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Molecular Basis for Serum Resistance in *Neisseria gonorrhoeae*

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The molecular basis for resistance to killing by normal human serum (NHS) exhibited by certain strains of *Neisseria gonorrhoeae* represents a composite of numerous phenomena. These include (i) the absence in human serum of bactericidal antibody directed against specific epitopes, the most important of which reside in lipooligosaccharide (LOS) antigens (11, 52, 59); and (ii) phenotypic shifting of LOS epitope expression (41, 59), such as may occur especially in vivo (10). This may result in the loss of epitopes that serve as appropriate receptors (bactericidal or lytic epitopes) for bactericidal antibodies and complement. Alternatively, new epitopes may be acquired in vivo, which may obscure underlying lytic epitopes, thereby preventing recognition of these by human bactericidal antibodies. In addition, (iii) blocking antibodies, directed at outer membrane protein antigens, modulate or down-regulate bacterial killing, mainly by preventing binding of bactericidal antibodies to lytic epitopes (26, 49). Finally, and least well understood, (iv) there is a partial failure to form the C5 convertase necessary for full development of membrane attack complexes (MACs or C5b-9 [11, 12]) but also a failure of MACs that do form to fully insert through the gonococcal outer membrane (22, 27). In addition, less C3 may bind to serum-resistant organisms, and C3 that does bind may not be hemolytically active (51).

IN VIVO (UNSTABLE) SERUM RESISTANCE

N. gonorrhoeae strains are phenotypically resistant to killing by NHS in vivo. When subcultured, many strains lose this property and become serum sensitive, hence the term unstable serum resistance. Studies performed by Ward et al. in 1970 demonstrated that *N. gonorrhoeae* harvested directly from a urethral exudate resisted killing by serum obtained from an infected patient as well as by immune sera prepared in rabbits (62). It has not been determined whether human sera with antibody directed against a putative bactericidal or lytic epitope(s) that may be expressed in vivo will overcome unstable serum resistance and kill these organisms. Nevertheless, *N. gonorrhoeae* organisms that maintain the resistant phenotype after in vitro subculture (stable serum resistance) can be killed by appropriate human immune sera, for example, serum samples from patients recovering from disseminated gonococcal infection (DGI) (48, 53).

In vivo serum resistance may be simulated in vitro by incubating serum-sensitive organisms in ultrafiltrates derived from guinea pig sera (36, 50) or human fluids, including sera (33, 34) and genital secretions (cervical secretions and seminal plasmas [35]). After incubation in these fluids, serum-sensitive organisms become serum resistant, having undergone changes in their LOSs. This is evidenced by shifts in LOS migration patterns on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (59), alteration of pyocin sensitivity patterns (63), and reduced binding of normal immunoglobulin M (IgM) to LOSs prepared from strains incubated in these fluids (59).

In vivo serum resistance may result from sialidation of gonococci in the genital tract. Support for this mechanism comes from recent evidence indicating that gonococci grown in vitro in the presence of cytidine 5′-monophospho-*N*-acetylneuraminic acid become serum resistant (42, 44). Gonococci are sialidated at an LOS site (C. A. Nairn, N. J. Parsons, P. V. Patel, J. A. Cole, E. L. Tan, J. R. C. Andrade, M. Goldner, and H. Smith, unpublished observations). Separate evidence that supports in vivo sialidation comes from studies demonstrating that a monoclonal antibody directed against a probable neuraminic acid determinant binds to LOS derived from in vivo- but not in vitro-grown gonococci (9).

IN VITRO (STABLE) SERUM RESISTANCE

Once isolated in culture, *N. gonorrhoeae* undergoes additional phenotypic shifting of antigens, in large part but not exclusively in LOS. At this time, strains assume a range in susceptibility to killing by NHS. This pattern of in vitro susceptibility, however, is relatively stable under constant growth conditions and is an important harbinger of the clinical potential of strains. *N. gonorrhoeae* strains are capable of causing a diverse array of syndromes in the hosts they infect. These syndromes range from the total absence of signs or symptoms at the local site of infection, as occurs commonly in DGI, to a marked local inflammatory response, as exemplified by acute salpingitis. The most solidly established attribute that bears on the inflammatory potential of the organism at the local site of infection is serum resistance (7, 11, 12, 43, 45, 48, 53). This property may confer upon the organism the ability to escape local defenses and enable it to penetrate mucosal barriers to reach the bloodstream and then to disseminate. Organisms with the stable serum-resistant phenotype often persist at local sites of infection without promoting clinically significant inflammation. This is evidenced by a disproportionate representation of these strains in asymptomatic men (7) and by the frequent lack of local symptoms that accompany disseminated infection (43, 45). Differences in stable serum resistance may subdivide DGI strains into two populations; those more resistant may cause arthralgias and tenosynovitis, and those less resistant may cause suppurative arthritis (43). In contrast, *N. gonorrhoeae* strains with the stable serum-sensitive phenotype are often associated with symptomatic local infection in both men and women, and sensitive organisms may be especially associated with the severity of the inflammatory response in women with acute salpingitis (48).

Serum-sensitive gonococci more rapidly generate C5a, the complement-derived neutrophil chemoattractant, when incubated in NHS than serum-resistant organisms do (12). They are also more rapidly and completely phagocytosed by polymorphonuclear leukocytes in vitro than serum-resistant strains are (51). Thus, generation of an inflammatory response by gonococci with the stable serum-sensitive phenotype appears to be conducive to the localization of disease and the prevention of bacteremic spread of infection, as evidenced clinically by the rarity with which acute salpingitis leads to DGI (3, 37, 43).

LOS

The structural and chemical makeup of gonococcal LOS molecules undoubtedly influence stable serum resistance. When gonococci are grown in vitro under glucose-limiting conditions, saccharide structures of gonococcal LOSs are modified. Serum-resistant organisms grown under these conditions in continuous culture remain serum resistant while high dilution rates are maintained. At lower dilution rates, LOS serotype antigen expression decreases and new LOS determinants appear, which may serve as bactericidal or lytic epitopes, and the organisms become more serum sensitive (41). In this case, serum sensitivity may result from newly formed determinants that serve as appropriate receptors for bactericidal antibody. Alternatively, bactericidal or lytic epitopes that previously were inaccessible or cryptic may become uncovered or more surface exposed. In either case, LOS structure undergoes measurable change, indicating that the oligosaccharide chemistry of these molecules is influenced by changes in growth conditions. Oligosaccharide preparations from LOSs of serum-sensitive *N. gonorrhoeae* strains, separated into larger *N*-acetylglucosamine-rich and smaller *N*-acetylglucosamine-poor components, have different effects in complement-dependent bactericidal systems. When used as immune inhibitors in IgM-dependent bactericidal assays, only the larger *N*-acetylglucosamine-containing molecules inhibit serum-bactericidal activity (20). This inhibition is due, in part, to the reduction of IgM LOS antibody by immune absorption, but activation of the alternative pathway of complement also occurs (24). Hexosamines containing LOS molecules are important activators of the alternative pathway (21).

Numerous studies have confirmed that specific antibodies directed against LOS antigens of *N. gonorrhoeae* are responsible for complement-dependent bactericidal activity (17, 46, 60, 61). This activity has been ascribed to LOS antibodies of the IgM class in NHS, directed against serum-sensitive strains (1, 2, 17). In addition, anti-protein I (PI) antibodies contribute to bactericidal activity, which is otherwise primarily influenced by LOS antibodies (23).

Serum-resistant strains also contain bactericidal epitopes. These may not be functional, because NHS lacks antibody to these sites (52) or because these epitopes may be cryptic (Rice et al., unpublished). Nevertheless, serum-resistant *N. gonorrhoeae* strains may be made serum sensitive with the appropriate antibody (32, 46, 53). At least two accessible LOS lytic or bactericidal epitopes are present on serum-resistant gonococci. One of these is defined by monoclonal antibody 2-1-L8, which binds to most strains that resist killing by NHS but to few that are sensitive. This determinant resides on a 3.6-kilodalton (kDa) species of LOS. Seemingly paradoxically, monoclonal antibody 2-1-L8 is bactericidal for serum-resistant strains (epitope present) but not for serum-sensitive strains (epitope absent) (52). Likewise, serum from DGI patients that contains antibody with 2-1-L8 specificity also kills serum-resistant gonococci (Rice et al., unpublished). A second LOS lytic epitope that permits killing of serum-resistant gonococci has been defined by immune serum obtained from an individual administered *Escherichia coli* J5 vaccine. This serum was capable of killing *N. gonorrhoeae* that resisted killing by NHS, but it lacked specificity for the 2-1-L8 epitope (8). In serum samples from both the DGI patients and the J5 vaccinee, bactericidal LOS antibody was IgG and not IgM (8).

Serum-resistant strains may harbor other epitopes that would serve as sites of recognition by bactericidal antibodies present in NHS if the epitopes were exposed. These epitopes may be obscured by LOS structures that prevent accessibility of antibody to these cryptic determinants. An examination of a series of pyocin mutants differing by sequential saccharide deletions in LOS (15) has revealed that as the saccharides shorten, exposure of the 2-1-L8 epitope increases. The organisms remain serum resistant as long as the 2-1-L8 epitope is present. As the 2-1-L8 epitope abruptly disappears from one mutant to another with a shorter LOS, the organisms convert to being serum sensitive as underlying serum-sensitive LOS epitopes become exposed. IgM binding to LOS also increases markedly as the 2-1-L8 epitope is lost and the cryptic epitopes emerge as exposed determinants (Rice et al., unpublished). These results have been corroborated by using the pyocin mutant LOS molecules themselves, isolated from whole organisms. Mutant LOSs of various molecular masses have been inserted into liposomes and used as antigenic targets. As the mass of LOS used in the liposomes increases beyond 3.6 kDa (where the 2-1-L8 epitope resides), the liposomes become more susceptible to antibody-mediated MAC insertion, despite the presence of the underlying 2-1-L8 epitope. If the liposome assay is used as an analog to serum killing of gonococci (Rice et al., unpublished), these results suggest that the presence of the 2-1-L8 epitope may not always preclude serum sensitivity. Indeed, certain pyocin mutants with longer oligosaccharide chains but also harboring the 2-1-L8 epitope may sometimes be serum sensitive (P. A. Rice and M. A. Apicella, unpublished observations). A genetic approach, i.e., selection of transformants on the basis of their strong reactivity with monoclonal antibody 2-1-L8, produces serum-resistant clones. However, the level of resistance is defined by the loss of higher-molecular-mass LOS components (56). Other reports have also indicated that changes in the molecular mass of LOS may alter serum resistance (40, 55, 57, 59). The loci on the gonococcal chromosome designated as *sac-1* (5, 6) and *sac-3* (54) are associated with serum resistance, and *sac-3* in particular has been shown to affect the molecular mass of LOS (57). Recently, a recombinant plasmid, called pWM3, was created from a cosmid gene library of *N. gonorrhoeae* and used to confer serum resistance upon transformation of a serum-sensitive strain (39). The deoxyribonucleic acid sequence of this cloned region has now been determined, and hybridization probes have shown the homologous gene sequences to be present in several strains of *N. gonorrhoeae*, including some that are phenotypically serum-sensitive (C. J. Conde-Glez, W. M. McShaw, S. Nowicki, S. I. Hull, and R. A. Hull, unpublished observations). These regions are probably separate from *sac-1* and *sac-3* loci.

PIII and Blocking Antibody

Natural antibodies of the IgG class may subvert adequate insertion of the MACs (or C5b-9) of complement and therefore contribute to serum resistance. These antibodies have been described for human sera and are termed blocking antibodies (26, 38, 47, 49). They interfere with the efficient insertion of the MAC by an as yet unknown mechanism(s); however, binding of these antibodies to particular antigenic targets on the surface of gonococci may divert the necessary localization of complement away from bactericidal sites (26). Recent studies with murine monoclonal antibodies have shown that an antibody specific for a gonococcal surface protein, protein III (PIII), is able to block killing of gonococci by bactericidal antibody directed against a separate

epitope (29). Human antibodies that are specific for PIII also block killing of *N. gonorrhoeae* by bactericidal antibody. Immunodepletion of PIII antibody from certain DGI sera that lack killing ability may restore their killing action for serum-resistant gonococci (49). In some instances the depletion of PIII antibody from NHS enables absorbed serum to kill gonococci that are otherwise serum resistant (P. A. Rice, S. Gulati, E. C. Gotschlich, L. M. Wetzler, and M. S. Blake, unpublished observations). This suggests that NHS may contain antibody that is bactericidal for all strains of *N. gonorrhoeae* but that this antibody may not always function. Through the use of genetically altered *N. gonorrhoeae*, a PIII deletional mutant (PIII$^-$) has been shown to be more serum sensitive than its parent. The relative difference in serum sensitivity between parent and mutant is directly related to the quantity of PIII antibody present in the sera used in the bactericidal assay (P. A. Rice, S. Gulati, E. C. Gotschlich, L. M. Wetzler, and M. S. Blake, unpublished). PIII not only appears to be present in all strains of gonococci (30, 31) but also appears to be biochemically and immunochemically identical among strains (31, 58). It is also a potent immunogen in humans. Contaminant amounts of PIII present in a PI vaccine preparation were capable of eliciting PIII blocking antibodies when administered to humans (F. Arminjon, M. Cadoz, S. A. Morse, J. D. Rock, and S. K. Sarafian, personal communication).

PIII has extensive homology to the enterobacterial OmpA protein (18) and class 4 meningococcal protein (K. P. Klugman, and E. C. Gotschlich, unpublished observations). Since members of the family *Enterobacteriaceae* and *N. meningitidis* are both common human commensal organisms, it is possible that PIII blocking antibodies arise as a result of exposure to cross-reacting proteins from other species. Human IgG directed at OmpA not only binds to gonococcal PIII but also promotes blocking activity (P. A. Rice, S. Gulati, E. C. Gotschlich, L. M. Wetzler, and M. S. Blake, unpublished). Hence, blocking antibodies may arise naturally from exposure to members of the family *Enterobacteriaceae* and perhaps to *N. meningitidis*.

Transformation of serum-sensitive *N. gonorrhoeae* strains with the plasmid pWM3 (see above) or subclones that encode only a 29-kDa protein enable the derivatives to bind blocking antibody. Although similar in size, the cloned 29-kDa protein and PIII are antigenically distinct, and sequences of deoxyribonucleic acid encoding PIII and the 29-kDa protein differ (Conde-Glez et al., unpublished). pWM3 also encodes a 17.5-kDa protein, but its role is presently unknown. The 17.5-kDa protein is not a binding site for blocking antibody (39).

Activation and Disposition of Complement

MACs (or C5b-9) form on the surfaces of both serum-sensitive and serum-resistant organisms (22, 27). Both sensitive and resistant strains consume equivalent amounts of C9 and bind similar numbers of C7 and C9 molecules when incubated in NHS (22, 27). However, two features distinguish the binding of C5b-9 to serum-sensitive versus serum-resistant strains (28). First, C5b-9 is more sensitive to trypsin on serum-resistant strains (>twofold), indicating that MACs are not inserted identically in serum-sensitive and serum-resistant organisms. There may be differences in the extent of insertion of C5b-9 complexes, variability in the capacity of trypsin-cleaved C5b-9 to remain surface bound, or differences in the extent of C9 incorporation into the C5b-9 complex. Second, the C5b-9 complex is bound in a different form on serum-sensitive than serum-resistant organisms. The major bactericidal form of C5b-9 on the surface of serum-sensitive *N. gonorrhoeae* strains sediments as a 33S complex when extracted with Zwittergent detergents, but nonbactericidal C5b-9 complexes on serum-resistant *N. gonorrhoeae* strains exist as larger aggregates of C5b-9 or as C5b-9 complexed to bacterial outer membranes. Presensitization of serum-resistant *N. gonorrhoeae* with immune rabbit serum that is bactericidal converts serum-resistant organisms to serum-sensitive ones, alters the release of C5b-9 by trypsin, and changes the sedimentation properties of C5b-9 complexes to coincide with those seen in serum-sensitive organisms incubated in NHS (27). Bactericidal and nonbactericidal complexes (C5b-9) bind to identical radiolabeled protein components of the outer membrane; however, bactericidal C5b-9 also binds to additional proteins (28). Taken together, these studies have suggested quantitatively but not qualitatively the equal presence of C5b on the surfaces of serum-sensitive and serum-resistant gonococci. Binding to other constituents of the outer membrane, particularly LOSs, has not been reported.

Separate studies have examined the generation of C5a; the formation of C5a is stoichiometrically related to C5b formation. These results suggest a different interpretation of the data from that indicated above. When phenotypically stable serum-resistant strains are incubated in NHS, C5a generation is significantly lower than when serum-sensitive strains are used (11), yet C5b binding is the same for both phenotypes (22, 27). Nevertheless, immune serum from DGI patients, particularly IgG LOS antibody that converts organisms from the serum-resistant phenotype to the serum-sensitive phenotype, permits additional generation of C5a by strains that otherwise minimally generate C5a and resist killing by NHS (11). Perhaps the discrepancy between unequal C5a generation by each of the two phenotypes versus equal C5b binding may be explained by differences in the C5 convertases present in each case. This difference may produce altered efficiency of C5b adherence that results in diminished C5b binding to serum-sensitive organisms, despite a given amount of C5a release. Effective MAC formation may also play a role in C5a generation. Perhaps incubation of serum-sensitive organisms in C8-deficient serum, which cannot kill gonococci, would result in decreased release of C5a compared with that obtained after incubation in NHS. Such is the case in analogous systems in which sensitized sheep erythrocytes are used instead of bacteria. Replacement of C8 in these systems restores the levels of released C5a (19).

The effect of MACs on earlier stages of the complement system is also reflected in the kinetics of factor B binding onto serum-sensitive organisms incubated in NHS versus C8-deficient human serum. Although both C3 and factor B binding to serum-sensitive gonococci start out equally in these sera, prolonged incubation (>5 min) results in a decay in factor B binding to serum-sensitive gonococci in NHS but not in C8-deficient serum, in which no effective C5b-9 is formed. C3 binding increases over time and is equal in both sera (14). Selective loss of factor B from gonococcal surfaces incubated in NHS compared with C8-deficient serum could reflect a role of the MAC in promoting the dissociation of the C3bBb-properidin complex or facilitating the action of factor H or I in the dissociating process that results in loss of factor B but maintenance of C3b. This suggests that events that transpire to create effective complement fixation may occur at the point of or before C3 fixation. The presence and quantity of bactericidal antibody are also important in this

case, because C3b can bind to antibody directly, and the function of C3b is thereby enhanced as a result of relative protection from cleavage by factors H and I (4, 16, 25).

The role of gonococcal activation of specific complement pathways has also been investigated. Gonococcal killing by NHS and human serum deficient in properidin proceeds with identical kinetics in both cases (13). Killing in human serum deficient in C2 occurs following a longer incubation period, but subsequently the rate of killing is similar to that observed in NHS and in properidin-deficient serum. These similar killing kinetics may reflect efficient activation of the classical pathway in both NHS and properidin-deficient serum. However, greater C3 fixation occurs in NHS and is due to recruitment of the alternative pathway (13). Gonococci correspondingly increase their ability to bind properidin as they become more serum sensitive (24). The addition of the alternative pathway increases by 40% the effective insertion of MACs by the classical pathway when LOS antigens prepared from serum-sensitive gonococci are inserted into liposomal membranes and used as targets for antibody-mediated insertion of MACs. The alternative pathway alone, however, achieves only 12% of the total insertion of MACs, indicating that the classical pathway facilitates activation of the alternative pathway in this system (M. L. Schulz, P. A. Dale, K. Y. Lung, and P. A. Rice, unpublished observations).

These findings together suggest that C3 may be deposited at different sites on serum-sensitive gonococci, particularly their LOS molecules. This may depend on the complement pathway that is active in the serum. Alternatively, C3 may be deposited at the same site by both pathways, but the efficiency with which it serves as a permissive site for additional C5 convertase formation may differ.

CONCLUSIONS

Resistance of *N. gonorrhoeae* to killing by NHS is probably exhibited by all strains in vivo. After in vitro subculture, however, some strains become serum sensitive, while others remain resistant (stable serum resistance). The molecular basis for resistance to NHS is multifactorial. In vivo resistance may result from sialidation, in vivo, of gonococcal LOSs, thereby providing the organism with a eucaryotic antigenic look against which there may be no human antibody. Strains subcultured in vitro with commonly used bacteriologic media are no longer sialidated but, nevertheless, may remain serum resistant (stable serum resistance). Stable (in vitro) resistance may also be due, in part, to the absence of antibody in NHS directed against surface-exposed LOS bactericidal or lytic sites. However, LOS sites on stable serum-resistant organisms may be suitable for recognition by antibodies present in immune gonococcal sera, thereby rendering these strains sensitive to immune sera. Serum-sensitive strains harbor exposed LOS lytic epitopes recognized from the outset by NHS after organisms are subcultured.

Other mechanisms also contribute to serum resistance. Blocking antibodies directed against outer membrane proteins, such as PIII, prevent binding by bactericidal antibodies and render *N. gonorrhoeae* serum resistant. Serum resistance does not prevent formation of MACs of complement, but, rather, MACs do not fully insert through the outer membranes of serum-resistant gonococci, and death of the organism does not ensue. In addition, the development of C5 convertases is reduced when serum-resistant organisms are incubated in NHS, and this may also contribute to serum resistance.

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