Meningococcal carriage and the immune response to colonization were studied in a group of military recruits undergoing basic training. Subtyping by determination of the class 1 protein sequence clearly differentiated between strains and demonstrated the dynamics of carriage and transmission. Expression of class 1 protein by each strain remained stable during prolonged carriage by different subjects. Following colonization, a marked increase in serum bactericidal response occurred, which was specific for the subtype of the acquired strain and was associated with an increase in reactivity by Western blot to the homologous class 1 protein. Subjects colonized by multiple strains showed evidence of a specific immune response to the class 1 protein of each strain acquired. The subtype specificity of the bactericidal response to meningococci and the stability of expression of the class 1 protein have important implications for the design of vaccines for prevention of serogroup B meningococcal disease.

Since early studies by Heist et al. [4], it has become generally accepted that immunity to meningococcal disease is dependent on the presence of serum bactericidal activity directed toward the infecting organism. Identification of the serogroup-specific polysaccharide capsules as targets for bactericidal activity [5, 6] led to the development of the first-generation meningococcal vaccines [7]. However such vaccines, based solely on capsular polysaccharide, provide only limited protection against serogroups A and C and offer no protection against serogroup B strains [8], which are the predominant cause of meningococcal disease in most temperate countries. The development of effective group B vaccines would be facilitated by understanding the molecular basis of natural immunity, in particular the identification of subcapsular antigens capable of inducing a protective immune response. Although the association between immunity to meningococcal infection and the presence of serum bactericidal activity has been long established, the mechanism by which such natural immunity develops is poorly understood. It is likely that persons can acquire protective antibodies in response to colonization by commensal neisseriae or meningococci of reduced virulence [9, 10]. However, the contribution that individual antigens make toward natural immunity to meningococcal disease is unknown, and little more is understood about the immune response to meningococcal colonization than was determined by Goldschneider et al. [5, 6] in the 1960s prior to major advances in the understanding of meningococcal surface structure.

With the advent of sophisticated molecular and biochemical techniques, the structure of the meningococcal outer membrane has been elucidated in detail [11]. Several antigens associated with the outer membrane have been shown to induce an immune response following invasive disease or vaccination with experimental outer-membrane vesicle (OMV) vaccines. These
antigens include lipooligosaccharide (LOS) [12] and several outer-membrane proteins [13, 14], notably the Opc [15] and class 1 proteins [16]. The class 1 porin protein, encoded by the porA gene, is a major constituent of the outer membrane and shows restricted antigenic diversity between strains; this forms the basis for the serosubtype classification of meningococci [17]. Recently, sequencing of the porA gene has revealed additional subtypes that were not previously identifiable by serologic methods [18–20]. Studies also suggest that the class 1 protein induces a bactericidal response following infection [13, 21], and sequence variations found in porA are presumably due to constant immune surveillance. Recently, experimental multivalent OMV vaccines have been produced from recombinant meningococci expressing multiple class 1 proteins and have been used in human trials [22].

In this study, samples were collected from a group of military recruits undergoing a 30-week course of basic training, to investigate both the dynamics of meningococcal carriage and the antigenic specificity of the humoral immune response. The isolates acquired also provided an opportunity to examine the stability of the class 1 porin during prolonged carriage.

Methods

Isolation of meningococci and collection of sera. Isolates of Neisseria meningitidis were obtained as part of a collaborative Public Health Laboratory Service (PHLS) study of meningococcal throat carriage in cohorts of military recruits undergoing basic training [23]. Individual recruits, training in troops of ~30 men, were enrolled at the start of a 30-week training program. Up to 8 throat swabs and 3 serum samples were collected over the course of training. Throat swabs were taken in weeks 1, 3, 6, 10, 15, 19, 24, and 29. Sera were taken in weeks 1, 10, and 29. Meningococci were isolated using standard techniques and were serologically characterized by the PHLS Meningococcal Reference Laboratory (Manchester, UK).

Determination of subtype by DNA sequencing. Meningococci were grown on chocolate agar for 18 h and harvested into PBS (pH 7.4); DNA was extracted using guanidium thiocyanate [24]. The regions of the porA gene spanning variable regions VR1 and VR2 were amplified by polymerase chain reaction using a series of oligonucleotide primers corresponding to known conserved areas of the gene as previously described [20]. Amplified DNA was purified by Gene-clean (Bio 101, La Jolla, CA) and sequenced using an Ampli-taq (BioRad, Hercules, CA) for 373A automated DNA sequencer (Applied Biosystems, Cambridge, UK) according to the manufacturer’s instructions. Sequences were analyzed by Lasergene software (DNASTar, Madison, WI).

Whole cell ELISA of human sera. The humoral immune response to meningococcal carriage was measured using a whole cell ELISA. Meningococci were suspended in PBS (pH 7.4), heat-inactivated at 56°C for 30 min, and the suspensions were adjusted to an A(660) value of 0.09–0.15 [25]. The suspensions (100 µL/well) were used to coat 96-well flat-bottomed polystyrene microtiter plates by incubation for 16 h at 37°C. The plates were incubated at 37°C for 1 h with test sera that had been serially diluted in PBS containing Tween 20 (0.05% vol/vol) and bovine serum albumin (1% wt/vol). After a wash, antibody binding was detected using horseradish peroxidase-conjugated goat anti-human immunoglobulins (Zymed, South San Francisco) with 3,3',5,5'-tetramethylbenzidine and H₂O₂ as enzyme substrate. Results were expressed as reciprocal serum dilutions giving an A(450) of 0.1 after 10 min of incubation with substrate.

SDS-PAGE and Western blotting. The immune response to individual meningococcal antigens was determined by Western blotting [18]. Whole cell lysates (250 µg of total protein) of meningococcal strains were separated by preparative SDS-PAGE on linear gradients of 10%–25% acrylamide. Proteins were transferred to nitrocellulose paper (BA85 0.45[m], Schleicher & Schuell, Dassel, Germany) using a Trans-Blot Semi-Dry transfer cell (Bio-Rad, Richmond, CA). The nitrocellulose sheet was washed twice in Tris-buffered saline (pH 7.5) containing 0.05% (vol/vol) Tween 20 (TBST) and blocked for 1 h at 25°C in TBST containing 5% (wt/vol) skim milk powder. After being washed, the nitrocellulose strips were reacted for 1 h at 25°C with Empigen BB (E-BB, 0.15% vol/vol; Albright & Wilson, Whitehaven, UK) diluted in Tris-buffered saline (TBS; pH 7.5) to renature transferred proteins [26]. After further washing, the strips were incubated for 18 h at 25°C with test sera diluted 1:100 in TBS containing 1% (wt/vol) gelatin. After another wash, the strips were incubated for 1 h at 25°C with alkaline phosphatase–conjugated goat anti-human immunoglobulins (Zymed) diluted 1:1000 in TBS containing 5% (wt/vol) skim milk powder.

Immunologic reactivity was detected with a substrate solution containing nitro-blue-tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Bio-Rad) according to the manufacturer’s instructions. The intensity of color reactivity was scored semiquantitatively from 0 to 6. Control antibodies were used to indicate the position of specific protein bands: rabbit polyclonal antibody raised to gonococcal porin, which cross-reacts with meningococcal class 1 proteins [16]. The class 1 porin protein, encoded by the porA gene has revealed incubation with substrate.

Bactericidal killing assay. The bactericidal activity of sera for meningococcal strains was determined essentially as described by Munkley et al. [30]. Meningococcal strains were harvested into Dulbecco B PBS (PBSB; Oxoid, Basingstoke, UK) containing 1% (vol/vol) heat-inactivated (56°C, 30 min) fetal calf serum (Gibco, Paisley, UK). Bacterial suspension (25 µL containing ~1000 cfu) was added to the wells of a sterile 96-well microtiter plate, followed by serial dilutions of heat-inactivated test sera in PBSB (10 µL). An exogenous source of complement was provided by using human plasma (courtesy of G. Carlone, CDC, Atlanta) or serum selected from among laboratory staff which lacked bactericidal activity to the specific meningococcal strain.

The optimal final complement concentration was determined to enable standard conditions to be used for all strains tested. Freshly thawed complement source or a heat-inactivated control was added to a final concentration of 5% (vol/vol), and the plates were agitated.
briefly to ensure thorough mixing. The plates were incubated at 37°C for 30 min in an atmosphere of 5% (vol/vol) CO₂, and 15-μL samples were removed for enumeration of surviving colony-forming units. All sera were assayed three times at each dilution. Serum bactericidal titers were expressed as the final serum dilution that resulted in a >50% reduction in surviving colony-forming units compared with an equivalent control containing heat-inactivated complement source.

Results

Patterns of meningococcal colonization. This study gave an opportunity to examine the dynamics of acquisition of meningococci within a troop of 31 military recruits over a 30-week period. Colonization by meningococci could be followed during periods of prolonged carriage by individual subjects and during transfer between different subjects. Initially, the meningococcal isolates obtained from recruits were analyzed with standard serologic reagents [17]. However, many of the isolates could not be identified readily by such methods, being classified as nongroupable (NG), nontypeable (NT), or nonsubtypeable (NST).

Nine subjects were selected for detailed study. These represented consenting subjects from the troop, who completed the training course and who demonstrated continuity of carriage as seen by ≥2 consecutive positive throat swabs. A meningococcal strain, characterized as X:4:NT, appeared to colonize a number of subjects in the early stages of the study and was subsequently acquired by others. In addition, serial X:4:P1.16 isolates were obtained from 1 subject (B). Carriage of X:4 meningococci was detected on more than one occasion in a total of 8 subjects (B–I). In addition, a further meningococcal strain could be identified (NG:1:P1.5), which initially colonized subject I, prior to being displaced by an X:4 strain, and was then acquired by another individual (subject J), causing prolonged carriage. Four other strains that were distinguishable by serologic methods were transiently carried by 3 subjects prior to being displaced by an X:4 strain.

In total, 35 meningococcal isolates were available from the 9 subjects. To determine the subtype of the isolates and to assess the stability of the porA gene on prolonged carriage, the amino acid sequences of VR1 and VR2 were derived by sequencing the porA gene. The dynamics of meningococcal colonization for the 9 subjects over the study period are shown in figure 1.

The X:4 meningococcal isolates could be clearly differentiated into 2 distinct strains on the basis of the subtype sequence. The first strain, X:4:P1.22,27a, was detected on 16 occasions in 7 subjects over the 30-week period. It was initially isolated from subject C and proceeded with time to colonize subjects D–I, displacing any resident meningococci. The second strain, X:4:P1.21,16, was detected only in subject B, who was colonized on entry and carried the strain throughout the 30-week study.

Only 1 other strain was found to colonize >1 individual—the NG:1:P1.5a,2c strain, which was obtained on 7 occasions from subjects I and J. Four other strains were identified, which colonized only individual subjects. Subjects D and G were colonized with Y:NT:P1.6 and NG:NT:P1.7d,1, respectively, before being colonized with the X:4:P1.22,27a strain. One subject showed a complex pattern of colonization: Subject F was initially colonized by B:NT:P1.7b,26 and subsequently the X:4:P1.22,27a was isolated; these were followed by W135:NT:P1.18a,3 and finally by reisolation of X:4:P1.22,27a.

The meningococcal isolates obtained during the course of the study therefore represented 7 strains with distinct subtypes. Despite the complex patterns of colonization shown in figure 1, including multiple acquisition, the DNA sequences of porA VR1 and VR2 of each strain remained unchanged during prolonged carriage by any individual subject and during transfer between subjects. Thus, there was no evidence of either mutation of the porA gene or horizontal exchange of genetic material between strains during the 30-week study period.

Immune response associated with meningococcal carriage. To assess the humoral immune response to nasopharyngeal acquisition and carriage of meningococci, serum antibody levels were determined by whole cell ELISA. For 8 subjects, sera were available from weeks 1, 10, and 29. For subject B, sera were available from only weeks 1 and 10. Antibody levels were determined against each of the different meningococcal strains isolated within the troop and to an additional control strain, H44/76 (B:15:P1.7,16). Titers to the specific meningococcal strains that were demonstrated to colonize an individual subject (homologous strains) and to the control strain are shown in figure 2. For each subject, titers to heterologous strains isolated from other subjects were comparable to those of H44/76 (data not shown).

In general, subjects who acquired meningococci during the study had low antibody levels to the homologous strain in sera taken prior to colonization. After colonization, a significant rise in serum antibody titer, specific to the colonizing strain, was observed. Titers to the control strain were low and showed no significant increases following colonization. For example, subject C had a low level of antibody to meningococcal strain X:4:P1.22,27a in week 1. Acquisition of that strain by week 6 was followed by a 10-fold rise in antibody levels to the homologous strain in serum from week 10. In contrast, antibody levels to strain H44/76 were low and showed no rise to accompany colonization.

Subjects who were colonized with >1 meningococcal strain during the course of the study showed a rise in serum antibody levels specific to each homologous strain following acquisition of that strain, while antibody levels to the control strain showed no significant rise (e.g., subject I). Subject F was colonized by 3 separate strains. Specific serum antibody to strains X:4:P1.22,27a and W135:NT:P1.18a,3 was detected following acquisition of each strain. However, antibody titers to B:NT:P1.7b,26 were high initially, prior to isolation of this strain at week 6, and remained unchanged in subsequent sera.

A different pattern of serum antibody titers was seen in subject B, who was colonized with meningococcal strain
X4:P1.21,16 throughout the study. He had high initial levels of antibody to the homologous strain in week 1 serum, that further increased by week 10. Lower levels of cross-reacting antibodies to the control strain, H44/76, could also be detected but showed no increase in titer during the study.

**Effect of carriage on immune response to individual meningococcal proteins.** To investigate the effect of carriage on the immune response to individual meningococcal proteins, sera from each subject were reacted in Western blot with the meningococcal strains that colonized that individual and also with strain H44/76 as a control (figure 3). In general, for subjects who became colonized during the study, precolonization sera demonstrated little or no reactivity to antigens from homologous or heterologous strains. Following colonization, marked reactivity to the homologous class 1 protein developed, along with weak reactivity to Opa and/or class 2/3 proteins. Reactivity to the control (H44/76) was not detected in sera from most subjects or was present at a low level to class 1 or class 3 proteins and did not show any increase in reactivity on colonization.

For example, week 1 serum from subject C showed no reactivity to the class 1 protein, class 3 protein, or Opa of the homologous strain. Following acquisition of the meningococci by week 6, serum from week 10 had high reactivity to the homologous class 1 protein and lesser reactivity to Opa and class 3 proteins. Reactivity to the class 1 and class 3 proteins of the control strain was present at low levels prior to colonization but showed no increase in reactivity following colonization.

Subject B, who was colonized throughout the study by a single strain (X4:P1.21,16), showed strong reactivity to homologous class 1 protein and lesser reactivity to class 3 protein and Opa in both available sera. In addition, these sera showed equivalent strong reactivity with the class 1 protein but not to class 3 or Opa proteins from the control strain. The control strain (H44/76) shares the common P1.16 epitope with the colonizing strain.

Some subjects produced strong reactivity by Western blot to specific outer-membrane proteins of homologous strains before the strain was cultured from throat swabs. This was only apparent for strains that colonized subjects early in the study (e.g., strain NG:1:P1.5a,2c in subject I), suggesting that initial colonization was not detectable by routine methods of culture. Sera from subject F, who was colonized by multiple strains, also showed high initial specific reactivity to class 1 protein and lesser reactivity to class 2/3 and Opa proteins, of the first colonizing strain (B:NT:P1.7b,26). Following subsequent colonization by W135:NT:P1.18a,3 and X4:P1.22,27a, reactivity developed to the class 1 proteins, and to a lesser extent class 2/3 and/or Opa proteins, of these strains.

Weak reactivity (blot score <2) to class 4 protein and pilin was seen in some subjects in addition to reactivity to the class 1, class 3, and Opa proteins. Such a response was seen only in subjects who entered the study with high levels of antibody to other outer-membrane proteins (subjects B, G, and I). No increase in reactivity to class 4 or pilin was observed in subsequent sera from these subjects (data not shown). No reactivity to class 4 protein or pilin was detected in any subject who acquired a strain during the course of the study. Of the meningococcal strains studied, only 2 weakly expressed Opc; W135:NT:P1.18a,3 and B:NT:P1.7b,26. No reactivity to Opc was detected in any sera collected during the study.
Figure 2. Effect of meningococcal carriage on immune response to homologous and heterologous strains. Immune response to nasopharyngeal acquisition of meningococci was determined by whole cell ELISA. Serum from each colonized individual was reacted with homologous colonizing strain(s) and also with control strain (H44/76). Bars show antibody titer at week 1 (shaded bars), week 10 (white bars), and week 29 (black bars).

Subject and Meningococcal Strain

Serum bactericidal activity to homologous and control meningococcal strains. The bactericidal activity of pre- and postcolonization sera was determined for subjects chosen as representatives of particular patterns of colonization, that is, colonized on entry (subject B), early (subject C), or late (subject H) or multiple colonization (subject F). For all of these subjects, there was a significant increase in bactericidal activity that was specific to the colonizing strain (table 1).

Subjects C and H, both colonized by the same single strain (X:4:P1.22,27a) during the course of the study, showed a >16-fold rise in bactericidal titer to that strain. Subject B, colonized on entry into the study with strain X:4:P1.21,16, similarly showed a 16-fold increase in bactericidal activity against his colonizing strain.

Subject F had a complex pattern of colonization by 3 separate strains. Bactericidal titer toward strain B:NT:P1.7b,26 was initially high, prior to isolation of the strain at week 6, and remained unchanged in serum taken at week 29. This is in accord with the total antibody and immunoblot data, which suggest he entered the study already carrying strain B:NT:P1.7b,26. Subsequently, subject F was colonized by strain X:4:P1.22,27a and developed a >16-fold increase in bactericidal activity specific for that strain. In addition, transient colonization with strain W135:NT:P1.18a,3 also produced a rise in specific bactericidal activity.

No increase in bactericidal activity was observed against any heterologous strain that did not share a common component with the homologous strain. In general, bactericidal titers to control strain H44/76 (B:15:P1.7,16) remained at low levels for all subjects and did not rise following colonization. The exception was subject B, whose serum was markedly bactericidal for H44/76, which shares the common subtype P1.16 epitope with the colonizing X:4:P1.21,16 strain. The colonization of different subjects by 2 X:4 strains of differing subtypes but common LOS (L3,7,9) permitted analysis of the relative contribution of antibodies to class 1 protein to bactericidal activity. Subjects C, F, and H all showed a 16-fold rise in homologous bactericidal activity after colonization with X:4:P1.22,27a. However, none developed more than a 4-fold rise in bactericidal activity to the heterologous X:4:P1.21,16.
strain, and subject H showed no rise at all. Conversely, subject B showed a 16-fold rise in bactericidal activity to the colonizing X:4:P1.21,16 strain but only a 4-fold rise to the heterologous X:4:P1.22,27a strain. Thus, it would appear that the major contribution to the bactericidal activity of sera from each of the subjects was subtype-specific.

Discussion

High levels of meningococcal acquisition and carriage have been demonstrated to occur commonly within semiclosed environments, such as military camps [2, 31]. Such communities therefore provide an ideal environment for studies on the dynamics of meningococcal carriage and the immune response to the carrier state. However, the majority of isolates that have previously been obtained from such studies have not been easily characterized by standard serologic typing methods, since they are frequently not typeable and/or subtypeable [2, 31, 32].

In this study, therefore, we applied molecular methods to study the dynamics of meningococcal carriage. A cluster of subjects from a single troop of military recruits were colonized at some time during their 30-week training period, apparently by a single meningococcal strain, which was characterized as X:4:NST. Although subtyping by serologic methods could not
be used to further characterize these isolates, subtyping by sequencing the porA gene clearly demonstrated that these were all of identical subtype (P1.22,27a) and demonstrated their continuity of carriage within any individual and transmission between subjects. Sequencing also clearly differentiated these isolates from the single X:4:P1.21,16 strain carried by subject B. Some subjects were initially colonized by other strains, but the X:4:P1.22,27a strain predominated within the troop, displacing resident meningococci; once acquired, it continued to colonize each subject for the remainder of the study. Sera from some subjects showed marked reactivity specific to homologous strains before the strain was cultured from throat swabs. This finding reflects the relative insensitivity of throat swabbing in detecting nasopharyngeal carriage of meningococci [31, 33].

Detailed molecular information on the antigenic profile of meningococcal isolates is also essential for analysis of the immune response to carriage. Early studies of antibodies induced by carriage showed antibody response to meningococcal capsular polysaccharide [5] but also that colonization by NG meningococci resulted in the development of bactericidal anti-bodies to capsulated pathogenic strains [10]. Such data indicated that a significant proportion of bactericidal antibodies induced by colonization was directed against subcapsular antigens, and these antibodies were predicted to form the basis of a large part of natural immunity [10, 34]. However, the meningococcal surface antigens that gave rise to such antibodies and the relative contribution of each antigen to the development of natural immunity could not be defined.

In our study, each subject had a detailed molecular profile of meningococcal colonization that permitted analysis of the specificity of the immune response to acquisition and carriage. In general, prior to colonization, subjects had low levels of serum antibodies specific to each homologous strain, demonstrated little or no reactivity to individual homologous meningococcal surface proteins, and showed minimal serum bactericidal activity against homologous strains. Following colonization, there was a marked increase in serum antibodies specific to the acquired strain; this was associated with a marked increase in reactivity by Western blot to the homologous class 1 protein and a lesser increase in reactivity to Opa and/or class 2/3 protein. Subjects colonized by multiple strains showed evidence of a specific immune response to the class 1 protein of each strain acquired during the study.

The most important correlate of protection against meningococcal infection is believed to be the presence of serum bactericidal activity against the invasive strain [6]. In the current study, a large rise in bactericidal activity specific for the homologous strain was observed following colonization. This may have been directed against a variety of components, including group, type, subtype, and LOS antigens. A subtype component to the response was demonstrated by the observation that the only subject with increased bactericidal activity to the control strain was colonized by a meningococcus that shared a common P1.16 epitope. Moreover, in each of the 4 subjects colonized by X:4 strains, bactericidal activity to their homologous X:4 strain rose 16-fold after acquisition, while bactericidal activity rose by ≤4-fold to the heterologous X:4 strain, which had common group, type, and LOS antigens but a different subtype.

The outer-membrane Opc protein has also been reported to induce bactericidal antibodies following vaccination and also after colonization by strains that express this protein at high levels [15]. In the current study however, only 2 strains weakly expressed Opc and were only transiently carried by 2 subjects who produced no detectable antibodies. Similarly, class 4 protein and pilin antibodies did not appear to be an important component of the immune response to colonization.

These data suggest that the majority of the increase in bactericidal activity was directed against the homologous class 1 protein. Thus, antibodies directed against the class 1 protein induced by colonization are likely to play an important role in the development of natural immunity to meningococcal disease.

Our studies on the specificity of immune response to carriage are also in accord with studies of the immune response to invasive disease, which have identified the class 1 protein as
an important immunogen [13, 16, 21]. In particular, meningococcal infections in recipients of an OMV vaccine were caused only by heterologous strains, indicating sero-subtype-specific protection [16]. No such protective effect was observed for type-specific antibodies directed against class 3 protein [35]. Serogroup B meningococcal vaccines based on OMVs containing multiple class 1 proteins are currently undergoing human clinical trials [22], and it is critical to the success of such vaccines that the class 1 proteins of circulating pathogenic meningococci do not undergo rapid change. A single amino acid change occurring at a critical site may dramatically alter the exposed epitope in the class 1 protein, with profound consequences for immune recognition [36]. Although evidence suggests that porA is not stable over a period of decades and multiple variants of individual subtypes have been detected [20], sequence differences observed between geographically or temporally unrelated meningococcal isolates cannot indicate the rate of change of porA.

By determining the DNA sequence of the variable regions of the porA gene from serial isolates obtained from the same persons over a defined time period, we were able to assess the stability of the porA gene. The porA gene remained stable for up to 30 weeks in 5 different meningococcal strains studied, during prolonged carriage by different subjects. This was despite evidence of colonization by multiple strains and the development of a subtype-specific bactericidal response, both of which are factors that would be expected to generate a selective pressure for mutation or recombination. This suggests that mutation of porA or horizontal exchange of genetic material is an uncommon event and that previously reported sequence variations of porA, detected in serologically indistinguishable meningococci isolated in close temporal proximity, are likely to represent the presence of distinct strains rather than a rapid mutation of the porA gene.

Thus, the data from this study, which show the importance of the subtype specificity of the bactericidal response to meningococcal colonization and the relative stability of the porA gene, support the rationale for the design of the current experimental multivalent class 1 protein vaccines for prevention of serogroup B meningococcal disease.

Acknowledgments

We thank the Meningococcal Reference Unit, Public Health Laboratory (Manchester) for performing serologic classification of isolates and G. Carlone for the human plasma complement source.

References


