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Elaboration of a 3.6-Kilodalton Lipooligosaccharide, Antibody against Which Is Absent from Human Sera, Is Associated with Serum Resistance of *Neisseria gonorrhoeae*†

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***Neisseria gonorrhoeae* strains that resist lysis by normal human sera (NHS) do so, in part, because NHS contain immunoglobulin M (IgM) specific for lipooligosaccharide (LOS) antigens of serum-sensitive strains, but lack antibodies for LOS antigens that can serve as loci for immune lysis of serum-resistant (ser^r) strains. We used a monoclonal antibody (McAb), specific for an epitope within a 3.6-kilodalton (kDa) component of *Neisseria meningitidis* L8 LOS, that binds a 3.6-kDa gonococcal LOS component so that we could explore further ser^r gonococcal strains. The McAb bound to the LOS of 6 of 7 ser^r of strains but not to the LOS of 0 of 14 serum-sensitive and serum-intermediate gonococcal strains of diverse origin. We studied three ser^r strains further. Strain 7134 does not elaborate the 3.6-kDa LOS component and does not bind the McAb; strains WR220 and WR302 do elaborate the 3.6-kDa LOS component. The titer (log_2) at which the McAb, diluted in NHS, lysed strain WR220 was 7.7; for WR302 it was 3.7, and for 7134 it was 0. Addition of McAb to NHS caused increased classical and alternative-pathway C3 deposition onto strain WR220, but only classical-pathway-activated C3 deposition onto strain WR302. The difference in lytic effectiveness of the McAb for the two strains, therefore, may result from differences in alternative-pathway augmentation of McAb-dependent classical-pathway activation on their surfaces. None of 40 randomly selected normal young adults had serum antibody that could compete with the McAb for binding to WR220 LOS in a solid-phase RIA. We conclude that the 3.6-kDa LOS component is commonly expressed by ser^r strains of *N. gonorrhoeae* and that antibody to it would be lytic if present in human serum, but that it is infrequently, if ever, present. As a result, strains elaborating this LOS are resistant to lysis by NHS.**

We have shown that resistance of certain strains of *Neisseria gonorrhoeae* to the bacteriolytic action of normal human sera (NHS) results from the absence from their lipooligosaccharides (LOS) of antigenic loci for the lytic immunoglobulin M (IgM) in most human sera (lytic loci) (28). Expressed as a function of the host, serum resistance (ser^r) may result from the absence from most NHS of lytic antibody directed against LOS epitopes that potentially can serve as loci for immune lysis.

Recently we found that gonococcal LOS contain multiple components, with M_rs ranging from 3,600 to 7,100, and that unique antigens are expressed on individual LOS components (29). In these studies, we used a monoclonal antibody (McAb) directed against a meningococcal L8 LOS epitope. This McAb bound to a single gonococcal LOS component with an M_r of 3,600, as estimated by coelectrophoresis of the sodium dodecyl sulfate (SDS)-disaggregated *Salmonella minnesota* isogenic rough mutant LOS through SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (29). We also noted that the LOS that bound the McAb were extracted from ser^r gonococci.

We extended these observations to a larger number of strains and used the McAb to better define the phenomenon of ser^r. The results are consistent with our earlier findings.

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MATERIALS AND METHODS

Microbiology. *N. gonorrhoeae* strains were from the collections of the Walter Reed Army Institute of Research and have been extensively characterized (12, 22, 25, 27–29, 31, 37). Strains were those used in previous studies by us and by others and are representative of commonly used study strains. They were not otherwise selected. They included all strains for which we had data on serum sensitivity (ser^s), including those in a preliminary publication (18) in which an alternative designation (SR) was used for strain WG. ser^s was characterized as described before (28). *Neisseria meningitidis* 355 and 7880, also from the Walter Reed Army Institute of Research collections, have also been characterized (7, 10, 40). Cultural conditions were as described previously (29). LOS were extracted from acetone-powdered organisms by the hot phenol-water method (2, 35).

SDS-PAGE. LOS were disaggregated in 2% SDS in 60 mM Tris hydrochloride–1.0 mM EDTA buffer, pH 6.8 (sample buffer), and were electrophoresed discontinuously through PAGE as previously described (16, 29). Electrophoresed LOS components were visualized by silver staining (32). M_rs were assigned to LOS components by reference to those of strains that had previously been coelectrophoresed with LOS of known M_r extracted from isogenic rough mutants of *S. minnesota* SF1111 (29). The log of the theoretical M_r of each *S. minnesota* LOS, as determined by summation of their chemical constituents, was linearly related to their

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distance of migration in SDS-PAGE. We used the regression function describing this relationship to estimate the M_r of neisserial LOS.

Serology. Production of a mouse monoclonal meningococcal strain L8 LOS antibody has been described before (40). This clone produces IgG3 antibody that binds staphylococcal protein A. McAbs 3F11 and 1-1-M have also been characterized (1, 18).

We used a modification of the Western blot transfer system to immunoblot electrophoresed LOS (5, 29). We first notched gels for orientation and then washed them with water for 30 min. We transferred LOS components from gels to a nitrocellulose medium (Bio-Rad Laboratories, Richmond, Calif.) overnight at 30 V and 10°C with a TE42 Transphor cell and a TE50 power lid (Hoefer Scientific Instruments, San Francisco, Calif.). The transfer buffer consisted of 20% (vol/vol) methanol in a 25 mM Tris–192 mM glycine buffer, pH 8.3. We placed the transblot in a washing buffer (10 mM Tris, 154 mM sodium chloride; pH 7.4) containing 5% (wt/vol) bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) for 30 min to saturate protein-binding sites and then reacted it for 1 h with McAb that was diluted (1:1,000) with bovine serum albumin washing buffer. The transblot was washed for 15-min intervals, once with washing buffer, twice with washing buffer containing 0.05% Nonidet P-40 (BDH, Poole, England), and once again with washing buffer. We reacted the washed blot with ^{125}I-labeled protein A (specific activity, 4,000 cpm/μg of protein) in bovine serum albumin washing buffer for 1 h and then washed it as described above. We autoradiographed the completed immunoblot at −70°C overnight on Kodak AR film (Eastman Kodak Co., Rochester, N.Y.).

We raised hyperimmune antisera (IRS) to ser^r strain WR220 and ser^s strain F62 in rabbits, using a previously described inoculation schedule (28).

We used a microassay (25) to assess bactericidal activity in NHS from a volunteer in one of our laboratories who did not admit to a history of infection with *N. gonorrhoeae*. His serum was processed at 4°C and stored in small aliquots at −70°C. We substituted Veronal (Winthrop Laboratories, Div. Sterling Drug Co., New York, N.Y.)-buffered saline with 0.1% gelatin (GVBS) containing 0.20 mM magnesium in 0.15 mM calcium for Geys balanced salt solution used previously in this assay. The microassay was modified to test McAb and heat-inactivated IRS for bactericidal activity. Each was first diluted to a 0.025-ml volume in twofold steps, after which similar volumes of freshly thawed NHS (diluted 1:4) and organisms were added sequentially. The final dilution of NHS in each reaction mixture was 1:12 (8.33% NHS). We took the bactericidal endpoint to be the highest dilution of serum that killed 95% of the inoculum.

We modified a previously described solid-phase radioimmunoassay (39) to detect the presence of antibodies of the same specificities as those of the McAbs in the sera of 40 military recruits who declined to participate in a field trial of an experimental meningococcal vaccine (11). The method was essentially similar to the enzyme-linked immunosorbent assay inhibition technique of Sarafian et al. (24). Briefly, we sensitized polyvinyl microtiter wells with that concentration of LOS from gonococcal strain WR220 that completely saturated LOS-binding sites in the wells as judged by the failure of higher concentrations to increase binding of McAb (17). We removed any unbound LOS and added a protein "filler" (0.5% casein, 0.5% bovine serum albumin, 0.2% sodium azide in Dulbecco phosphate-buffered saline, pH 7.4) to block any protein-binding sites. We incubated serial doubling dilutions of each serum in the wells for 3 h and then added an equal volume of the lowest concentration of each McAb that provided saturated binding to sensitized wells, as described above. We incubated this mixture in the plates overnight, removed unbound antibody, washed the wells, and then added ^{125}I-labeled goat anti-mouse IgG or IgM, depending on the isotype of the McAb. We incubated the plates for an additional 4 h, removed the goat antibody, washed the wells, cut the wells from the plates, and counted them in a gamma radiation counter.

We diluted the polyclonal and monoclonal antibodies and the ^{125}I-labeled goat antibodies in filler. We included IRS as a positive control (i.e., serum known to contain LOS antibody), and McAbs 3F11 and 1-1-M (both IgM) as comparisons for McAb 2-1-L8.

Quantitation of complement components. We assessed C3 and factor B deposition onto the surfaces of WR220 and WR302 by a modification of the method of Verbrugh et al. (33). We chelated NHS with 10 mM ethylene glycol-bis (β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA; Sigma, St. Louis, Mo.) in Dulbecco phosphate-buffered saline without Ca^{2+} or Mg^{2+}, or with 10 mM $MgCl_2$ (MgEGTA) (4, 20). Chelated serum was unable to lyse antibody-coated sheep erythrocytes but did lyse unsensitized rabbit erythrocytes (19). We prepared standard inocula from 18-h-old solid-medium cultures of gonococci that were suspended in GVBS (20) to 0.685 A_{660} and pelleted at 12,500 × *g*. Either native or MgEGTA-chelated (1.0 ml) NHS was added to the washed (GVBS) gonococcal pellets to effect a final serum concentration of 25% in GVBS. After incubating mixtures with rotation (8 rpm) at 37°C for 30 min, we pelleted the bacteria at 12,500 × *g*, washed them three times with GVBS, and reacted them with fluorescein-conjugated antisera raised to either human C3 (Tago, Inc., Burlingame, Calif.) or factor B (Atlantic Antibodies, Scarborough, Maine). After we washed them in GVBS three additional times, we suspended the gonococcal pellets in 1.0 ml of 0.1 N NaOH. We measured the intensity of fluorescence in a fluorescence spectrophotometer (model 203; Perkin-Elmer Corp., Norwalk, Conn.) with excitation and emission wavelengths of 485 and 525 nm, respectively. We used heat-inactivated NHS as a control for background trapping of fluorescent antibody and subtracted this value from the fluorescence intensity of each sample. For each organism, we arbitrarily assigned unity to the fluorescence on WR302 without McAb and compared all other values to it. The significance of differences was assessed by use of Student's *t* test for independent population means (38).

RESULTS

Figure 1 is a composite immunoblot analysis of homologous (L8) meningococcal LOS (355), heterologous meningococcal LOS (7880), and seven gonococcal LOS. WR213 and 1342 are ser^s; the remaining gonococcal strains are ser^r. WR213-1 LOS is WR213 LOS that had degraded during storage for 6 weeks in sample buffer; WR213 was freshly dissolved in sample buffer. All of the LOS except WR213 and 1342 (ser^s) contained a component that comigrated with a single component of strain WR220 and that bound the L8 McAb. To reference the McAb-binding component in the polyacrylamide gel, we reacted a transblot of selected gonococcal LOS with both the L8 and 3F11 McAbs (Fig. 2). L8 McAb bound the fastest-migrating component (lowest M_r) of strains WR220 and 135 LOS; 3F11 McAb bound a higher-M_r component. The L8 McAb bound a quantitatively minor component of strain WR302 LOS that

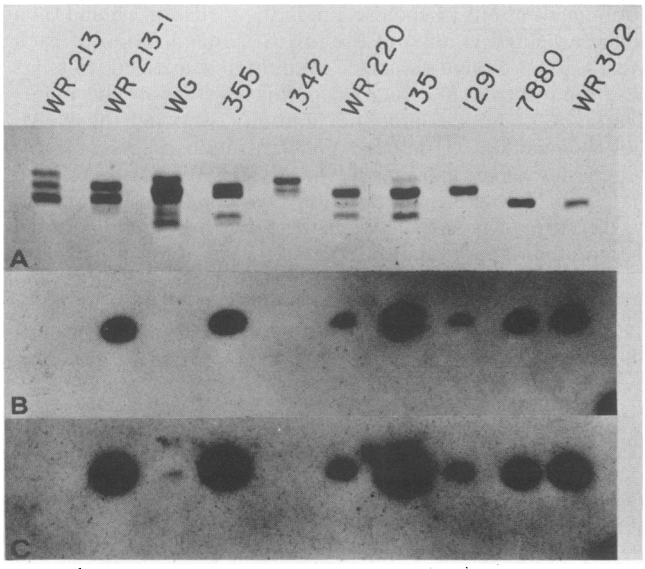

FIG. 1. Immunoblot analysis of reactivity of meningococcal L8 McAb with the homologous meningococcal LOS (355) and with LOS from a heterologous meningococcal strain (7880) and from seven gonococcal strains. A 750-ng amount of each LOS was applied to each lane. Panels: A, silver-stained gel of LOS samples after SDS-PAGE; B, autoradiograph of immunoblot prepared from a replica of gel A after 48 h of exposure; C, the same autoradiograph after 5 days of exposure. WR213 and 1342 are ser^s; other gonococcal strains are ser^r. WR213-1 is WR213 that had degraded during storage in sample buffer.

was observed within a low-M_r doublet (Fig. 2); in subsequent gels, it could just be separated from the other component and was found to comigrate with the 3.6-kilodalton (kDa) components of strain WR220 and 135 LOS. The M_r of the L8 McAb-binding component was estimated to be 3,600 by reference to the theoretical M_rs of the *S. minnesota* mutant LOS; that of the 3F11 McAb-binding component was estimated to be 5,200. (In a preliminary report [26] we incorrectly estimated the M_r of the L8 McAb-binding component to be 4,800.) After degradation, WR213 LOS (WR213-1) also expressed a 3.6-kDa component that bound the McAb.

The L8 McAb bound to the LOS of 6 of 7 ser^r strains, but to the LOS of 0 of 14 ser^s and serum-intermediate (ser^i) gonococcal strains of diverse origin. We studied three ser^r strains further. Figure 3 shows that strains WR220 and WR302 elaborated different quantities of the 3.6-kDa LOS component that bound the L8 McAb, whereas LOS from strain 7134 was devoid of the 3.6-kDa component and did not bind the L8 McAb.

None of the three strains was lysed by undiluted NHS (50% final concentration), whereas all three were lysed by IRS diluted in NHS (Table 1). The McAb was also bacteriolytic, but only for the two strains that expressed the 3.6-kDa LOS component. The titer at which the two strains were sensitive to the McAb and human complement paralleled the quantitative expression of the 3.6-kDa component.

Binding of C3 was greater in native NHS than in chelated NHS for both WR220 and WR302; binding of factor B was greater in chelated than in unchelated serum. Strain WR220 bound 35% more C3 in native NHS ($P = 0.001$) but 8% less in chelated NHS ($P < 0.05$) than did strain WR302. Binding of factor B was equivalent for the two strains in both native and chelated NHS ($P > 0.1$). Addition of L8 McAb significantly and equivalently increased the binding of C3 to both strains; as a result, C3 deposition onto WR220 remained significantly higher (30%; $P = 0.001$) than that onto WR302. Lysis was dependent upon the presence of antibody and was independent of the quantity of complement components bound per se. This is indicated by the fact that at nearly equivalent C3 and factor B deposition (Table 2), strain WR302 (with McAb) was lysed (Table 1), whereas strain WR220 (without McAb) was not (Table 1). Although WR302 bound more C3 in the presence of McAb than did WR220

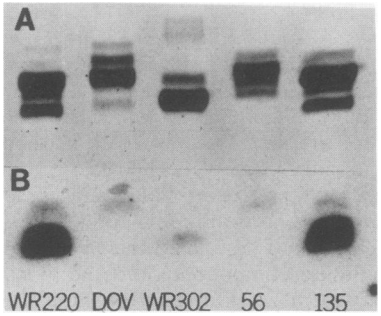

FIG. 2. Immunoblot analysis of reactivity of L8 and 3F11 McAbs with LOS from gonococcal strains selected for binding of 3F11 McAb (DOV and 56), L8 McAb (WR302), or both McAbs (WR220 and 135). A and B were duplicate gels. A was silver stained after SDS-PAGE, and B was first electroblotted and then reacted with McAb. B is the autoradiograph of this immunoblot.

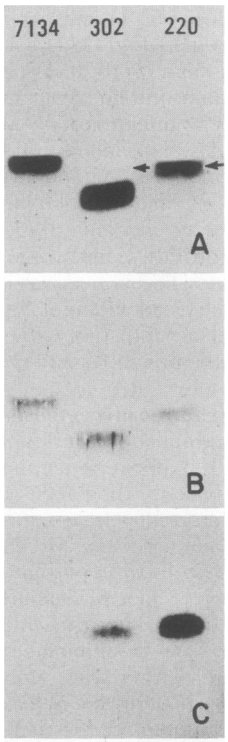

FIG. 3. Immunoblot analysis of reactivity of L8 McAb with LOS from three ser^r gonococcal strains. A and B were duplicate gels. A was silver stained after SDS-PAGE, and B was first electroblotted and then stained. C is an autoradiograph of the immunoblot prepared from B. Arrows in panel A should be disregarded.

TABLE 1. Correlation among expression of a 3.6-kDa LOS component by ser^r gonococcal strains, binding of strain L8 McAb by LOS extracted from these strains, and lysis of them by NHS without and with the addition of that McAb or IRS[a]

Strain	Expression of 3.6-kDa LOS	Binding of L8 McAb	Lytic titer[b] of:		
			NHS	NHS + McAb	NHS + IRS
WR220	+++	+++	None	7.7	8.0
WR302	+	+	None	3.7	5.7
7134	0	−	None	None	3.3

[a] IRS prepared against strain WR220.
[b] Geometric (log_2) mean titer (three replicate assays) of NHS, of ascitic fluid (McAb) diluted in a 1:4 dilution of NHS, and of IRS, also diluted in 1:4 NHS. The final concentration of NHS was 50% for NHS alone and 8.3% for assays in which exogenous antibody was added.

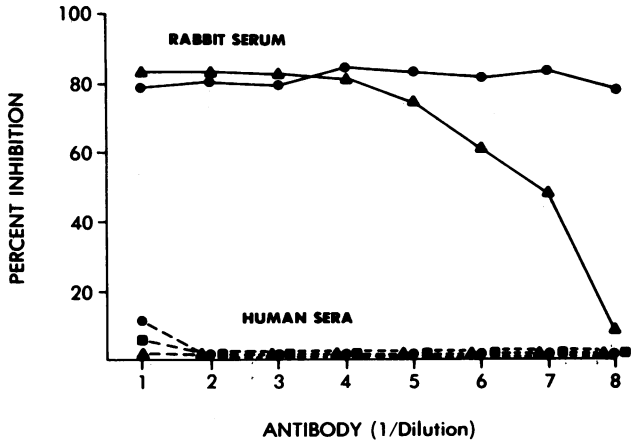

FIG. 4. Competition by antibodies in polyclonal sera with LOS binding of McAb. Forty sera from normal young adults were tested individually for their ability to compete with 3F11 (●), 1-1-M (▲), and 2-1-L8 (■) McAb binding to WR220, F62, and WR220 LOS, respectively. Data are the means of the 40 determinations. IRS to F62, whose LOS expresses both 3F11- and 1-1-M-defined epitopes, was tested with 3F11 (●) and 1-1-M (▲) McAbs as a positive control.

without McAb (162% versus 135%), WR302 was also lysed in the presence of antibody in 12.5% serum, in which C3 binding would be quantitatively less. The McAb-dependent increase in C3 binding in native serum was accompanied by a significant increase in alternative-pathway deposition of C3 (chelated serum) only onto strain WR220 (Table 2). Factor B deposition onto strain WR302 in native serum was significantly decreased by the McAb (Table 2).

None of 40 sera from normal young adults contained antibody that competed with either L8 or 3F11 McAb for binding to WR220 LOS, or with the binding of 1-1-M McAb to the LOS of ser^s strain F62 (Fig. 4). In contrast, IRS contained high titers of antibody that interfered with binding of 3F11 and 1-1-M McAbs to F62 LOS (Fig. 4).

DISCUSSION

These data extend our previous finding that NHS lack antibody specific for lytic loci within the LOS of ser^r gonococci that would render them ser^s, but contain IgM specific for LOS lytic loci of ser^s strains (28). The 3.6-kDa LOS component is commonly expressed by ser^r strains, although its quantitative expression varies among such strains. Human sera seldom, if ever, contain antibody against this LOS (Fig. 4); when antibody specific for it (Table 1) is added to NHS, it readily initiates lysis mediated by human complement.

The 3.6-kDa component is only one of several LOS components expressed by ser^r strains. Some ser^r strains do not express it, and others express very little. For example, the quantitative expression of the 3.6-kDa component by strain WG was so minor that prolonged exposure of the Immunoblot was necessary to reveal it (Fig. 1), and it could not be detected by a less sensitive solid-phase radioimmunoassay (18). No attempt was made to determine the prevalence of expression of the 3.6-kDa component among ser^r strains shown to be clonally discrete by other markers. Because our strains were initially characterized in several different laboratories in different cities, and because they elaborate LOS that are quite different physically, it seems unlikely to us that they represent multiple copies of a single clonotype or that the association of the 3.6-kDa LOS with ser^r is a fortuitous result of nonindependent sampling. However, strains WR220 and 135 do seem to be similar, if not identical, on the basis of the physical characteristics of their LOS alone. We would need to study more extensive and carefully selected samples before generalizing these observations.

We assume that the failure of other LOS components to serve as lytic loci also relates to the absence of antibody against them in NHS (Fig. 4). The addition of either polyclonal rabbit antibody (Table 1) or its $(NH_4)_2SO_4$-precipitated immunoglobulin (28) to NHS results in initiation of lysis of strains that do not elaborate the 3.6-kDa component (28).

C3 in NHS is deposited through the classical pathway (CP)

TABLE 2. Effect of L8 McAb on binding of NHS C3 and factor B to two ser^r *N. gonorrhoeae* strains that quantitatively differ in expression of a 3.6-kDa LOS component

Strain	McAb, presence (+) or absence (−)	% Binding[a] (mean ± SD)			
		NHS (CP and ACP)		Chelated NHS (ACP)	
		C3	Factor B	C3	Factor B
WR220	+	213 ± 0 ($P < 0.001$[c])	98 ± 3 ($P > 0.05$)	137 ± 0 ($P < 0.001$)	103.5 ± 2 ($P > 0.05$)
	−	135 ± 1	101 ± 2	92 ± .5	97 ± 4
WR302	+	162 ± 2 ($P < 0.001$)	87 ± 1 ($P < 0.05$)	105 ± 2 ($P > 0.05$)	110 ± .4 ($P < 0.05$)
	−	100	100	100	100

[a] Expressed as percent binding of that by strain WR302 without McAb, within each assay. Data are mean ± standard deviation of four replicate determinations.
[b] NHS is a 25% concentration; chelated NHS is 25% NHS with MgEGTA.
[c] Values of P for the comparison between the presence and absence of McAb for each strain are shown in parentheses.

onto ser^r strains. This indicates either that C1 activation by ser^r strains is antibody independent (6, 8) or that NHS contain antibodies against surface antigens of ser^r strains that activate complement without promoting immune lysis; i.e., they bind to nonlytic loci. In either event, the deposition of C3 onto their surfaces does not result in lysis of ser^r strains unless such deposition is initiated by antibody binding to a lytic locus. Our finding that it is not the quantity of C3 deposited onto the gonococcal surface that determines whether lysis ensues, but the mechanism of its deposition, is consistent with the finding that the C5-9 membrane attack complex is not stably assembled onto the surface of ser^r gonococci (15). Ineffective activation of complement and deposition of the membrane attack complex have also been found to explain ser^r of enteric bacteria that elaborate considerably larger lipopolysaccharide components (14, 23).

Quantitation of complement-component deposition showed that the McAb effectively augmented ACP deposition of C3 onto WR220 (chelated NHS; Table 2), but had no effect on its deposition onto WR302 and even diminished factor B deposition on the latter. Augmentation of complement activation at low antibody concentration by recruitment of ACP activity has been described for many target cells, including bacteria (3, 34, 36), and the fact that ACP augmentation varies among different strains has also been shown (9, 34). We conclude that the surfaces of the two organisms vary in their capacity to activate the ACP, with the surface of WR302 being relatively nonactivating as compared with that of WR220. We have shown previously that the concentration of antibody required to effect equivalent lysis of ser^s gonococcal strains is a function of the ability of the strain to activate the ACP rather than of the specificity of the initiating antibody (12). Ser^s strains that are relatively poor ACP activators require correspondingly more antibody for equivalent lysis than do strains that readily activate the ACP (12). It should also be noted that ACP deposition of C3 onto strain WR220 was independent of factor B deposition. We have no explanation for this phenomenon, but it has also been observed for immune lysis of group B *N. meningitidis* (13).

The degradation of WR213 LOS in sample buffer is another curious phenomenon of uncertain explanation. It reproducibly occurred, but for only one of four separate lots of WR213 LOS (30). We have also observed degradation of a lot of WR220 LOS with neo-expression of a different McAb-defined epitope. Although it is still speculative, we suspect that the EDTA in the sample buffer chelates divalent cations from the LOS, leading to their replacement by the monovalent Tris cation. Divalent cations are critical for epitope expression in gonococcal LOS (17), and replacement of divalent cations by Tris alters ser^s of *S. typhimurium* by permitting stable association of C5b with the cell membrane (21, 28).

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