**MNC extracted from PT are a representative population**

Immunostaining of PT sections revealed the presence of CD20+ B cells, CD4+, and CD8+ T cells (Fig. 1, a–f). B cells were localized primarily in follicles while T cells predominated in the interfollicular zones. However, there was also clear evidence of the presence of both B and T cells infiltrating the tonsillar epithelium. Class II MHC expression was readily observed on B cells within the follicles as well as on numerous cells in the interfollicular zones and in the epithelium where some cells had a morphology characteristic of dendritic cells (Fig. 1, g and h).

Isolated populations of PT cells contained all of the cell types observed in situ. The majority of cells in suspensions from children’s PT were CD19+HLADR+ B cells (51.3 ± 2.7%, n = 5). Similar levels of CD4+ T cells (40.7 ± 3.5%) were detected and a lesser number of CD8+ T cells (12.5 ± 1.3%). Adult’s PT (n = 8) demonstrated on average fewer B cells (32.1 ± 3.2%, p < 0.005) and relatively more CD4+ T cells than those of children (54.5 ± 4.5%, p < 0.05), with comparable CD8+ T cell levels (9.5 ± 1.2%). Expression of CD25 on CD4+ and CD8+ T cells occurred at a higher level in adults than in children (8.0 ± 0.6% compared with 5.2 ± 0.5%, p < 0.005 and 3.7 ± 0.8% compared with 1.7 ± 0.6%, p < 0.05 for CD4+ and CD8+ cells, respectively).

**PT MNC respond to Neisseria meningitidis OMV in vitro**

Tonsillar-derived MNC populations were stimulated with meningococcal OMV or control Ags and the kinetics of proliferation were examined. Responses to secondary control recall Ag, TT, were detected in tonsil cell cultures, with peak responses typically between days 4 and 6 after stimulation (Fig. 2a). Primary control Ag, KLH, induced responses in a small number of individuals with classically later and smaller responses, reaching a peak between days 7 and 9 after stimulation (Fig. 2a). In vitro culture of PT MNC with H44/76, TR52, TR4, and TR10 OMV was also associated with detectable proliferation above background, which frequently reached a peak between 4 and 6 days after stimulation (Fig. 2b). Importantly, the responses to the control Ags demonstrate that both primary and secondary responses can be detected in PT MNC in vitro. PT MNC responses against Men-B OMV generally exhibited secondary-type kinetics.

**OMV-induced PT cell proliferation is T cell and OMP dependent**

Before studying the precise nature of the tonsillar cell responses, it was important to confirm that the proliferation observed resulted from T cells responding to the protein constituents of the OMV. It
is noteworthy that conventional OMV preparations contain 5–10% LPS relative to protein (45), a known B cell mitogen. To examine the proliferative contribution of B cells, samples of PT MNC from two adults and two children were depleted of B cells before initiation of the cultures and compared with undepleted PT MNC. The results of a representative experiment are shown in Fig. 3. Despite depletion of CD19+ cell numbers from 23.4 to 3.3%, no marked suppression of the OMV-induced proliferative response was detected. Indeed, the peak response to all of the OMV preparations occurred 1–2 days earlier in the B cell-depleted population. This shift in peak response probably results from a compensatory increase in the numbers of T cells present in the cultures and is in line with previous cell dose-response curves. Even in children’s tonsils, where 54% of MNC were CD19+, depletion of B cells to 2.3% did not prevent the OMV-induced proliferative responses.

In additional experiments using PT from three donors (one adult), the responses to OMV were compared with those against outer membranes isolated from the LpxA− strain (LPS deficient) to determine the effect of membrane-bound LPS on the level of OMV-induced MNC proliferation. In all cases, stronger responses to the LpxA− membranes were detected (Fig. 4e) than with the parent OMV (H44/76). Electrophoretic separation and silver staining (Fig. 4b) confirmed the absence of LPS in the LpxA− membrane preparations used in our assays.

**OMV-responsive T cells have both CD45RA+ and CD45RO+ phenotypes**

To further examine the in vitro responses to the OMV, immunomagnetic CD45RA+ and CD45RO− negative depletions were performed on MNC from three adult PT and proliferation assays were performed with OMV and control Ags as previously described. In whole PT MNC preparations, CD45RA+ T cells predominated (Fig. 5a) (57.2, 61.7, and 73.6%) over the CD45RO+ T cells (18.4, 37.5, and 41.5%). Depletions resulted in CD45RO+ populations of 99.6–99.9% purity and CD45RA+ populations of 98.3–99.9% purity, as determined by flow cytometric analysis (Fig. 5, c and e).

The proliferation of unseparated adult PT MNC in response to OMV reached a peak on day 5 (Fig. 5b). Unusually, in the individual shown, the TT response reached a later peak (day 8) than the OMV responses. Examination of CD45RO− and CD45RA+ depletions demonstrated both populations proliferated to the OMV. CD45RA+ cells responded with late primary-type kinetics to OMV and TT (Fig. 5, c and d), reaching a peak on day 8. CD45RO− cells reached a peak on days 4–5 with early recall-type kinetics to OMV (Fig. 5, e and f). Similar CD45RO+ and CD45RA+ responses and kinetics were observed against OMV in the other PT MNC. These data confirm that the tonsil harbors both CD45RA+ and CD45RO+ T cells, which are capable of responding to all of the OMV tested.

**Acquisition of high-level responses to OMV is age dependent**

The relationship between the magnitude of the peak response against each Ag treatment and age of the subject was examined.
correlation with age was observed. The majority of responses to KLH were low, with most giving $<9,440\text{ cpm}$ (Fig. 6b). Each of the OMV (TR4, TR10, TR52, and H44/76), containing different PorA Ags, induced a biphasic age response, with high levels observed in teenagers (33,624–44,081 cpm) followed by a steady rise toward adulthood (Fig. 6, d–g). Overall, a clear positive correlation of OMV response and age was detected, with responses to TR4, TR52, and H44/76 OMV reaching statistical significance (Rho = 0.408–0.451, p < 0.05). A positive correlation was also detected in responses against the PorA-negative OMV (Rho = 0.54, p < 0.05) (Fig. 6c).

OMV responses demonstrate both PorA-dependent and PorA-independent variation

Peak proliferative responses induced by OMV over a period of 9 days were examined for adults and children to determine whether there were clear differences in the levels of the response to the different PorA types in the population tested. Adults demonstrated significant responses against all OMV (p < 0.005, n = 16; Fig. 7a). Children similarly demonstrated significant responses (p < 0.05, n = 12; Fig. 7b) but of a smaller magnitude. Overall, some PorA-dependent variations in response were observed. Both TR10 and H44/76 demonstrated larger interindividual variations in response than the other OMV. Some differences in kinetics were also observed between OMV (data not shown), with TR4 inducing the most responses between days 3 and 7 (n = 21) and TR52 the least responses between days 3 and 7 (n = 18).

Comparison of responses against the PorA-negative OMV (median, 16,237; range, 1,815–19,878 cpm; data not shown) with TR4 inducing the response than the other OMV. Some differences in kinetics were also observed between OMV (data not shown) with TR4 inducing the most responses between days 3 and 7 (n = 21) and TR52 the least responses between days 3 and 7 (n = 18).

T cell responses are not associated with SBA

OMV-specific systemic Abs were quantified by ELISA. Results were compared against a serum standard selected from a vaccinated individual with protective titers, giving results as arbitrary

FIGURE 5. Proliferative responses to meningococcal Ags are mediated by both CD45RA$^+$ and CD45RA$^+$ T cells. a, Levels of CD45RO$^+$ (PE) and CD45RA$^+$ (FITC) expression in tonsil MNC. b, Proliferation of tonsillar MNC against 100 ng/ml TT, TR10, TR52 OMV, PorA-negative OMV, and complete medium control. c, Similar MNC against 100 ng/ml TT, TR10, TR52 OMV, PorA-negative OMV, and complete medium control. Levels of CD45RO$^+$ (PE) and CD45RA$^+$ (FITC) expression in tonsil MNC depleted of CD45RA$^+$ cells. d, Proliferation of CD45RO$^-$-depleted cells against 100 ng/ml TT, TR10, and TR52 OMV, PorA-negative OMV, and complete medium control. e, Levels of CD45RO$^+$ (PE) and CD45RA$^+$ (FITC) expression in tonsil MNC depleted of CD45RA$^+$ cells. f, Proliferation of CD45RA$^-$-depleted cells against 100 ng/ml TT, TR10, and TR52 OMV, PorA-negative OMV, and complete medium control. Data represent one of three separate experiments (donor age, 19 years 5 mo). Means demonstrate <5% SEM.

FIGURE 6. Peak tonsil proliferative MNC responses to meningococcal OMV correlate with donor age. a, Secondary control Ag, TT (n = 30), b, Primary control Ag, KLH (n = 30), c, PorA-negative OMV (B:15.P1.7-8.4; n = 14), and H44/76(B:15.P1.7.16.L3.7,9); d, TR4 (P1.7-8.4); e, TR52 (P1.5, 2); g, TR10 (P1.5-2,10; n = 28). Each point represents the mean of triplicate data with <5% SEM.

FIGURE 7. The peak and range of tonsil MNC proliferation is Ag dependent. a, Peak adult tonsil responses (n = 16); age range, 18–36 years. b, Peak child tonsil responses (n = 12); age range, 3–14 years. Responses were measured against control Ags (KLH, TT, and LPS) and OMV.H44/76 (B:15: P1.7.16.L3.7,9), TR4 (P1.7-8.4), TR10 (P1.5-2,10), and TR52 (P1.5.2) and represent the peak response generated over 9 days of stimulation.
units. OMV-specific IgG was demonstrated in all samples, with values ranging from 3,000–60,000 arbitrary units. Detectable Ab in all individuals was confirmed by Western blotting against H44/76. Only a limited number of IgG-positive samples also exhibited SBA positivity, but in most cases this correlated with high IgG values (Fig. 8). No relationship was observed between peak proliferative responses and SBA (data not shown); but IgG values demonstrated a similar age distribution to proliferation data, with the lowest levels in children and a peak in teenagers (data not shown).

Comparison of SBA in adults and children demonstrated only one child with a positive titer (>4) against TR4, TR10, TR52, and/or H44/76 (n = 14) compared with half of all adults (n = 18; data not shown).

Discussion

PT are thought to act as a site of induction of cellular immune responses to Ags encountered in the nasopharynx (46, 47) but whether long-term memory responses reside in the PT is unclear (48). Human PT are an important mucosal associated lymphoid tissue (26, 49, 50) which is naturally colonized by N. meningitidis (4). In line with previous work (48), we found both intraepithelial and subepithelial CD4+ and CD8+ T cells, B cells, and HLA-DR, -DP, -DQ-positive cells with dendritic cell morphology within the PT that may have important roles in surveillance against colonizing meningococci. In this study, we demonstrate for the first time that T cells resident in the PT are capable of a range of proliferative responses to meningococcal OMV, with evidence of age-related acquisition of immunity and immunological memory but no relationship with SBA.

The PT MNC proliferative responses to control and meningococcal Ags seen in this study appeared to be largely T cell mediated. CD19+ B cell depletion experiments demonstrated that neither LPS-induced mitogenic stimulation of B cells (51–53) nor porin-induced B cell stimulation (54) accounted for the OMV-induced proliferations observed. Given that intraepithelial cells proliferate minimally in response to Ag and mitogen alike (55) and that we used exogenous Ags (MHC class II restricted) in our study, intraepithelial cells and CD8+ cells are also unlikely to contribute to these responses.

Proliferation response kinetics, control Ag responses (KLH and TT) and cell depletion experiments demonstrated the presence of naive and memory populations of T cells responsive to N. meningitidis in the PT. It has been suggested that the CD45RA+ primary-type responses observed (56) may be memory T cells in the PT that have lost CD45RO expression (57). Given that priming through either TT vaccination or meningococcal carriage in the older subjects was likely to have occurred many months or years previously, our data suggest that the antimeningococcal memory responses are sustained in the medium to long term.

The overall increase in level of Men-B proliferative responses with age, suggestive of an increase in memory-type responses, supports the idea of “acquired” mucosal immunity (58). This pattern of mucosal cellular immunity appears complex and possibly bimodal, with similarities to the classic profile of SBA acquisition in the population (11, 59). Pollard et al. (29) demonstrated a comparable age-related acquisition of peripheral blood MNC responses to meningococcal Ags among children convalescing from meningococcal disease. Such memory-type responses may be maintained through repeated exposure to neisserial Ags from nasopharyngeal carriage and cross-reactive Ags from other sources, such as enteric flora (60).

The interaction between the meningococcus and host cells is modulated by meningococcal endotoxin, LPS, which interacts with cognate Toll-like receptors (61), typically Toll-like receptor 4 (62), and has both mitogenic and adjuvant properties (53). We, and others, have previously observed the influence of LPS on meningococcal-host interactions in other cell systems (63). To examine the importance of LPS-mediated cosignaling in the PT proliferative responses, we have used an endotoxin-negative mutant of N. meningitidis, H44/76, in which a key enzyme in the LPS biosynthetic pathway, LpxA−, has been inactivated by allelic replacement (37, 64). Outer membranes derived from the LpxA− mutant strain not only induced PT T cell proliferation in the absence of LPS cosignaling but the response was frequently more marked when compared with wild-type H44/76 OMV of equivalent protein concentration. Whether this reflects LPS toxicity in vitro or is related to inherent differences between H44/76 OMV and LpxA− membranes, such as the relative quantity of immunodominant Ags, the composition of outer membrane phospholipids (37), the expression of iron limitation-inducible cell surface lipoproteins (37), or greater access to Ags in the membranes, remains to be determined. In this study, we focused predominantly on PorA, a major meningococcal OMP which forms the basis of meningococcal classification or serosubtyping (65, 66) and a candidate vaccine Ag which is capable of inducing SBA (67, 68) and eliciting T cell immunity (24, 69). A greater variation in responses was observed against the less common U.K. serosubtypes (TR10 and H44/76) and a higher prevalence of memory-type responses with the most common U.K. serosubtype (TR4; data not shown). This may reflect the frequency of previous colonization by these serosubtypes or the degree of HLA class II restriction of the immunodominant PorA epitope (24, 70). However, the responses predominantly demonstrated cross-reactive T cell mucosal immunity to the range of isogenic OMV expressing common PorAs such as TR4 (1.7-8.4) or less common serosubtypes circulating in the U.K. (H44/76 (P1.7,16), TR10 (p1.5-2.10), and TR52 (P1.5,2)). This may be explained by the location of T cell epitopes largely within the conserved region of meningococcal OMP, enabling cross-reactivity to other serosubtypes (70, 71). Alternatively, the cross-reactive immunity may be induced by carriage of related commensal species such as N. lactamica (8, 72), which typically colonizes earlier in childhood than N. meningitidis (73) and does not express PorA (72). Indeed, our detection of responses to mutant OMV without PorA (data not shown) suggests that other OMP such as PorB, Rmp, Opc, Opa, Tbps, or OMP85 are involved in the T cell stimulation (9, 24).
The methodological approach taken in this study has the potential to provide important insights into the mucosal cellular immunological response to meningococcal Ags. However, the assay does not account for the influence of supportive cells such as stromal cells, the natural cytokine milieu within the PT, or artifacts induced by in vitro culture, e.g., apoptosis of IL-4-producing germinal center B cells (74). Additionally, although none of the subjects had clinical tonsillitis at the time of operation, there are limitations to the use of material taken from subjects with recurrent tonsillar infection. The relatively high background proliferation displayed by PT MNC was comparable to previous reports (48, 50). Whether natural or vaccine-induced mucosal immunity protects against invasive meningococcal disease remains to be determined.

In summary, our findings demonstrate high levels of T cell proliferation to Men-B at a mucosal site, reflecting an age-associated acquisition of response. This novel in vitro system has enabled us to successfully investigate mucosal cellular immunity to Men-B and lends itself to further analysis of the underlying mechanisms and assessment of alternative candidate vaccine Ags.

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References

12. Chernev, I. M. Feavers, A. Robinson, K. Cartwright, and A. R. Gorringe. 2002. Meningococcal carriage by the meningococcus has been widely considered to lead to systemic immunity (typically measured by SBA) and therefore protection. In our study, the high frequency of mucosal T cell proliferative responses coincided with OMV-specific IgG Abs, including those against PorA, but frequently an absence of SBA, particularly in children. Recent vaccine trials, using intranasal vaccination to mimic the natural route of immunological priming, have demonstrated limited success in inducing SBA (78, 79).

Whether natural or vaccine-induced mucosal immunity protects against invasive meningococcal disease remains to be determined.

Acknowledgments

We are grateful to Prof. Muntaz Virji (University of Bristol, Bristol, U.K.) for valuable comments on this manuscript and to Dr. Robert Copland (University of Bristol, Bristol, U.K.) for helpful discussions. We gratefully acknowledge the expert help and support of Rachel Horton, Dr. Christopher Hobbs, Dr. Jolanta Bernatoniene, and Dave Copland (University of Bristol, Bristol, U.K.) for providing the material used in this study. We are grateful to Prof. Mumtaz Virji (University of Bristol, Bristol, U.K.) for providing the material used in this study.

References

MUCOSAL IMMUNITY TO Neisseria meningitidis


