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Selection of Opa$^+$ *Neisseria gonorrhoeae* by Limited Availability of Normal Human Serum

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Experimental infections of human male volunteers with *Neisseria gonorrhoeae* have provided valuable insights into the early stages of gonorrheal disease. Bacterial variants expressing outer membrane opacity (Opa) proteins appear to be selected from the inoculum during a period in which total recoverable numbers of bacteria decrease rapidly. This apparent survival advantage occurs simultaneously with the onset of an inflammatory response, characterized by local production of interleukin 6 (IL-6) and IL-8 and the appearance of leukocytes in urine. Since the inflammatory response may also result in the presence of serum factors on the mucosal surface, we investigated the possibility that killing in normal human serum (NHS) leads to the selection of Opa$^+$ variants. We therefore studied killing of separate populations and mixtures of Opa$^-$ and Opa$^+$ *N. gonorrhoeae* MS11mk in NHS. Expression of an Opa protein conferred a survival advantage upon the organism; i.e., the Opa$^+$ variants were more serum resistant than their isogenic Opa$^-$ counterparts, resulting in a selection for Opa$^+$ phenotypes when a mixture of Opa$^+$ and Opa$^-$ gonococci (GC) was exposed to submaximal doses of NHS. This selection was observed in three different lipooligosaccharide (LOS) backgrounds, indicating that it was not due to a difference in LOS expression between Opa$^-$ and Opa$^+$ phenotypes. Incubation in NHS of sialylated GC resulted in a similar selection for Opa$^+$ variants. The presence of normal human urine during the serum killing assay had no effect on the selection phenomenon but drastically depleted NHS of bactericidal activity, which was found to be at least partly due to complement inhibition. The results suggest that serum killing may contribute to the transition from Opa$^-$ to Opa$^+$ phenotypes during the early stages of infection of the male urethra.

The specificity of *Neisseria gonorrhoeae* for its human host and the lack of a suitable animal model have led to the use of a human male intraurethral challenge model to study gonococcal pathogenesis. Several human volunteer studies have shown that after instillation, most of the input gonococci (GC) disappear rapidly; i.e., they are not recoverable from urine samples (8, 23, 26). After variable time periods, the number of GC that can be isolated from urine increases. However, the variation in phenotypes of the reisolated GC was found to be drastically higher than the variation found when the inoculum was cultured in vitro. Increased phenotypic variation in the reisolated organisms was found for the outer membrane opacity (Opa) proteins. When Opa$^-$ organisms were used to inoculate volunteers, predominantly Opa$^+$ GC were isolated over the course of the infection (8, 26). Thus, if initially most input organisms do not survive in the urethra, the presence of an Opa protein seems to be advantageous for survival in the early stage of infection.

The rapid decrease in recoverable organisms as observed in the human male volunteer studies is accompanied by an inflammatory response, as indicated by the local production of interleukin 6 (IL-6) and IL-8 (20). In accordance with IL-8 induction, increased numbers of neutrophils were found in urine soon after infection (20). The recruitment of neutrophils may lead to the extravasation of serum factors that play a role in the killing of the colonizing organisms at the urogenital mucosal surface. Whether indeed bactericidal antibodies and an intact complement system are present in the male urinary tract during the early stage of GC infection is presently not clear. However, the presence of complement on cervical mucosa has been established (19), and naturally occurring antibodies were shown to exist in the male reproductive tract (2), indicating that the components required for serum killing can be present on urogenital mucosae. Furthermore, transient plasma exudation through the epithelial layer has been shown to take place very rapidly after provocation on another mucosal surface, the respiratory tract, and is considered to play an important role in the first-line mucosal defense (17). During an inflammatory response, which has been shown to occur rapidly after GC infection, serum bactericidal activity may very well play a role in the early host defense. Thus, serum killing may be one of the phenomena responsible for the disappearance of the inoculum.

Serum sensitivity of GC is determined by a number of different phenomena (reviewed by Rice [21]). In normal human serum (NHS), bactericidal immunoglobulin M (IgM) antibodies that recognize epitopes on certain neisserial lipooligosaccharide (LOS) molecules are present (5). However, organisms taken directly from urethral exudates are generally resistant to NHS (29), but they often become serum sensitive upon in vitro subculture. This unstable type of serum resistance is due to sialylation of LOS. Some LOS structures can be sialylated in the presence of cytidine 5′-monophospho-*N*-acetylneuraminic acid (CMP-NANA) (13), which is present in urogenital secretions, and this results in an increased serum resistance of the organism (24). This enhanced serum resistance has been attributed to an inhibition of complement by sialic acid residues (30) and/or by a decrease in antibody binding (3, 16). Stable serum resistance is partially determined by the type of outer membrane porin expressed, which is stable in a given strain. In general, organisms expressing PorA have been reported to be

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more serum resistant than those expressing PorB (21). Another determinant of stable serum resistance is the outer membrane protein Rmp, against which IgG antibodies in NHS are present. These so-called blocking antibodies interfere with the proper insertion of complement membrane attack complexes (MACs) and compete with bactericidal antibodies for binding to GC (11). Since naturally occurring GC strains express a structurally conserved Rmp, the contribution of blocking antibodies to serum resistance will be stable for a given strain (21).

We set out to address the apparent survival advantage of Opa^+ GC in vivo by studying in vitro the effect of NHS on survival of different GC phenotypes. In addition, we also investigated the effect of environmental factors, such as the presence of urine and the effect of sialylation of GC, on selective survival in NHS.

MATERIALS AND METHODS

Bacterial strains. *N. gonorrhoeae* MS11mk (26) was propagated on GC clear typing agar (GCTM) (25) or on GC HEPES-agarose medium (GCHA) to alleviate clumping of Opa^+ GC (27). Phenotypes of GC strains expressing Opa proteins were monitored with a binocular microscope. Opa expression was confirmed in all cases by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with monoclonal antibody 4B12 (a generous gift of M. Blake), which recognizes all Opa proteins from *N. gonorrhoeae* MS11mk (26). Opa protein nomenclature is that used by Swanson et al. (26). LOS phenotypes were determined by Tricine-SDS-PAGE (TSDS-PAGE) in a Bio-Rad Mini-Protean cell system followed by silver staining, as described by Lesse et al. (12). Samples were obtained by spinning down approximately 3×10^8 bacteria for 3 min at 10,000 $\times$ *g*. Bacterial pellets were resuspended in 80 μl of sample buffer (63 mM Tris-HCl [pH 6.8], 10% glycerol, 2% SDS, 0.7 M 2-mercaptoethanol, and 0.1% bromophenol blue) and boiled for 10 min. They were then treated with proteinase K (0.5 mg/ml, 60 min, 55°C) in sample buffer, and finally 1 mM EDTA (final concentration) was added and samples were boiled for 10 min. Three to 5 μl of sample was run per lane. When Opa^- and Opa^+ phenotypes were compared, the Opa^- culture was derived directly from the Opa^+ culture and plated separately overnight, to ensure, as far as possible, that the variants differed only in Opa expression. In all cases, nonpiliated strains were used.

NHS and NHU. NHS was collected intravenously from five volunteers with no history of GC infection. Aliquots of the serum were heated at 56°C for 30 min as a source of control, complement-inactivated, serum. Sera were pooled and stored in aliquots at −70°C. Normal human urine (NHU) was collected as midmorning samples from healthy male volunteers, centrifuged for 10 min at 1,000 $\times$ *g*, filtered through a 0.22-μm-pore-size filter, and kept at 4°C.

Serum killing assay. A slight modification of a previously described assay (1) was used. GC were grown on GCHA plates for 14 to 16 h. Bacteria were suspended in Hanks' balanced salt solution (137 mM NaCl, 5.4 mM KCl, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 5.6 mM glucose, 1.26 mM $CaCl_2$, 0.4 mM $MgSO_4$, 0.49 mM $MgCl_2$, pH 7.3) to an optical density of 0.5 at 540 nm and diluted 1,000× in RPMI (Gibco Life Sciences). Diluted bacterial suspensions (25 μl; representing 1.5×10^4 organisms) were mixed with an equal volume of serum (diluted in RPMI) and incubated for 60 min at 37°C in 5% CO_2 in a 96-well plate. Control assays were done identically with heat-inactivated serum. After performance of a 1:20 dilution of the mixtures in Hanks' balanced salt solution, the number of viable bacteria was calculated by plating and culturing for 18 h on GCTM. Serum resistance levels were determined by comparing the number of surviving bacteria treated with NHS with that of the survivors treated with heat-inactivated NHS (defined as 100%). We did not observe significant levels of bacterial growth in the heat-inactivated serum assay. When killing of Opa^--Opa^+ mixtures was studied, dilutions were made so that at least 25 surviving colonies were available for microscopic screening of opaque versus transparent colonies. Killing of serum-resistant organisms, expressing a 3.6-kDa LOS (LOSa) (22), was established by supplementing NHS with a mouse monoclonal antibody against this LOS. This antibody was raised in our laboratory against whole gonococci. The antibody recognizes the same 3.6-kDa band in a Western blot assay (1) as antibody 2-1-L8, characterized by Schneider et al. (23).

Sialylation studies. To obtain sialylated organisms, GCHA plates were spread with 0.2 ml of CMP-NANA (Sigma) (0.4 mM), before GC were plated for overnight culture. The degree of sialylation of different organisms was estimated by growing bacteria in 1 ml of liquid GCTM for 3 h in the presence of [^{14}C]CMP-NANA (0.4 mM, 315 mCi/mmol [NEN]). Suspensions were standardized to a common optical density at 540 nm (approximately 0.3), 0.5 ml was spun down for 3 min at 10,000 $\times$ *g*, and incorporated radioactivity in the bacterial pellet was determined by liquid scintillation counting. Sialylation was verified by TSDS-PAGE and silver staining of LOS bands.

Complement activity assay. Approximately 1.5×10^7 antibody-coated sheep erythrocytes (Sigma) in 0.1 ml of gelatin-Veronal buffer (141 mM NaCl, 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, 0.1% gelatin, 1.8 mM sodium barbital, and 3.1 mM barbituric acid, pH 7.4) were incubated with 0.1 ml of test substances at 37°C with vigorous shaking. After 20 min, samples were put on ice, 3 ml of ice-cold Veronal-buffered EDTA (10 mM) was added, samples were spun down (10 min at 500 $\times$ *g*), and the absorbance at 415 nm of the supernatant was determined. To control for spontaneous and maximal lysis, cells were incubated with 0.1 ml of gelatin-Veronal buffer or 3.1 ml of Na_2CO_3, respectively.

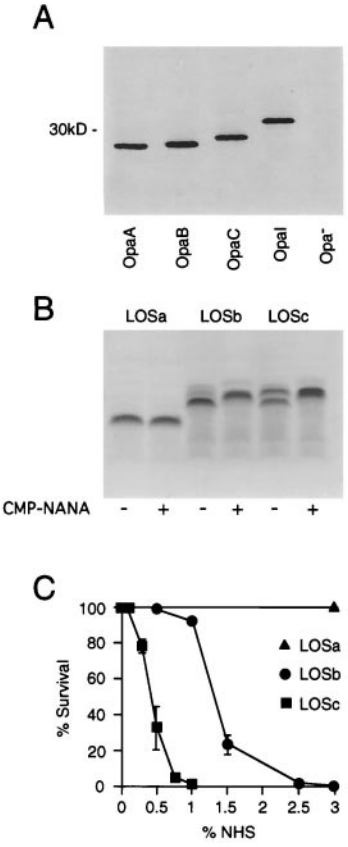

FIG. 1. Characterization of Opa and LOS phenotypes of the MS11mk variants used in this study. (A) Representative Western blot of Opa proteins, probed with 4B12 antibody. (B) Silver-stained TSDS-PAGE pattern of LOSa, -b, and -c phenotypes (all Opa^-); the LOS variants were grown in the absence (− CMP-NANA) or presence (+ CMP-NANA) of CMP-NANA. (C) Dose-response curves for serum sensitivity of LOS phenotypes (all are Opa^- variants). The LOSa phenotype was not killed by doses as high as 70% NHS. Data are means ± standard deviations of triplicate determinations in one experiment and are representative of three independent experiments.

RESULTS

Effect of LOS phenotypes of MS11mk on serum sensitivity. In order to study the effect of Opa protein expression on serum killing, we selected Opa variants of MS11mk in different LOS backgrounds. The Opa protein and LOS profiles of the variants are shown in Fig. 1. Since LOS has a major impact on serum sensitivity, we first determined the effect of NHS on the LOS variants in the absence of Opa.

We examined three different LOS phenotypes, designated LOSa, -b, and -c on the basis of their migration patterns. LOSa migrated as a single 3.6-kDa molecule and did not change its migration after growth of GC in CMP-NANA (Fig. 1B). GC expressing only this LOS type were virtually serum resistant (Fig. 1C; no killing occurred in up to 70% NHS). However, they are killed when a monoclonal anti-LOSa antibody is added to NHS. LOSb is characterized by one major band, migrating at about 4.5 kDa, and two minor bands of higher M_rs

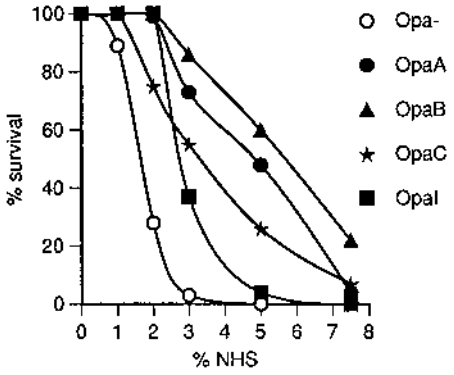

FIG. 2. Survival in NHS of separate suspensions of Opa variants of MS11 LOSb GC. Incubations were in RPMI at 37°C for 1 h. For clarity, standard deviations are not shown, but they did not exceed 10%. Data are means of duplicate determinations in one experiment and are representative of at least three independent experiments.

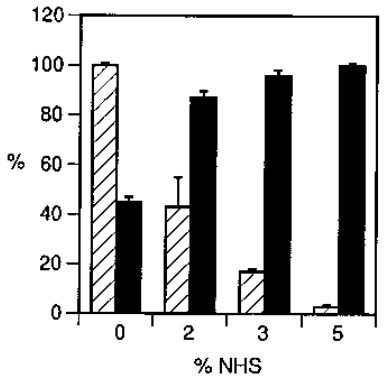

FIG. 3. Selection for Opa+ phenotypes during serum killing of an Opa−-OpaI mixture of MS11 LOSb GC. Mixtures containing 45% OpaI and 55% Opa− organisms were incubated in RPMI plus NHS or heat-inactivated NHS at 37°C for 1 h. After serial dilution, plating, and overnight culture, Opa-positive colonies were scored by microscopic monitoring of colony opacity. Percentages of Opa+ colonies in control incubations with heat-inactivated serum were equal to those found in nontreated mixtures, which were plated immediately after preparation. Hatched bars represent percent survival of the total mixture; filled bars represent percent Opa+ colonies among the survivors. The *y* axis label stands for both percent survival and percent Opa+ colonies. Data are means ± standard deviations of three determinations in one experiment and are representative of at least three independent experiments.

(Fig. 1B). GC expressing this LOS were moderately serum resistant (Fig. 1C). The 4.5-kDa band was sialylated upon growth in CMP-NANA and subsequently migrated more slowly in TSDS-PAGE (Fig. 1B). This sialylation led to a complete serum resistance of the LOSb organism (data not shown). LOSc contains two prominent bands at 4.5 and 4.8 kDa and a faint band of higher M_r. After growth in CMP-NANA, the lower band shifts upwards (Fig. 1B). GC expressing LOSc were highly serum sensitive (Fig. 1C; 50% survival at 0.4% NHS), and sialylation rendered them more, but not completely, serum resistant (50% survival at 1.8% NHS).

Opa protein expression as a determinant of serum sensitivity. In order to determine whether Opa protein expression influenced serum sensitivity, we incubated isogenic variants expressing none or a single defined Opa protein with various concentrations (0 to 7.5%) of NHS. After 60 min, the incubation was terminated and the number of viable bacteria was determined by serial dilution and enumeration of CFU. Bacteria incubated with heat-inactivated NHS served as controls. All variants expressed the same LOSb phenotype as determined by TSDS-PAGE and silver staining. These experiments clearly demonstrated that GC expressing an Opa protein required more NHS for killing than the Opa-negative variant (Fig. 2). This effect was found for all Opa variants that were examined. Half-maximal killing of Opa− GC was obtained with 1.7% NHS while Opa+ variants needed 1.7 (OpaI) to 3.4 (OpaB) times as much NHS to get killing of 50% of the population.

Selection for Opa+ phenotypes during serum killing. The difference in serum sensitivity of Opa variants led us to examine whether NHS could serve as an agent for selection of Opa+ phenotypes. Approximately equal numbers of Opa− and Opa+ LOSb organisms were mixed and monitored for survival in 0 to 5% NHS. Surviving bacteria were scored for Opa expression by colony opacity. These experiments showed a distinct selection for Opa+ gonococci at submaximal concentrations of NHS. At 3% NHS, more than 95% of surviving colonies were Opa+ (Fig. 3), while in a parallel experiment using heat-inactivated NHS, the same distribution of Opa− and Opa+ was found as in the original mixture. The selection for Opa+ bacteria was found for all Opa variants tested (OpaA, -B, -C, and -I; only data for OpaI are shown). When only Opa− bacteria were studied in a dose-response serum killing assay, we never found more opaque colonies among the survivors than a number that could be expected from the normal rate of phase variation for Opa expression (about 15 CFU of 15,000 bacteria), indicating that the increased percentage of Opa+ colonies among the survivors of a mixture was not due to an increased rate of phase variation.

Selection for Opa+ phenotypes in NHS occurs with different LOS phenotypes. To examine the impact of LOS variation on the increased serum resistance of Opa+ GC, we determined serum sensitivity of Opa variants in different LOS backgrounds. Serum killing of Opa− and Opa+ mixtures of the various LOS variants resulted in selection for Opa+ organisms in both the highly sensitive LOSc background (Fig. 4B) and the serum-resistant LOSa background, in which killing was established by addition of a monoclonal anti-LOSa antibody (Fig. 4A). Thus, the protective effect of Opa proteins was not re-

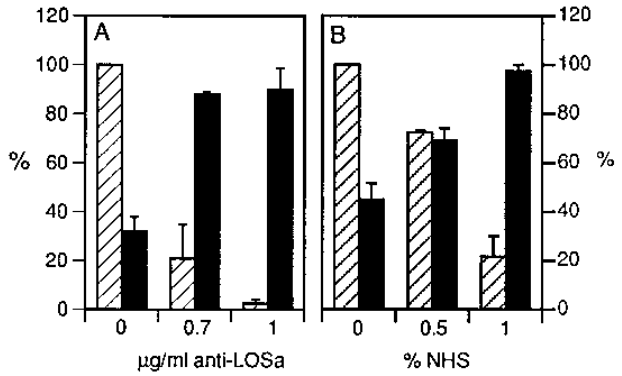

FIG. 4. Selection for Opa+ phenotypes during serum killing of Opa−-Opa+ mixtures in different LOS backgrounds. Hatched bars represent percent survival of the total mixture; filled bars represent percent Opa+ colonies among the survivors. (A) Survival of a mixture of Opa− and OpaI MS11 LOSa GC. Killing was established by adding increasing amounts of a mouse anti-LOSa monoclonal antibody to 10% NHS. (B) Survival of a mixture of Opa− and OpaA MS11 LOSc GC in different concentrations of NHS. Mixtures were incubated for 1 h in RPMI containing NHS or heat-inactivated NHS at 37°C. Data are means ± standard deviations of duplicate determinations in one experiment and are representative of three independent experiments. See the legend to Fig. 3 for the definitions of the *y* axes.

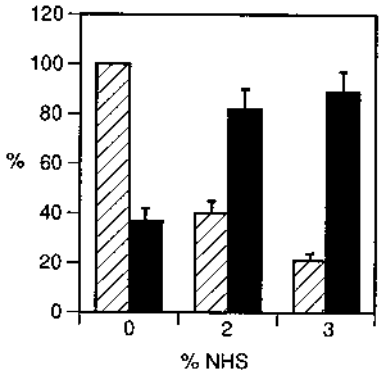

FIG. 5. Effect of LOS sialylation on Opa$^+$ selection during serum killing of an Opa$^-$-OpaA mixture of LOSc MS11. Hatched bars represent percent survival of the total mixture; filled bars represent percent Opa$^+$ colonies among the survivors. Organisms were grown separately overnight in the presence of CMP-NANA. Mixtures were incubated in RPMI with NHS or heat-inactivated NHS for 1 h at 37°C. After serial dilution, plating, and overnight culture, Opa-positive colonies were scored by microscopic monitoring of colony opacity. Data are means ± standard deviations of two determinations in one experiment and are representative of at least three independent experiments. For the definition of the *y* axis, see the legend to Fig. 3.

stricted to a certain LOS background or to the effect of antibodies naturally present in human serum.

Role of LOS sialylation in selection of gonococcal variants. During the natural infection, GC can sialylate their LOS by using host-derived CMP-NANA as a sialyl donor. This sialylation has been related to increased serum resistance (reviewed by Smith et al. [24]). In order to test the effect of sialylation status on Opa$^+$ selection in NHS, we studied the two LOS types that can be sialylated, LOSb and LOSc. As mentioned above, induction of serum resistance by CMP-NANA was much more pronounced in the LOSb variant (which became virtually serum resistant) than in the LOSc GC, which became only partially resistant to NHS. This reduced effect of CMP-NANA in LOSc organisms corresponded with lower incorporation of [^{14}C]CMP-NANA into LOSc (40% less incorporation than in LOSb).

When mixtures of sialylated LOSc bacteria were studied in serum killing assays, we found that more serum was required to get significant killing, but that selection for Opa$^+$ variants was again evident (Fig. 5).

Effect of urine on selection for Opa$^+$ in NHS. In addition to serum factors, the special microenvironment in the male urethra may have profound effects on the survival of GC. We therefore examined killing of Opa variants by urine. We did not find any difference in killing of Opa$^+$ versus Opa$^-$ phenotypes by NHU (data not shown). We then determined the effect of urine on the serum-induced Opa$^+$ selection. Urine by itself, in the concentration (15%) and time period (1 h) tested, did not kill GC and did not induce any selection (data not shown). When 15% NHU was added in a serum killing assay of a mixture of Opa$^-$-OpaI LOSb organisms, we observed a similar selection for Opa$^+$ as seen without urine. However, considerably more serum was needed in these circumstances to get substantial killing of GC (Fig. 6A). This increased serum resistance was observed for both Opa$^-$ and Opa$^+$ phenotypes, suggesting that it could be due to inhibition of complement activity by NHU. In order to test this, we examined different NHU batches in an erythrocyte lysis test, which measures classical pathway complement activity. We found a linear increase in erythrocyte lysis with serum concentrations between 0.1 and 0.6% (data not shown). When we added increasing amounts of NHU to 0.4% NHS in this test, we found a dose-dependent inhibition of complement activity by NHU (Fig. 6B). These data imply that NHU may contribute to the observed selection of gonococcal phenotypes by limiting the availability of complement.

DISCUSSION

Serum sensitivity of *N. gonorrhoeae* is determined by phenotypic traits such as the expression of outer membrane components LOS, Por, and Rmp (21) or components that mediate properdin binding (6). We have studied the effect of Opa protein expression on serum sensitivity and found that Opa expression enhances serum resistance in three different LOS backgrounds and after sialylation of LOS. Previous reports on the relationship between Opa expression and serum sensitivity gave conflicting results (4, 7, 28). These studies, however, did not focus on submaximal NHS concentrations or on the effect of LOS variation on serum killing. Our data indicate that LOS expression is of paramount importance in determining serum sensitivities of gonococcal variants, since minor differences (e.g., LOSb versus LOSc expression) have major effects on serum sensitivity of GC. It is also evident from our data that the Opa-related serum resistance is observed only in the pres-

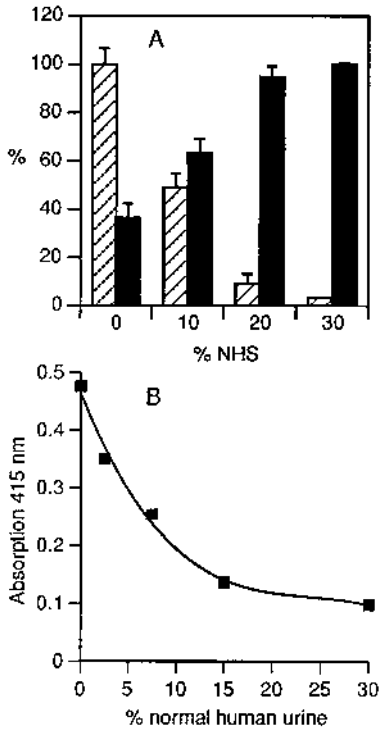

FIG. 6. Effect of NHU on Opa$^+$ selection during serum killing. (A) Mixtures of Opa$^-$ and OpaI LOSb bacteria were incubated in RPMI containing 15% NHU and NHS or heat-inactivated NHS for 1 h at 37°C. After serial dilution, plating, and overnight culture, Opa-positive colonies were scored by microscopic monitoring of colony opacity. Hatched bars represent percent survival of the total mixture; filled bars represent percent Opa$^+$ colonies among the survivors. Data are means ± standard deviations of two determinations in one experiment and are representative of at least three independent experiments. For the definition of the *y* axis, see the legend to Fig. 3. (B) Effect of urine on classical pathway complement activity in NHS. Antibody-coated sheep erythrocytes were incubated with 0.4% NHS and increasing concentrations of NHU. After removal of unlysed cells by centrifugation, absorbance at 415 nm was measured in the supernatant, as an estimate of complement-dependent erythrocyte lysis. Data are means ± standard deviations of two determinations and are representative of three independent experiments.

ence of limiting amounts of bactericidal activity, since bacteria with Opa$^+$ phenotypes were also killed when sufficient serum was present. This may have relevance to the in vivo situation, in which, depending on the stage of the infection, GC may encounter very differing levels of serum bactericidal activity. Hence, in the early stage of infection, when serum killing activity may not be maximal, Opa$^+$ GC may have a survival advantage over Opa$^-$ organisms.

This study reemphasizes the major contribution of LOS to serum resistance. The major structural component of the LOSb phenotype represents the lacto-*N*-neotetraose moiety, which can be expressed by all pathogenic *Neisseria* spp. (5). This LOS molecule may or may not be extended by either a galactosamine (GalNac), creating an epitope for bactericidal IgM, or a sialic acid residue, leading to enhanced serum resistance (5). The higher-M_r component, migrating just above the 4.5-kDa lacto-*N*-neotetraose band, most likely represents the GalNac-extended lacto-*N*-neotetraose. The highly serum-sensitive LOSc phenotype clearly possesses more of this molecule than the LOSb phenotype (Fig. 1B). This corroborates the data of Griffiss et al. (6) which point to the importance of the terminal GalNac in recognition by bactericidal IgM. Frangipane and Rest (4) found a similar relationship between this particular LOS SDS-PAGE pattern and serum sensitivity. They showed that the LOSc phenotype occurs in strain F62 predominantly under aerobic conditions and that the LOSb phenotype predominates in an anaerobic environment. However, we had no problem maintaining both phenotypes of MS11 in an aerobic milieu.

Several mechanisms may account for the Opa-associated enhanced serum resistance. Different microbial strategies for evasion of complement-mediated killing have been described by Joiner (9). Opa protein expression may somehow mask the target for bactericidal antibody. However, assuming that the major target is LOS, our results indicate that this masking occurs very similarly with different LOS molecules and different antibodies (serum derived versus mouse hybridoma derived), making this possibility less likely to be responsible for our results. The Opa protein could also interfere with the activation and disposition of complement. Gonococcal serum resistance has been linked to a difference in insertion of MACs in serum-resistant versus serum-sensitive variants, in such a manner that insufficient MAC insertion in the serum-resistant GC does not lead to killing of the organism (10). The Opa protein may interfere with the complement cascade at many possible sites, such as at the binding of complement control proteins or at the proper insertion of the MAC.

Outer membrane blebbing might also play a role in the serum resistance associated with Opa expression. Opa$^+$ organisms in general bleb more readily than Opa$^-$ GC when exposed to certain compounds such as polyanions (25a). When the target for bactericidal antibodies in NHS is shed from the bacterial outer membrane (i.e., bleb formation), binding of bactericidal serum factors to the blebs depletes bactericidal activity for the whole organism. However, the data show that in a mixture of Opa$^+$ and Opa$^-$, when both phenotypes experience the same extracellular environment, the Opa$^+$ GC maintain a survival advantage over Opa$^-$ GC, indicating that the phenomenon is not related to an overall depletion of bactericidal serum factors. Blebbing could, however, still account for the Opa$^+$ selection if the blebs contain more of the bactericidal target than the outer membrane of the intact organism. The organism producing blebs is left with less target for serum bactericidal activity than is the nonblebbing organism. There is evidence that naturally elaborated blebs from *N. gonorrhoeae* can have a different composition than the cell surface they came from (18). Alternatively, the process of blebbing could alter the alignment of inner and outer membranes such that insertion of MACs is ineffective.

The selection for Opa$^+$ phenotypes occurs only at submaximal concentrations of serum. In this respect, our data indicating that urine inhibits complement activity may be highly relevant. Reducing availability of complement at the mucosal surface may result in levels of bactericidal activity that favor survival of Opa$^+$ bacteria. Urine by itself has been shown to be bactericidal for GC only when it is sufficiently acidic and concentrated (14, 15). Our data showed no differences in sensitivity to killing by urine for Opa phenotypes and are in agreement with previous studies (8, 14).

In summary, we have shown that the presence of an Opa protein confers an advantage upon the gonococcus for survival under conditions of limited serum bactericidal activity. This effect is independent of the gonococcal LOS background. This survival advantage is due to an enhanced serum resistance of the Opa$^+$ organisms and leads to selection for Opa$^+$ GC in a mixture of Opa$^-$ and Opa$^+$ phenotypes in the presence of limiting amounts of NHS. Taking into account the inhibitory effect of urine on complement activity that we have reported here, complement is very likely present only in limiting concentrations in the male urethra. Thus, the selection of Opa$^+$ phenotypes by NHS observed in vitro may be a phenomenon that contributes to the Opa$^+$ selection observed in vivo.

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