Neisseria meningitidis Escape from the Bactericidal Activity of a Monoclonal Antibody Is Mediated by Phase Variation of lgtG and Enhanced by a Mutator Phenotype

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Bacteria adapt to environmental changes through high-frequency switches in expression of specific phenotypes. Localized hypermutation mediated by simple sequence repeats is an important mechanism of such phase variation (PV) in Neisseria meningitidis. Loss or gain of nucleotides in a poly(C) tract located in the reading frame results in switches in expression of lgtG and determines whether a glucose or a phosphoethanolamine (PEtn) is added at a specific position in the inner core lipopolysaccharide (LPS). Monoclonal antibody (Mab) B5 is bactericidal for N. meningitidis strain 8047 when PEtn is present in the inner core LPS and lgtG is switched “off.” Escape from the bactericidal activity of this antibody was examined by subjecting strain 8047 to multiple cycles of growth in the presence of Mab B5 and human serum. Escape variants with alterations in the lgtG repeat tract rapidly accumulated in bacterial populations during selection with this antibody. Strain 8047 was outcompeted in this assay by the 8047 ΔmutS strain due to the elevated PV rate of this mismatch repair mutant and hence the greater proportion of preexisting phase variants of lgtG in the inoculum. This ΔmutS mutant was also more virulent than strain 8047 during escape from passive protection by Mab B5 in an in vivo infant rat model of bacteremia. These results provide an example of how PV rates can modulate the occurrence and severity of infection and have important implications for understanding the evolution of bacterial fitness in species subject to environmental variations that occur during persistence within and transmission between hosts.

One determinant of the mutation rate of a DNA molecule is the nucleotide sequence (8). Evolution has acted on this intrinsic feature of different DNA sequences to focus the production of genetic variation in regions of genomes subject to intense environmental selection (28). One outcome of such localized hypermutation is phase variation (PV) (27, 40) or the rapid and reversible generation of variants exhibiting different expression states (e.g., “on” and “off”) of a particular phenotype. Mutations in simple sequence repeat tracts, also termed microsatellites, are a widespread mechanism responsible for PV and are thought to mediate adaptation to specific environmental alterations (6). The mutation rates of these repeat tracts are likely, therefore, to have a major impact on the fitness of the bacterial commensals and pathogens in which this mechanism of PV occurs.

Neisseria meningitidis is a commensal of the upper respiratory tract of humans and has the potential to cause serious invasive diseases, such as septicemia and meningitis. Like a number of other pathogens and commensals, this species contains multiple loci (~40 loci/genome) which are subject to PV due to simple sequence repeat tracts (26, 39). Most of these loci encode surface proteins (e.g., adhesins, iron acquisition proteins, and porins) or enzymes involved in biosynthesis of surface molecules, such as lipopolysaccharide (LPS) (18, 37). The fitness advantages associated with the different PV states (“on” and “off”) have been demonstrated for some of these loci and include significant roles in adhesion, resistance to the bactericidal activities of human serum, iron acquisition, and other phenotypes critical for host adaptation (references 5, 27, 33, and 40 and references therein). In many cases, it is assumed that specific antibodies drive selection for one of the PV states (usually the “off” phenotype). While PV can mediate evasion of antibody-mediated killing (for example, PV of LPS sialylation that results in general resistance to antibody-mediated killing due to dysfunctional activation of complement [41]), escape due to alterations in the structure or expression of the binding site for a specific antibody is also likely to be important. Escape from specific antibodies by PV in N. meningitidis has not been robustly documented even though bactericidal antibodies are associated with protection against this pathogen and antibodies specific for phase-variable surface structures are present in sera from patients and carriers (7, 17, 19, 21, 29). A major determinant of adaptation by PV is likely to be the rate of generation of phase variants. The majority of the phase-variable loci of meningococci contain poly(C) or poly(G) tracts consisting of more than seven repeat units whose mutation rates are controlled by the mismatch repair proteins MutS and MutL (25, 26, 34). High numbers of mismatch repair mutants with elevated PV rates have been observed among epidemic isolates of serogroup A strains of N. meningitidis (35). This
finding was interpreted as an indication that a meningococcal mutator phenotype increases the transmission and spread of this bacterial pathogen during epidemics.

Monoclonal antibody (MAb) B5 (also designated L3B5) recognizes an inner core LPS epitope and has an absolute requirement for a phosphoethanolamine (PEtn) moiety attached at the 3′ position of the second heptose (PEtn-3) (32). Attachment of PEtn is mediated by the product of lgtG (22). Binding of MAb B5 to the LPS of some meningococcal strains is subject to PV, and for strain BZ157, loss of binding was correlated with alterations to an in-frame number of repeats in the mononucleotide repeat tract of lgtG (22). The gonococcal lgtG gene is 96% identical to the N. meningitidis lgtG gene and mediates the phase-variable addition of a glucose to the 3′ position of the second heptose (4). Attachment of this glucose prevents addition of PEtn-3 such that a gain of expression of lgtG is associated with loss of the MAb B5 epitope. Some meningococcal isolates are subject to MAb B5-mediated bactericidal activity and opsonophagocytosis, and these activities are most pronounced in strains, such as 8047, which possess truncated LPS glycoforms and PEtn-3 (31). In addition, MAb B5 was demonstrated to protect infant rats against challenge with strain 8047 (31). Antibodies specific for PEtn-3 are present in sera from both meningococcal patients and healthy carriers (29), and recently Cox et al. (9) showed that a protective response can be induced using conjugate LPS molecules carrying this epitope. These results indicate that the PEtn-3-containing epitope is an important potential target for deriving protection against meningococcal infection. Thus, MAb B5 provides a good reagent for studying the contribution of phase-variable switches in expression of surface epitopes to escape acquired immunity. We utilized this antibody and a modified serum bactericidal assay to test the hypotheses that PV can mediate escape from an adaptive immune response and that the elevated PV rate of a mutator strain provides an adaptive advantage to meningococci under these conditions.

MATERIALS AND METHODS

Bacterial strains and growth conditions. N. meningitidis strain 8047 has been described previously (31). A mutS mutant of strain 8047 (8047 ΔmutS) was derived by transformation with genomic DNA from an MC58 ΔmutS mutant in which the gene had been disrupted by insertion of a kanamycin resistance cassette (25). Strains were grown overnight on brain heart infusion (BHI) media supplemented with Levinalthal broth (10%, vol/vol) or on chocolate agar plates at 37°C in an atmosphere containing 5% CO2. Plates were supplemented with kanamycin (75 µg/ml) when it was appropriate.

Serum bactericidal escape and selection assays. In the two versions of the assay utilized in this study (Fig. 1 and 2), the first passage involved preparation of an inoculum (whose size was variable) from an overnight bacterial culture grown on solid medium. The inoculum was prepared by suspending cells scraped from agar plates in 1 to 2 ml of PBSB (phosphate-buffered saline [PBS] supplemented with 0.5 mM MgCl2 and 0.9 mM CaCl2 [pH 7.4]), estimating the concentration of cells using the optical densities at 260 nm of known dilutions of cells lysed in 1% sodium dodecyl sulfate-0.1 M NaOH, and preparing dilutions in PBSB. A 50-µl aliquot of the dilution of bacteria that resulted in the desired inoculum was mixed with 500 µl of PBSB-0.1% glucose and put into one well of a 24-well tissue culture plate. This inoculum was mixed with 500 µl of PBSB containing 0.1% glucose, 10% human serum (either adult pooled serum from healthy carriers or adult serum with a low level of intrinsic bactericidal activity),
and various amounts of either MAb B5 ascites fluid or purified MAb B5. The plate was then incubated for 2 to 3 h (protocol 1) or for 2 h (protocol 2) at 37°C in an atmosphere containing 5% CO₂. During the incubation period, samples were removed from the well at different time points, serially diluted, and then plated onto BHI agar plates prior to overnight incubation. These procedures constituted the first passage. For protocol 1, the inoculum for the second passage was prepared on the next day using the method that was used for the first passage for one of the sample time points (usually 80 min), using the plate containing the highest density of bacteria. In protocol 2, the second passage was performed on the same day by removing 500 µl of the first-passage culture and mixing it with 500 µl of a fresh preparation of human serum and MAb B5. For each protocol, subsequent passages were performed like the second passage.

The numbers of CFU in each inoculum were estimated by using appropriate dilutions grown overnight on solid media. The numbers of phase variants in each inoculum were determined by probing colony immunoblots with MAb B5 (see below). The changes in the number of bacterial cells and the proportion of phase variants occurring during each passage were estimated in a similar fashion by plating dilutions of samples taken at the indicated time points (Fig. 1 and 2) or at the end of the passage.

Detection of MAb B5 phase variants and analysis of the lgtG repeat tract lengths of phase variants. Phase variants were detected using MAb B5 and a colony immunoblotting protocol as described previously (26). Briefly, colonies were transferred to nitrocellulose filters, which were then incubated in blocking buffer for 1 h. The filters were washed three times prior to incubation for 2 h with a 1-in-2,000 dilution of a 1-mg/ml stock solution of MAb B5 in PBS containing 0.1% Tween 20 and 2% (wt/vol) bovine serum albumin. The filters were then washed three times prior to incubation for 1.5 h with a 1-in-2,000 dilution of a secondary antibody, anti-mouse immunoglobulin G alkaline phosphatase-conjugated goat antibody (Sigma), in PBS containing 0.1% Tween 20 and 2% (wt/vol) bovine serum albumin. The filters were washed three times, and bound antibody was detected with a solution containing 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium (Perkin Elmer Life Sciences).

Colonies representative of specific PV phenotypes were identified using the stained filters. In order to analyze the repeat tracts of phase variants, plates were reincubated for ~6 h after transfer of colonies to filters. Phase variant colonies identified from the filters were restreaked onto fresh BHI medium plates and incubated overnight. Genomic DNA was isolated from sweeps of these plates by using a cetyltrimethylammonium bromide protocol (3). The lgtG gene was amplified by PCR using primers lgtG-C1 (5'-CTCAAAATAGACATTGCAACC) and lgtG-CB1 (5'-TACGCCGACCTCCTCATCTG), and the repeat tract was then sequenced using an internal primer (lgtG-CB1 [5'-TACGCCGACCTCCTCATCTG]) and BigDye sequencing reagents (Perkin Elmer), followed by electrophoresis and analysis with an autosampler. The presence or absence of an inactivated mutS gene was determined by PCR as described previously (25).

Assessment of escape from passive protection in an infant rat model of infection. The relative abilities of strains 8047 (mutS⁻) and 8047 ΔmutS (ΔmutS) to escape from passive protection by MAb B5 were assessed using an infant rat model of infection as described previously (31). The bacterial strains were grown overnight on BHI medium plates, the resulting cultures were used to prepare bacterial suspensions, and 1.35 × 10⁹ CFU of each suspension was inoculated into 9 ml of Mueller-Hinton broth containing 0.25% glucose. The broth cultures were incubated for 2.5 h at 37°C in the presence of 5% CO₂ with gentle agitation until the optical density at 620 nm was ~0.5. Bacterial cells were recovered from 2 ml of culture by centrifugation for 5 min. The pellets were resuspended in 200 µl of PBS and used for preparation of bacterial suspensions containing 1 × 10⁸ CFU/ml. Appropriate working dilutions of these stocks and of MAb B5 were then prepared in PBS-1% bovine serum albumin. The inoculum (100 µl/animal) for each group of animals was prepared from these stocks immediately prior to administration by the intraperitoneal route. Bacterial cells and MAb B5 were mixed and injected simultaneously, as reported previously (31). Each group of
animals consisted of five 5- to 8-day-old outbred Wistar rats. Group 1 received 2,500 CFU of strain 8047, 2,500 CFU of strain 8047 ΔmutS, and 2 µg of purified MAb B5. Group 2 received 5,000 CFU of strain 8047, 5,000 CFU of strain 8047 ΔmutS, and 2 µg of purified MAb B5. Group 3 received 2,500 CFU of strain 8047 and 2,500 CFU of strain 8047 ΔmutS. Group 4 received 5,000 CFU of strain 8047 and 500 CFU of strain 8047 ΔmutS. Blood (~5 µl) was obtained at 6 h postinfection from the tail vein of each animal and mixed with 50 µl PBS, and then 25-µl portions were plated onto BHI medium plates with and without kanamycin. Animals were euthanized at 18 h postinfection, and blood was obtained by cardiac puncture. Aliquots of serial dilutions were plated onto BHI medium plates with and without kanamycin, which were then incubated overnight at 37°C in the presence of 5% CO₂. The numbers of CFU per ml of blood medium plates with and without kanamycin. Animals were euthanized at 18 h postinfection, and blood was obtained by cardiac puncture. Aliquots of serial dilutions were plated onto BHI medium plates with and without kanamycin, which were then incubated overnight at 37°C in the presence of 5% CO₂. The numbers of CFU per ml of blood were determined for both the mutS⁻ and ΔmutS strains and used to calculate output ratios. These output ratios (mutS⁻/ΔmutS) were then divided by the input ratios (mutS⁺/ΔmutS), which were derived from colony counts obtained using appropriate dilutions of each inoculum plated on BHI medium plates with and without kanamycin, to generate a competitive index (CI). The frequency of nonreactive MAb B5 phase variants in the populations and the status of the lgtG repeat tract in phase variants were determined as described above.

RESULTS

Phase variants of lgtG mediate escape from the bactericidal activity of MAb B5. Many surface structures of N. meningitidis are subject to PV and are capable of eliciting bactericidal antibodies during an infection such that there is a significant potential for PV to mediate escape from killing by specific antibodies. In order to investigate this phenomenon in detail, we utilized MAb B5, which was previously shown to mediate killing of N. meningitidis strains through binding to a phase-variable LPS epitope (22, 31), and two novel versions of the serum bactericidal assay. The two assays both involved subjecting bacterial populations to sequential cycles of killing by specific antibody, and they differed only in whether cycles or passages were interrupted by nonselective growth on media or were continuous (Fig. 1 and 2). Interrupted selection mimics periodic exposure to a specific immune response, while continuous selection mimics an ongoing immune response in which antibody is constantly replenished. These assays enabled us to examine the role of PV in escape from killing by a specific immune response and to analyze the determinants of the rate of escape.

N. meningitidis strain 8047 was chosen for these assays as this strain reacts with MAb B5, is highly sensitive to MAb B5-mediated killing, and contains lgtG, the known correlate for PV of the MAb B5 phenotype (22, 31). The lgtG gene of our stock of strain 8047 was analyzed and found to contain a repeat tract consisting of 12 C residues, which is an “off” number of repeats and correlates with an MAb B5 “on” phenotype (data not shown). Colony immunoblotting experiments indicated that the frequency of switching from “on” to “off” for the MAb B5 phenotype was ~3.8 × 10⁻⁷, similar to the value previously reported for strain MC58, 2.6 × 10⁻⁷ (25). Based on this result we predicted that a population of ~1 × 10⁶ CFU would contain ~40 MAb B5 nonreactive variants and could escape killing by this antibody.

Escape from MAb B5 antibody-mediated killing was examined using strain 8047 in both the interrupted (Fig. 1) and continuous (Fig. 2) assays. In both cases, a large inoculum (~1 × 10⁷ CFU) was incubated with a level of MAb B5 that had previously been shown to mediate significant bactericidal activity (data not shown). In the first passage, strong bactericidal activity of MAb B5 was observed after 40 min of incubation with antibody (e.g., there were 74- and 140-fold reductions in the total number of CFU for the interrupted and continuous assays, respectively [Fig. 1 and 2]) but not after incubation without antibody (Fig. 2). In subsequent passages with the same amount of antibody, the reductions in the total sizes of the populations were smaller (e.g., 13- and 2-fold for both assays), indicating that the population had become resistant to MAb B5-mediated serum bactericidal activity. The passaged populations were then examined by colony immunoblotting in order to determine whether the development of resistance was due to the accumulation of MAb B5 nonreactive variants. The frequency of MAb B5 nonreactive variants increased from an undetectable level in the inoculum (~2.5 × 10⁻⁴) to 1.0 and 0.83 in passage 3 for the interrupted and continuous assays, respectively (Fig. 1 and 2). In the absence of MAb B5 (Fig. 2) or during selection with MAb P1.2 (data not shown), which recognizes a PorA epitope present in an outer membrane protein of strain 8047, there was no detectable change in the proportion of MAb B5 nonreactive variants. These findings indicated that the increase in the proportion of MAb B5 non-reactive phase variants was not due to these variants being resistant to nonspecific killing by human serum or other antibodies. Thus, we demonstrated that repeated cycles of growth of N. meningitidis strain 8047 in the presence of MAb B5 and a complement source (i.e., human serum) lead to development of resistance to killing by this antibody, which is correlated with a rapid and dramatic increase in the proportion of MAb B5 nonreactive phase variants.

The increase in the proportion of MAb B5 nonreactive phase variants could have been due to PV during selection with the antibody or outgrowth of preexisting phase variants from the inoculum. The inocula for all experiments performed with strain 8047 were examined by colony immunoblotting and were found to contain levels of variants below the level of detection (i.e., ~2 × 10⁻⁶). The frequencies of MAb B5 nonreactive variants in the inocula for the two experiments described above (Fig. 1 and 2) were, therefore, estimated from the growth rates (an analysis of the growth rates in the absence of antibody suggested that the doubling time was ~35 min [Fig. 2]) and the PV frequencies in the first cycle. Frequencies of 7 × 10⁻³ and 1 × 10⁻² were obtained for the interrupted (Fig. 1) and continuous (Fig. 2) assays, respectively (these frequencies translated into 270 and 10 variants in each inoculum). In all subsequent experiments with this strain, the inoculum was assumed to contain MAb B5 nonreactive phase variants at frequencies of ~4 × 10⁻³. These frequencies were similar to previous estimates (see above) for the frequency of MAb B5 nonreactive variants for this strain, suggesting that development of resistance to killing by this antibody was associated with survival and outgrowth of small numbers of preexisting MAb B5 nonreactive variants present in the inoculum.

PV of the MAb B5 phenotype is associated with alterations in expression of lgtG. Two MAb B5 nonreactive variants of strain 8047 from an interrupted assay (Fig. 1) and nine variants of this strain from a continuous assay (Fig. 2) were examined. All these variants had 11 C residues in the lgtG repeat tract, an “on” number of repeats, demonstrating that switching “off” of the MAb B5 reactive phenotype was due to switching “on” of expression of lgtG and indicating that escape from MAb B5-mediated killing was associated with PV of this gene.
The concentration of bactericidal antibody and the size of the bacterial inoculum affect the dynamics of escape from bacterial clearance by PV. The kinetics with which bacterial populations evade or escape from an acquired antibody response are influenced by factors such as the amounts, affinities, and functionalities of the bacterial specific antibodies and the size of the bacterial population. The effects of different antibody levels on escape from a specific antibody response were examined by performing the continuous assay using various levels of MAb B5 (Fig. 3). The frequencies of MAb B5 nonreactive variants present following three rounds of selection were dependent on the amount of antibody used for selection; for example, selection with 0.6 and 1.5 μg of antibody resulted in frequencies of 0.06 and 0.89, respectively (note that the inoculum contained variants at a frequency of ~4 × 10⁻³). The structures of the populations also differed during the course of selection. With the two highest concentrations of MAb B5, the population was reduced to a minimal size (140 to 200 CFU) before it recovered, yielding a final population composed entirely of variants possessing an MAb B5 nonreactive phenotype. A similar frequency of such variants was obtained with a lower level of MAb (i.e., 1.5 μg), but in this case the size of the total population was always more than ~1,000 CFU. These experiments were repeated on two other occasions using an inoculum consisting of 5 × 10⁴ CFU and either 0.6 and 1.2 μg of antibody or 0, 0.3, 1.2, and 3 μg of antibody. Three passages in the presence of 1.2, 1.2, and 3 μg of antibody produced populations consisting of 6 × 10⁴, 7 × 10⁴, and 6 × 10⁵ CFU containing MAb B5-negative variants at frequencies of 0.05, 0.07, and 0.94, respectively. Lower levels of antibody (0 to 0.6 μg) did not result in detectable changes in the proportions of variants. The requirement for a higher level of antibody to generate a similar change in the proportion of phase variants in the latter experiments compared to the results shown Fig. 3 reflects differences in the activities of aliquots of purified MAb B5 and/or a loss of activity due to prolonged storage at 4°C. Overall, the results of these experiments mirrored the results described above in that a 2.5-fold increase in level of antibody resulted in a 15-fold increase in the level of MAb B5-negative phase variants after three passages.

The influence of inoculum size on escape from MAb B5 was also examined (Fig. 4). Escape was observed with inocula containing ~7 × 10⁴ CFU, while with an inoculum containing ~7,000 CFU the level was less than the detectable level after two passages, indicating that the latter inoculum was too small to contain phase variants and therefore could not survive MAb B5-mediated killing. The former inoculum was predicted to contain three MAb B5-negative phase variants (assuming a PV frequency of 4 × 10⁻⁵ [see above]), indicating that very small numbers of phase variants can mediate escape. In repetitions of this experiment, using inocula containing 5 × 10³, 5 × 10⁴, and 5 × 10⁵ CFU subjected to three rounds of selection with >0.6 μg of antibody resulted in surviving populations consisting of 20, <3, and 80 CFU. In these cases all surviving cells were MAb B5 reactive, meaning that escape had not occurred. The absence of escape may have reflected stochastic variations in the presence of variants in such small populations or an inability of small numbers of variants to reproducibly mediate escape from other bottlenecks and survive. As observed for the various levels of antibody, the various inoculum sizes influenced the kinetics of escape. Thus, a high frequency of MAb B5 nonreactive phase variants was obtained after three passages with an inoculum consisting of 7 × 10⁵ CFU, but four passages were required with an inoculum consisting of 7 × 10⁶ CFU, although in the latter case the total population size was never less than 1 × 10⁴ CFU (Fig. 4). These experiments demonstrated that inoculum size is a critical determinant of the ability of meningococcal populations to escape from killing by a bactericidal antibody and that the kinetics of escape are

**FIG. 3.** Influence of high levels of antibody on escape from MAb B5-mediated serum bactericidal activity. Strain 8047 was passaged in a continuous selection assay as described in the legend to Fig. 2 using various amounts of purified MAb B5. The total number of viable cells was plotted for the inoculum and each passage. The frequencies of MAb B5 nonreactive variants (Freq. B5-ves) present at the end of the assay are indicated on the right. Squares, 0.6 μg of MAb B5; diamonds, 1.5 μg of MAb B5; triangles, 6 μg of MAb B5; circles, 12 μg of MAb B5.
influenced by the relative levels of bacterial cells and bactericidal antibody.

A mutS mutation increases the PV rate and provides a competitive advantage during escape from MAb B5-mediated serum killing. Another determinant of the dynamics of PV-mediated escape from killing by specific antibodies is likely to be the rate of PV. The influence of the PV rate was examined by constructing a mutation in the mutS gene, a known determinant of the MAb B5 PV frequency (25). Insertion of a mutS mutation into strain 8047 resulted in an ~1,000-fold increase in the frequency of MAb B5 nonreactive variants to $2.4 \times 10^{-2}$. Escape from MAb B5-mediated killing by the 8047 ΔmutS mutant was examined using both the interrupted (Fig. 1) and continuous (Fig. 2) assays. The mutant rapidly became resistant to MAb B5-mediated serum bactericidal activity. The development of resistance was accompanied by a switch to a population composed entirely of MAb B5 nonreactive variants in both assays (Fig. 1 and 2) and by a change in the length of the lgtG repeat tract (two and six MAb B5 nonreactive variants for each assay were found to have repeat tracts consisting of 11 C residues). In the absence of antibody, the proportion of MAb B5 nonreactive variants remained low (Fig. 2). The effects of both antibody concentration and inoculum size on changes in the proportion of MAb B5 nonreactive escape variants for the mutS mutant were also examined. The proportion of variants increased rapidly during assays with high levels (1.2 and 3 μg) of MAb B5, but increases were not observed with low levels of antibody or no antibody (data not shown). Similarly, large ($2.7 \times 10^6$ CFU), intermediate ($2.7 \times 10^5$ CFU), and small ($2.7 \times 10^3$ CFU) inocula of 8047 ΔmutS resulted in increased frequencies of variants (0.48, 0.61, and 0.8, respectively) after one passage, suggesting that the size of the inoculum influenced the dynamics of change in these populations (data not shown). Notably, an inoculum consisting of only 2,700 CFU (expected to contain 64 MAb B5 nonreactive variants) mediated robust escape from MAb B5 killing (data not shown). The lgtG repeat tracts of two strain 8047 and six mutS mutant MAb B5 nonreactive escape variants from the latter assay were analyzed, and seven of these variants had a tract consisting of 11 C residues, while the results for the eighth were indeterminate. These experiments indicated that the 8047 ΔmutS mutant rapidly escaped from killing by MAb B5 due to outgrowth from the inoculum of phase variants with an MAb B5 nonreactive phenotype and a repeat number permissive for translation of lgtG.

The assays described above indicated that the higher PV rate of the 8047 ΔmutS mutant populations would permit this mutant to outcompete the wild-type parental strain during escape from killing mediated by MAb B5. A competition assay was performed using 13- and 127-fold excesses of strain 8047 (mutS+) over the 8047 ΔmutS mutant (ΔmutS) in three passages in the continuous selection assay (Fig. 5). In the absence of antibody there was no significant change in the ratio of the two strains, indicating that the mutS mutation did not confer a growth advantage (Fig. 5), and no change in the number of MAb B5 variants (data not shown). In the presence of antibody, the final mutS+/ΔmutS ratios were <0.3, indicating that the mutS mutant had outcompeted the parental strain (Fig. 5). In both cases the populations had changed so that the bacteria had a fully MAb B5 nonreactive phenotype (data not shown). This assay was repeated in triplicate, and a similar competitive advantage was observed for the mutS mutant (data not shown). Competition between strains 8047 and 8047 ΔmutS was also examined using the interrupted assay (Fig. 6). In assays initiated with either an 18- or 180-fold excess of the mutS+ strain over the ΔmutS strain, two passages resulted in a final ratio of <0.2:1, indicating that the mutS mutant had outcompeted the parental strain. In both cases this was accompanied by a switch to a fully MAb B5 nonreactive population. In assays with 1,800- and 18,000-fold excesses of mutS+, the final mutS+/ΔmutS ratios after two passages were 0.6:1 and 17:1, respectively, and the frequencies of MAb B5 nonreactive variants were 0.89 and
outcompete strain 8047 during in vivo selection, the experience simultaneously test whether the 8047 to an MAb B5 nonreactive phenotype was possible and to Plested, unpublished data). In order to confirm that escape due bacteremic cells were shown to have a MAb B5 nonreactive (31). Intriguingly, low levels of bacteremia were observed in increased to 0.96. Competition in the interrupted assay was re-

mutS 8047. The inocula for the first passage contained different mixtures of strains 8047 (mutS+) and 8047 ΔmutS (ΔmutS). The ratios for subsequent time points were determined using serial dilutions grown overnight on BHI agar plates with and without kanamycin. Open squares, mutS+/ΔmutS ratio of 127:1 without MAb B5; open circles, mutS+/ΔmutS ratio of 13:1 without MAb B5; filled squares, mutS+/ΔmutS ratio of 127:1 with MAb B5; open circles, mutS+/ΔmutS ratio of 13:1 with MAb B5.

Four groups of five animals were inoculated with mixed populations of strains 8047 and 8047 ΔmutS (at a ratio of 1:1 for groups 1 to 3 and at a ratio of 10:1 for group 4). For two groups (groups 1 and 2), the bacterial inocula were mixed with 2 μg of MAb B5 immediately prior to injection. Bacteremia was assessed at both 6 and 18 h postinfection. At 6 h, there was a significant difference in the levels of bacteremia between groups that received antibody (the geometric mean bacteremia [GMB] levels were 0.9 × 10^3 and 3.3 × 10^3 CFU/ml for culture-positive animals in groups 1 and 2, respectively) and groups that did not receive antibody (the GMB levels were 8.5 × 10^4 and 6.6 × 10^4 CFU/ml for animals in groups 3 and 4, respectively) (P < 0.001 for a comparison of the levels of bacteremia of group 1 and 2 animals and the levels of bacteremia of group animals 3 and 4, as determined using a Mann-Whitney rank sum test). Differences in the levels of bacteremia were also seen at 18 h (the GMB levels were 0.9 × 10^3, 1.8 × 10^3, 2.4 × 10^3, and 7.1 × 10^3 CFU/ml for groups 1 to 4, respectively), but they were not significant (P = 0.09). A CI could not be calculated for the 6-h time point for animals that received antibody due to the small size of the output populations, but for the groups that did not receive antibody (groups 3 and 4) the geometric mean CIs were both 0.7 (standard deviations, 0.1 and 0.23, respectively). At 18 h, CIs were determined for the eight culture-positive animals inoculated with antibody and the nine culture-positive animals not inoculated with antibody (Fig. 7). The geometric mean CIs were 12.7 (standard deviation, 164) for the combined values obtained for groups 1 and 2 and 0.9 (standard deviation, 1.7) for the combined values obtained for groups 3 and 4. Only two of the animals treated with antibody had CIs of <3, and in both these cases the levels of bacteremia were low and 10/10 variants recovered from non-selective plates were kanamycin resistant, indicating that the CIs were underestimates of the actual levels of competition.

The inocula for the first passage contained different mixtures of strains 8047 (mutS+) and 8047 ΔmutS (ΔmutS). The inocula for passages 2 and 3 were derived from overnight cultures on plates at the 40- and 80-min time points of the previous passage, respectively. Ratios were determined as described in the legend to Fig. 5. Diamonds, mutS+/ΔmutS ratio of 18,000:1; triangles, mutS+/ΔmutS ratio of 1,806:1; squares, mutS+/ΔmutS ratio of 180:1; circles, mutS+/ΔmutS ratio of 18:1.

A mutS mutant outcompetes a nonmutator strain during infection of infant rats passively protected with MAb B5. High doses of MAb B5 were previously shown to completely protect infant rats against challenge with N. meningitidis strain 8047 (31). Intriguingly, low levels of bacteremia were observed in some animals given lower doses of antibody. In some cases the bacteremic cells were shown to have a MAb B5 nonreactive phenotype, indicating that strain 8047 could escape from the passive protection provided by this MAb (J. C. Hoe and J. S. Plested, unpublished data). In order to confirm that escape due to an MAb B5 nonreactive phenotype was possible and to simultaneously test whether the 8047 ΔmutS mutant could outcompete strain 8047 during in vivo selection, the experiments were repeated using mixed infections.
animals inoculated with strains 8047 and 8047 ∆mutS, respectively [referred to as groups 1 and 2]). CIs for using a total inoculum containing either 4,200 or 7,200 CFU (four and determined following inoculation of a 1:1 mixture of the two strains /H9004
mutS
stream. Overall, these results demonstrated that the 8047 mediated killing) from the peritoneal cavity into the blood-
numbers of bacterial cells (i.e., the cells that escaped antibody-
ing the levels of mutant and nonmutant cells and/or may have
animals may have reflected technical difficulties with compar-
The wide variation in the CIs for the other antibody-treated animals may have reflected technical difficulties with comparing the levels of mutant and nonmutant cells and/or may have reflected the bottlenecks associated with the transfer of small numbers of bacterial cells (i.e., the cells that escaped antibody-mediated killing) from the peritoneal cavity into the bloodstream. Overall, these results demonstrated that the 8047 ∆mutS mutant can outcompete strain 8047 in an in vivo model when MAb B5 is present but not when it is absent.

The MAb B5 phenotypes of the bacterial populations present at 18 h postinoculation were investigated by colony immunoblotting for equal numbers (n = 7) of animals that received antibody and animals that did not receive antibody. The frequencies of MAb B5 nonreactive variants were >0.95 (range, 0.95 to 1) for all animals treated with antibody but <0.07 (range, 0.002 to 0.06) for the animals which did not receive antibody (data not shown). The inocula for groups 1 to 5,900 CFU and either a 1:1 or 10:1 mixture of the two strains (five and four animals, respectively [referred to as groups 1 and 2]). CIs for animals inoculated with strain 8047, strain 8047 ∆mutS, and MAb B5 (mutS+;∆mutS Plus mAb B5) were determined following inoculation of a 1:1 mixture of the two strains using a total inoculum containing either 4,200 or 7,200 CFU (four and three animals, respectively [referred to as groups 1 and 2]). CIs for animals inoculated with strains 8047 and 8047 ∆mutS but no MAb B5 (mutS+;∆mutS No mAb B5) were determined for animals inoculated with 5,900 CFU and either a 1:1 or 10:1 mixture of the two strains (five and four animals, respectively [referred to as groups 3 and 4]). A statistical comparison of the CIs of the two groups was performed using a Mann-Whitney rank sum test.

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DISCUSSION

In this paper we describe a method for investigating the role of PV in mediating escape from a specific antibody response. Using this assay, meningococcal populations were shown to escape from MAb B5-dependent bactericidal activity when phase variants with a variation in a single gene, lgtG, were present in the population prior to initiation of selection. We then investigated whether the PV rate provided an adaptive advantage by examining the escape of a meningococcal strain carrying a mutation in a mismatch repair gene. Below, we discuss the specific impact of PV on immune clearance of meningococci and the general relevance of our findings for bacterial pathogens. Our findings also have a bearing on the prevalence of mutator strains in bacterial populations and on the evolution of mechanisms for generating localized variations in the mutation rate.

PV-mediated escape from a specific immune response. Microbial escape from an adaptive host immune response is a phenomenon common to most bacterial pathogens, but it occurs with reduced efficiency compared to virus escape (16) due to the relatively lower mutation rates of bacteria. The high frequency of antigenic switching associated with PV provides a mechanism to increase escape from adaptive immune responses. Using in vitro and in vivo models of escape from the bactericidal activity of an MAb (MAb B5) due to survival and outgrowth of phase variants lacking an LPS epitope (22, 31), we found that escape by meningococcal populations was rapid; for example, it occurred within ~5 h (Fig. 2, 3, and 4). Indeed, we calculated by extrapolation of the data shown in Fig. 4 and using four rounds of selection followed by 12 40-min rounds of replication that only 15 h would be required to generate a large meningococcal population (i.e., 6 × 10^6 organisms) consisting of 97% nonreactive MAb B5 variants from a population that was a similar size and consisted of >99% MAb B5 reactive organisms. Many of the nonreactive MAb B5 phase variants present in the meningococcal populations after selection were analyzed and found to contain an in-frame number of repeats in the lgtG gene that indicated that they were formed by off-to-on PV of this gene (note that switching this gene “on” is associated with loss of the MAb B5 reactive epitope [see Introduction]). These findings provide a formal experimental demonstration of the ability of repeat-driven PV to mediate
Our results demonstrated that there was a direct link between certain epitopes and correlation of the appearance of specific immune responses. In preventing carriage and spread of meningococci expressing a specific antigen (23). Phase variants have been detected during epidemics (1, 11) or natural carriage of meningococci (2, 15, 42), but the appearance of these variants has not been correlated with the presence of specific immune responses. Our results demonstrated that there was a direct link between a specific immune response and selection of lgtG phase variants, implying that an immune response to a meningococcal LPS epitope containing PEtn-3 drives selection for an off-to-on switch in this gene. The amounts of MAB B5 and core LPS antibodies present in sera from meningococcal patients have been compared (30). Levels of MAB B5 that are 4- to 10-fold-lower than those used in our study produce levels of activity comparable to the levels of activity obtained with native levels of antibody; e.g., 30% phagocytosis was observed in an opsonophagocytosis assay with either 0.15 μg/ml of MAB B5 or 10 arbitrary units of serum immunoglobulin G antibodies that bound galE LPS and are found in acute and convalescent-phase sera. Determining whether our experimental findings are relevant to the evolution of PV requires demonstration of a natural human mucosal immune response to PEtn-3 LPS epitopes and correlation of the appearance of such mucosal immunity with the generation of switching in lgtG or a similar correlation for antibodies to another phase-variable meningococcal antigen.

**Implications arising from the importance of the strength of selection and population size for escape from an adaptive immune response.** Both gradual and dramatic increases and decreases in titers of antibodies to either whole bacterial cells or specific surface molecules have been observed during long-term meningococcal carriage (19, 36), but it is not known how these changes affect the proportions of phase variants present in a bacterial population. Here we show that the rate of accumulation of MAB B5 nonreactive phase variants in a population was dependent on the relative levels of antibodies and bacterial cells utilized during selection. The gradual turnover of a population from one phase variant type to another may have important implications for bacterial populations in their natural environments. Thus, a gradual increase in the titer of antibody to a specific phase-variable antigen imposes selection for a slow accumulation of phase variants lacking expression of that antigen in a meningococcal population (Fig. 8b). The high number of phase variants present in such a preexposed population facilitates survival and recovery from a subsequent high-titer antibody response to the antigen because it results in a surviving population that is larger than the previously nonexposed population (Fig. 8, compare panels b and a). This larger surviving population buffers against other selective events, such as the mechanical effects of the movement of saliva or mucus across mucosal surfaces, and nonselective reductions in the size of the population.

The size of the population subjected to selection is another important determinant of the ability of a population to escape from an adaptive immune response. It is shown here that PV permits small bacterial populations (~1 × 10^2 CFU for wild-type strains and ~1 × 10^3 CFU for mutS mutants) to escape killing by a specific immune response (Fig. 4). Transmission of *N. meningitidis* occurs through droplets or salivary contact and is likely to involve small numbers of organisms (i.e., <10,000 cells). Small inocula rarely contain mutants with point mutations resistant to a specific immune response due to the low basal mutation rate, and so inoculum size becomes a limiting factor during transmission in a population of immune individuals. Our results indicate that PV increases the likelihood of a small population surviving a specific immune response (by increasing the number of immune resistant variants in such a population) and hence may increase the number of times that transmission between immune individuals is mediated by such populations, a characteristic with the potential to have a major impact upon meningococcal spread during, for example, the latter stages of epidemics, when many individuals are immune. **Implications for the prevalence of mutators in N. meningitidis populations.** Richardson et al. (35) interpreted the high prevalence of mutator strains with “high” PV rates among isolates of epidemic serogroup A *N. meningitidis* as an indication that an elevated PV rate is associated with efficient transmissibility (fitness) and could evolve during an epidemic through selection. We found that an *N. meningitidis* mutator phenotype could indeed confer a competitive advantage to a
strain over a nonmutator strain and facilitate escape from an adaptive immune response (Fig. 5, 6, and 7). As the PV rates of many other meningococcal surface structures are elevated in a mutator strain (25, 35), this competitive advantage could be due to PV of \( \text{lgtG} \) (whose expression is directly correlated with loss of the MAB B5 phenotype), PV of genes that modify presentation of the MAB B5 reactive epitope, PV of genes which cause dysfunction of antibody-mediated killing (41), or PV of a combination of genes. The vast majority of cells in the output populations in these experiments exhibited an MAB B5 nonreactive phenotype, and all of these cells that were tested had alterations in the \( \text{lgtG} \) repeat tract. Importantly, the competitive advantage was proportional to the difference in the “on-to-off” MAB B5 PV rate (i.e., \(-1,000\)-fold) between the wild-type and mutator strains (Fig. 6), indicating that it resulted from an elevated PV rate for \( \text{lgtG} \) alone and not from switching of \( \text{lgtG} \) and another gene, which would have occurred at a much lower frequency. While escape by some phase variants of the mutator strain may have been due to PV of other genes, these results suggest that the competitive advantage of the mutator strain during selection with MAB B5 was primarily dependent on the elevated PV rate of \( \text{lgtG} \) (Fig. 8c). One extrapolation of these results is that the adaptive immune response may be the selective pressure responsible for the higher prevalence of mutators observed for epidemic serogroup A meningococcal strains (35). Thus, during a meningococcal epidemic, the number of hosts with antibodies to currently circulating surface structures of the bacterium would increase and there would be selection for bacterial strains in which these structures are absent. These selective pressures would exert strong secondary selection for elevated PV rates and hence for a higher prevalence of strains with a mutant phenotype.

We also observed that the meningococcal mutator strain outcompeted the nonmutator parental strain for escape from a specific immune response, followed by transfer to and replication within the bloodstream. No competitive advantage was observed in the absence of selection with antibody. This result implies that meningococcal mutator strains may have a major influence on infections in partially immune individuals, in which the propensity to escape from acquired immune responses may be an important step preceding initiation of a disease-causing infection. The potential association between a mutator phenotype and pathogenesis deserves further attention, particularly in view of our limited knowledge of the epidemiological prevalence of mutators in meningococcal populations. These considerations could also be relevant to multivalent meningococcal vaccines, where protective antigens are known to be phase variable (14, 24).

The rate of localized hypermutation confers an adaptive advantage. Our model differs from previous studies of mutator phenotypes (13, 20, 38) in that it mimics a recurrent natural selective pressure (i.e., an adaptive immune response) and selection acts on a specific sequence whose evolution was driven by a requirement for mutability in a specific gene. Thus, the ability of the \( N. meningitidis \) \( \text{mutS} \) mutant to outcompete the nonmutator strain was related to the number of preexisting variants with alterations in the repeat tract of \( \text{lgtG} \) and hence to the difference in the mutability of this specific region of the genome, as opposed to a generalized increase in the mutation rate. This aspect of our model is critical as a mutator phenotype is accompanied by an increase in generation of deleterious mutations and hence a loss of fitness (12). However, the advantage conferred by the \( \text{mutS} \) mutation in our assays could also be achieved through the heightened rates of mutation associated with the differences in repeat tract length (10) that are known to exist in different meningococcal isolates (10, 35). A correlation between repeat mutability and competitive advantage, as indicated here, is a prerequisite for evolution of localized hypermutation as a mechanism for production of phenotypic variation. Our results, therefore, provide experimental evidence of the powerful impact of localized hypermutation on the fitness of a bacterial pathogen subject to the dynamic and stringent selective pressures exerted by an adaptive host immune response.

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