

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 Δ *mutS* 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 *mutS* 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

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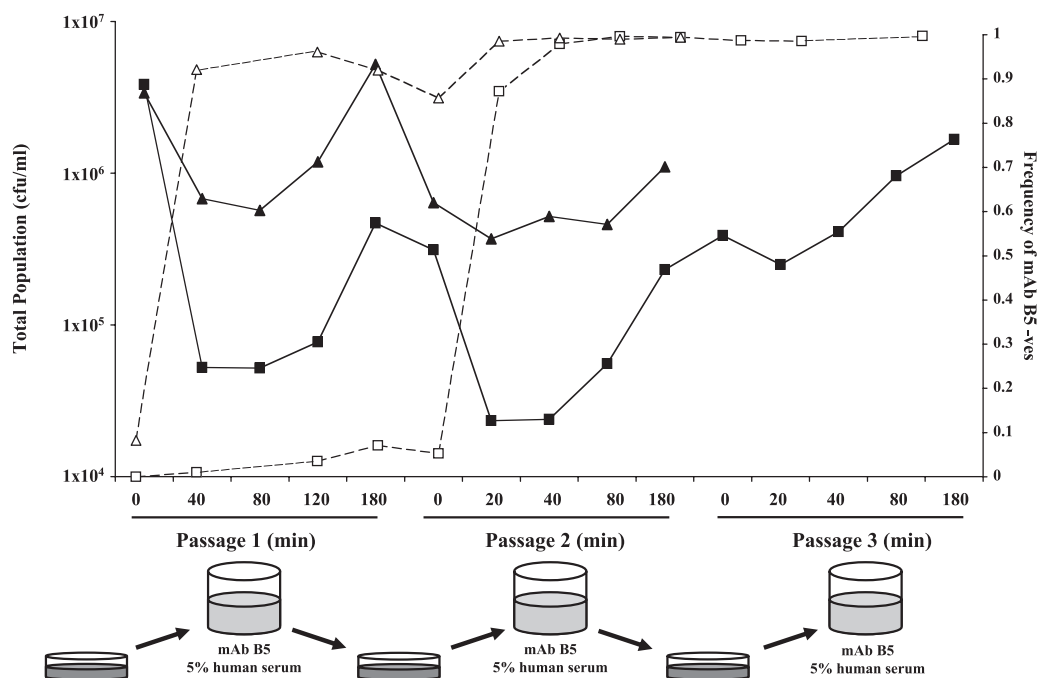


FIG. 1. Escape of *N. meningitidis* strain 8047 from MAb B5-mediated serum bactericidal activity in an interrupted selection assay. Strain 8047 or 8047 $\Delta mutS$ was incubated in 1 ml of PBSB containing 0.1% glucose and 5% pooled human serum supplemented with 15 μ l of a 1:50 dilution of MAb B5 ascites fluid. Three 180-min passages were performed. The inocula for each passage were prepared following overnight growth of bacteria on BHI medium plates (as indicated at the bottom). The inocula for passages 2 and 3 were prepared from plates inoculated with bacterial cells present at the 80-min time point in the previous passage. The data are the numbers of viable cells present in the inoculum and each passage, which were determined using serial dilutions of appropriate samples, and the frequencies of MAb B5 nonreactive variants (mAb B5-ves), determined by probing colony immunoblots with MAb B5. Dashed lines indicate the frequency of MAb B5 nonreactive variants, and solid lines indicate the size of the total population. Squares, strain 8047; triangles, strain 8047 $\Delta mutS$.

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 *lpt3* (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 $\Delta mutS$) was derived by transformation with genomic DNA from an MC58 $\Delta mutS$ 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 Levinthal broth (10%, vol/vol) or on chocolate agar plates at 37°C in an atmosphere containing 5% CO₂. Plates were supplemented with kanamycin (75 μ g/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- μ l aliquot of the dilution of bacteria that resulted in the desired inoculum was mixed with 500 μ l of PBSB-0.1% glucose and put into one well of a 24-well tissue culture plate. This inoculum was mixed with 500 μ l 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),

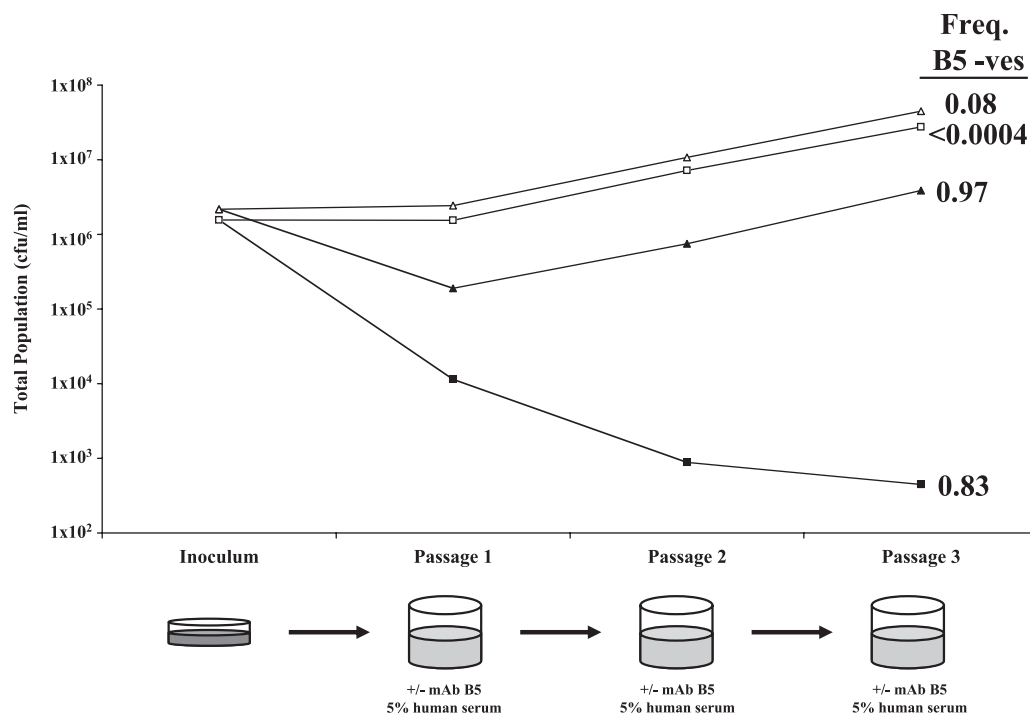


FIG. 2. Escape of *N. meningitidis* strain 8047 from MAb B5-mediated serum bactericidal activity in a continuous selection assay. Strain 8047 or the 8047 $\Delta mutS$ mutant was incubated in 1 ml of PBSB containing 0.1% glucose and 5% human serum either with or without MAb B5. Two 120-min passages and one 40-min passage were performed. The inoculum for the first passage was prepared following overnight growth of bacteria on BHI medium plates, while for subsequent passages a 500- μ l sample from the previous passage was mixed with an equal volume of PBSB containing 1% glucose, 5% human serum, and antibody (as indicated at the bottom). The data are the numbers of viable cells present in the inoculum and each passage, which were determined by plating serial dilutions of appropriate samples. Samples for passages were collected after 40 min of incubation. The frequencies of MAb B5 nonreactive variants (Freq. B5-ves) present in the final passage were determined using colony immunoblots probed with MAb B5 and are indicated on the right. Filled squares, strain 8047 and 1.2 μ g of purified MAb B5; open squares, strain 8047 and no MAb B5; filled triangles, strain 8047 $\Delta mutS$ and 1.2 μ g of purified MAb B5; open triangles, strain 8047 $\Delta mutS$ and no MAb B5.

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 μ l of the first-passage culture and mixing it with 500 μ l 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*-F (5'-GAACTCGTCCCCGATTTT), and the repeat tract was then sequenced using an internal primer (*lgtG*-CBI [5'-TACGCCGACCTCCTCAT CTGC]) and BigDye sequencing reagents (Perkin Elmer), followed by electrophoresis and analysis with an autosequencer. 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 $\Delta mutS$ ($\Delta mutS$) 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 \times 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 each culture by centrifugation for 5 min. The pellets were resuspended in 200 μ l of PBS and used for preparation of bacterial suspensions containing 1 \times 10⁹ CFU/ml. Appropriate working dilutions of these stocks and of MAb B5 were then prepared in PBS-1% bovine serum albumin. The inoculum (100 μ l/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 $\Delta mutS$, and 2 μg of purified MAb B5. Group 2 received 5,000 CFU of strain 8047, 5,000 CFU of strain 8047 $\Delta mutS$, and 2 μg of purified MAb B5. Group 3 received 2,500 CFU of strain 8047 and 2,500 CFU of strain 8047 $\Delta mutS$. Group 4 received 5,000 CFU of strain 8047 and 500 CFU of strain 8047 $\Delta mutS$. Blood ($\sim 5 \mu\text{l}$) 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 were determined for both the $mutS^+$ and $\Delta mutS$ strains and used to calculate output ratios. These output ratios ($mutS^+/\Delta mutS$) were then divided by the input ratios ($mutS^+/\Delta mutS$), 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 on-going 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 $< 3.8 \times 10^{-5}$, similar to the value previously reported for strain MC58, 2.6×10^{-5} (25). Based on this result we predicted that a population of $\sim 1 \times 10^6$ 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 ($> 1 \times 10^6$ 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 ($< 2.5 \times 10^{-4}$) 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 nonreactive 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., $\sim 2 \times 10^{-4}$). 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^{-5} and 1×10^{-5} 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 $\sim 4 \times 10^{-5}$. 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.

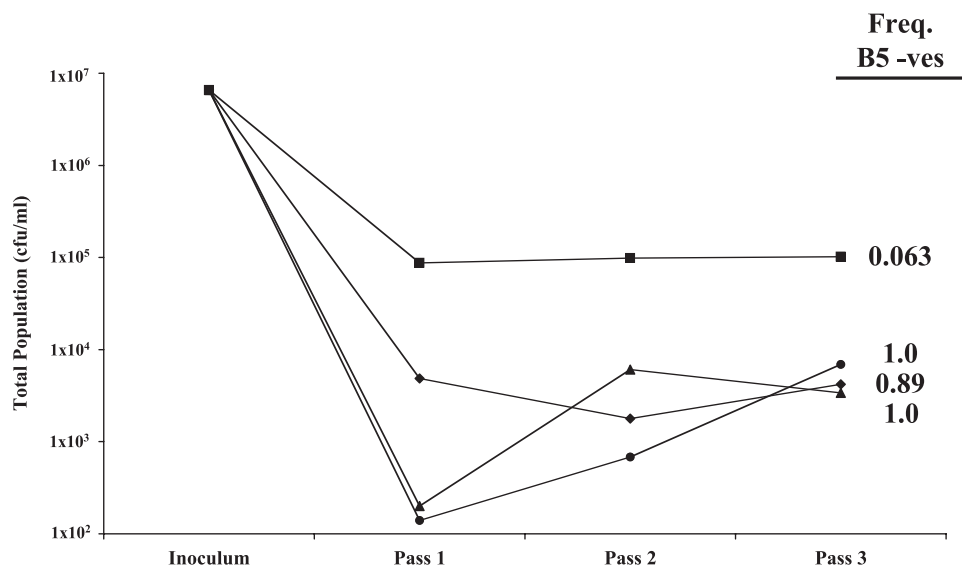


FIG. 3. Influence of high levels of antibody on escape from MAb B5-mediated serum bactericidal activity. Strain 8047 was passaged in a continuous selection assay as described in the legend to Fig. 2 using various amounts of purified MAb B5. The total number of viable cells was plotted for the inoculum 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.

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 $\sim 4 \times 10^{-5}$). 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 $\sim 1,000$ CFU. These experiments were repeated on two other occasions using an inoculum consisting of 5×10^6 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 6×10^4 , 7×10^4 , and 6×10^3 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 $\sim 7 \times 10^4$ CFU, while with an inoculum containing $\sim 7,000$ 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 5×10^3 , 5×10^4 , and 5×10^4 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 7×10^5 CFU, but four passages were required with an inoculum consisting of 7×10^6 CFU, although in the latter case the total population size was never less than 1×10^4 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

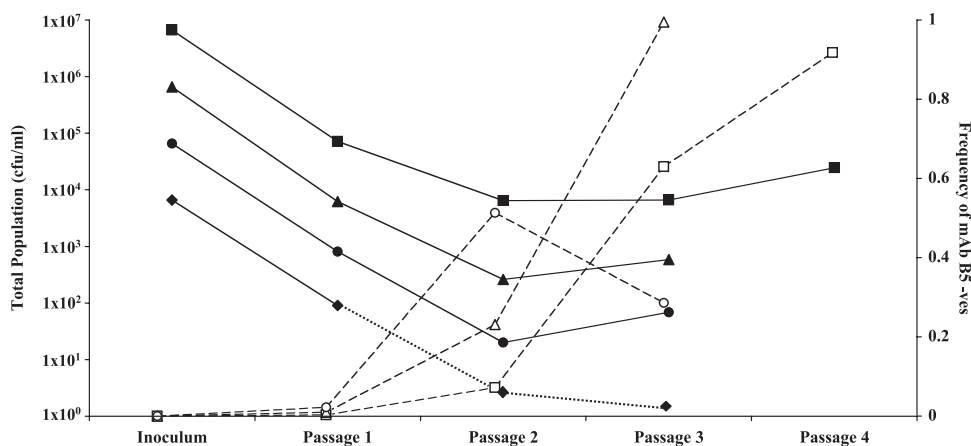


FIG. 4. Influence of inoculum size on escape from MAb B5-mediated serum bactericidal activity. Assays were performed and results were analyzed as described in the legend to Fig. 2, using 1.2 μg of purified MAb B5 and different inoculum sizes for strain 8047 for the first passage. The data are the total number of viable cells present in the inoculum or after 40 min of incubation for each passage and the frequencies of MAb B5 nonreactive phase variants (mAb B5-ves). The dotted line indicates the upper limit of detection for samples in which no cells were detected. Solid lines and filled symbols, number of CFU/ml; dashed lines and open symbols, frequency of MAb B5 nonreactive phase variants. Squares, inoculum containing 6.6×10^6 CFU; triangles, inoculum containing 6.6×10^5 CFU; circles, inoculum containing 6.6×10^4 CFU; diamonds, inoculum containing 6.6×10^3 CFU.

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 $\sim 1,000$ -fold increase in the frequency of MAb B5 nonreactive variants to 2.4×10^{-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 *igtG* 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 (2.7×10^6 CFU), intermediate (2.7×10^4 CFU), and small (2.7×10^3 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 *igtG* 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 *igtG*.

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

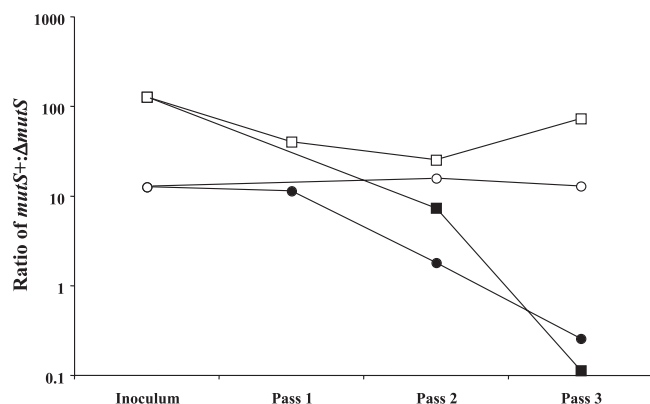


FIG. 5. Competition between strains 8047 and 8047 $\Delta mutS$ for escape from MAb B5-mediated serum bactericidal activity in an assay with continuous selection. Assays were performed and results were analyzed as described in the legend to Fig. 2, using either 3 μg or no purified MAb B5 and an inoculum containing 9×10^6 CFU of strain 8047. The inocula for the first passage contained different mixtures of strains 8047 ($mutS^+$) and 8047 $\Delta mutS$ ($\Delta mutS$). The ratios for subsequent time points were determined using serial dilutions grown overnight on BHI agar plates with and without kanamycin. Open squares, $mutS^+/\Delta mutS$ ratio of 127:1 without MAb B5; open circles, $mutS^+/\Delta mutS$ ratio of 13:1 without MAb B5; filled squares, $mutS^+/\Delta mutS$ ratio of 127:1 with MAb B5; open circles, $mutS^+/\Delta mutS$ ratio of 13:1 with MAb B5.

0.25, respectively. The ratios remained unchanged in the third passage, while the PV frequencies of both populations increased to 0.96. Competition in the interrupted assay was repeated using 9- and 1,027-fold excesses of the $mutS^+$ strain. Ratios of 0.07:1 and 2.2:1, respectively, and a switch to a fully MAb B5 nonreactive phenotype were observed after two passages in the presence of antibody (data not shown). Twelve MAb B5 nonreactive escape variants (two $mutS^+$ variants and ten $\Delta mutS$ variants) from the latter experiment and the previous competition experiments were analyzed, and all of them contained 11 C residues in the *lgtG* repeat tract. These experiments indicated that the $mutS$ mutant had a $\sim 1,000$ -fold competitive advantage over the parental strain. This competitive advantage of the $mutS$ mutant was probably due to the presence of a 1,000-fold excess of $mutS$ mutant-associated MAb B5 nonreactive variants in the inoculum. As these variants had alterations in the *lgtG* repeat tract, this finding suggests that 8047 $\Delta mutS$ outcompeted strain 8047 due to the effect of the $mutS$ mutation on repeat-mediated PV of *lgtG*.

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 $\Delta mutS$ mutant could outcompete strain 8047 during in vivo selection, the experiments were repeated using mixed infections.

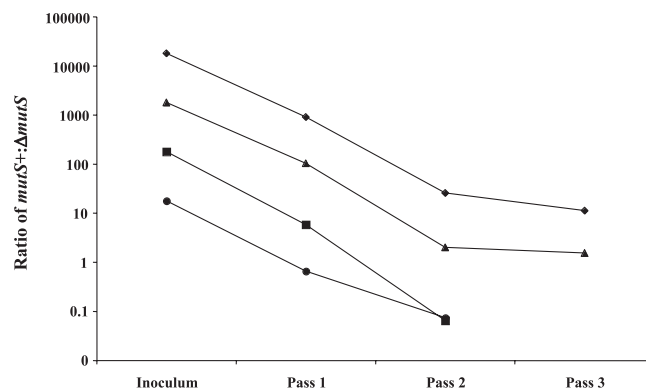


FIG. 6. Competition between strains 8047 and 8047 $\Delta mutS$ for escape from MAb B5-mediated serum bactericidal activity in an assay with interrupted selection. Assays were performed and results were analyzed as described in the legend to Fig. 1, using 0.6 μg of purified MAb B5 and an inoculum containing 5×10^6 CFU of strain 8047. The inocula for the first passage contained different mixtures of strains 8047 ($mutS^+$) and 8047 $\Delta mutS$ ($\Delta mutS$). 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, $mutS^+/\Delta mutS$ ratio of 18,000:1; triangles, $mutS^+/\Delta mutS$ ratio of 1,800:1; squares, $mutS^+/\Delta mutS$ ratio of 180:1; circles, $mutS^+/\Delta mutS$ ratio of 18:1.

Four groups of five animals were inoculated with mixed populations of strains 8047 and 8047 $\Delta mutS$ (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 , 2.4×10^4 , 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 < 5 , 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.

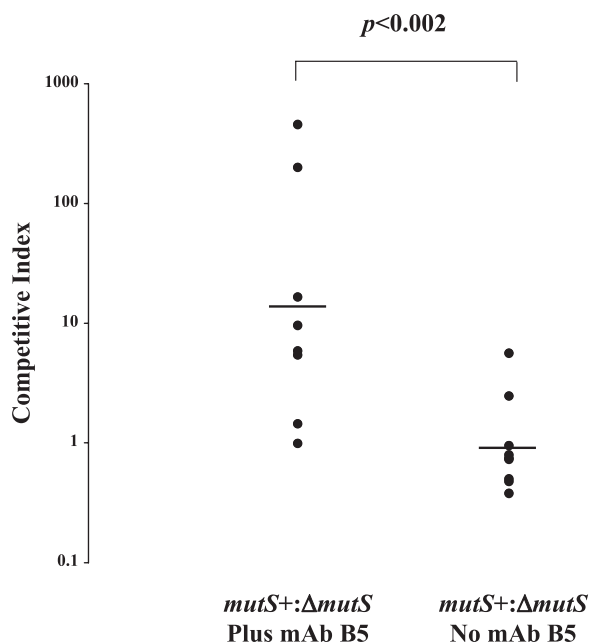


FIG. 7. Competition between strains 8047 and 8047 $\Delta mutS$ for escape from MAb B5-mediated passive protection in an infant rat model of infection. Infant rats were inoculated by the intraperitoneal route in the presence or absence of 2 μg of purified MAb B5 with mixtures of strains 8047 ($mutS^+$) and 8047 $\Delta mutS$ ($\Delta mutS$). Bacteremia was measured at 18 h postinfection, and the “output” ratio of the two strains was assessed by plating serial dilutions of blood on BHI agar plates with and without kanamycin. A CI was derived by dividing the output ratio by the input ratio. CIs for animals inoculated with strain 8047, strain 8047 $\Delta mutS$, and MAb B5 ($mutS^+:\Delta mutS$ 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 $\Delta mutS$ but no MAb B5 ($mutS^+:\Delta mutS$ 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.

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 $\Delta mutS$ 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 4 contained MAb B5 nonreactive variants at frequencies of 0.04, 0.05, 0.05, and 0.007, respectively, and selective plating with or without kanamycin indicated that all these variants

were derived from the $mutS$ mutant. The repeat tract lengths of $lgtG$ were examined in nonreactive and reactive (where available) MAb B5 variants obtained from animals treated with antibody. All 21 of the MAb B5 nonreactive variants contained tracts consisting of 11 C residues, while the two MAb B5 reactive variants had tracts consisting of 12 and 10 C residues. Examination of variants derived from the inocula showed that the MAb B5 nonreactive variants ($n = 2$) had tracts consisting of 11 C residues and the MAb B5 reactive variants ($n = 2$) had tracts consisting of 12 C residues. These results demonstrated that PV-mediated escape from passive protection by MAb B5 is frequent and is associated with mutations in the $lgtG$ repeat tract. Furthermore, these findings imply that the competitive advantage of the mutator strain in this assay was due to the destabilizing effect of the $mutS$ mutation on the mononucleotide repeat tract located within $lgtG$.

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

escape from a specific immune response but also indicate the rapidity with which bacterial populations can change in the face of an adaptive immune response.

The role of the adaptive immune response in driving evolution of meningococcal genomes is currently unclear. Protection against meningococcal infection is correlated with the titer of serum bactericidal antibodies (7), but this correlation may be relevant only to bacteremic invasive infections. The introduction of the MenC vaccine in the United Kingdom was associated with a 66% reduction in carriage of serogroup C isolates, suggesting that adaptive immune responses have a major role 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 $\mu\text{g/ml}$ 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

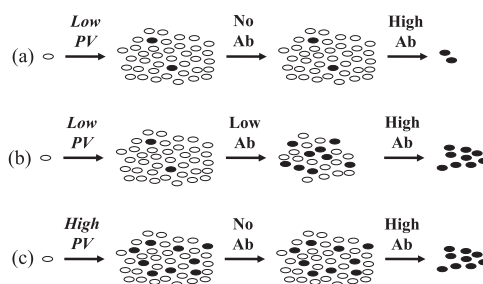


FIG. 8. Survival in the presence of an immune response to a phase-variable epitope is facilitated by preexposure to low levels of antibody or an increased PV rate. The model shows a bacterial population containing phase variants for a specific surface epitope that are subjected to selection with a bactericidal antibody specific for the phase-variable epitope. The initial population is generated from a single cell with a reactive phenotype (i.e., “on” variants) (open ellipses). The numbers of nonreactive phase variants (i.e., “off” variants) (filled ellipses) in the first population are determined by the PV rate. The PV rate may be determined by the length of the repeat tract in the phase-variable gene or the activity of *trans*-acting factors. In the case of mononucleotide repeat tracts (such as those present in the meningococcal *lgtG* gene) this includes components of the mismatch repair system. The number of phase variants in the initial population is low for a nonmutator strain (a and b) and high for a mutator strain (c). If the initial population is subjected to selection with low levels of specific antibody, there is an increase in the proportion of phase variants (b), while in the absence of antibody there is no change in the proportion of variants (a). If each of the populations is then subjected to selection with a high level of antibody, the surviving population is larger for a preexposed population (b) or for a strain with a higher PV rate (c). Ab, antibody.

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 ($\sim 1 \times 10^5$ CFU for wild-type strains and $\sim 1 \times 10^3$ 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 *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 *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 *lgtG* alone and not from switching of *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 *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 mutator 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* *mutS* mutant to outcompete the nonmutator strain was related to the number of preexisting variants with alterations in the repeat tract of *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 *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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