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Complement Consumption by Gonococcal Peptidoglycan

B. H. PETERSEN[1] AND R. S. ROSENTHAL[2]*

Lilly Laboratory for Clinical Research, Eli Lilly & Company,[1] and Department of Microbiology and Immunology, Indiana University School of Medicine,[2] Indianapolis, Indiana 46223

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Purified peptidoglycan (PG) obtained from *Neisseria gonorrhoeae* was tested for the ability to consume complement in normal human sera. Sonicated PG (S-PG), a heterogeneous mixture of soluble fragments (molecular weight, $>10^6$), as well as intact (insoluble) PG, reduced the level of whole hemolytic complement in a pool of four human sera. The minimal concentration of S-PG required for this activity was approximately 500 μg of S-PG per ml of serum. Complete lysozyme digestion of S-PG, yielding PG fragments of $<10^4$ molecular weight, eliminated complement-consuming activity. S-PG–mediated complement consumption resulted in depletion of the individual complement components C4 and C3. Consumption of complement did not occur when C4-deficient human serum or normal human sera treated with Mg^{2+}–(ethylene glycol-bis(β-aminoethyl ether)-*N*, *N*-tetraacetic acid to specifically impair classical complement pathway activity were used. The addition of rabbit anti-PG antibody greatly enhanced gonococcal PG-mediated complement consumption. Together, the data suggested that gonococcal PG-mediated complement consumption occurred via the classical complement pathway, was dependent on the presence of anti-PG antibody, and required glycosidically linked polymers of PG. Individual human sera varied widely in the extent of gonococcal PG-mediated reduction of complement levels, presumably a reflection of either different amounts of natural antibody to gonococcal PG, different levels of human PG hydrolase(s) capable of degrading PG to inactive fragments, or both.

The bacterial surface is a mosaic of macromolecules that constitutes the interface between the organism and its external environment. As such, these surface molecules collectively exert an important influence on the bacterium's interaction with host tissues, cells, and molecules. One aspect of this interaction of current interest is the ability of cell wall peptidoglycan (PG) (see reference 6 for review of structure) to activate complement as a means of generating mediators of inflammation. In one well-developed study, several groups concluded that PG plays a key role in the complement-dependent opsonization of staphylococci in the presence of normal human sera (17, 30–33, 36, 37) and may contribute to the acute inflammatory response to staphylococci (23, 35) and to other gram-positive bacteria (2, 8, 26).

The consumption of complement by PG derived from gram-negative bacteria has not been studied, probably because gram-negative PG is not regarded as an exposed site on the external surface. Yet, the opportunity for direct interaction between PG from gram-negative bacteria and host components could be quite extensive. First, PG could be liberated into host tissues as a result of host PG hydrolase activity, e.g., lysozyme, accompanying bacterial degradation in phagocytic cells and in various host fluids. PG fragments released in vivo by such a mechanism might vary considerably in complexity and in size, a reflection of the extent of enzymatic digestion achievable under a given set of circumstances. Second, PG fragments could be released from living bacteria as a result of PG turnover during growth, i.e., solubilization of native PG by the action of bacterial PG hydrolases (autolysins) (5). These PG products would reflect the specificities of endogenous autolysins and might also be quite heterogeneous in size and structure.

Although extensive turnover of PG during growth does not appear to be common among gram-negative bacteria studied to date, *Neisseria gonorrhoeae* is a notable exception. Gonococci turn over approximately 35% of their existing PG per generation during exponential growth in vitro (7, 10, 20) and release the soluble PG fragments into the medium (20, 25). If this activity occurs in vivo, it could represent an important mechanism for increasing the opportunity for interaction between gonococcal PG and host tissues.

We are testing the hypothesis that release of

gonococcal PG, mediated by bacterial and host PG hydrolase activities, contributes to the intense inflammatory reactions associated with gonococcal infections. The current studies evaluated the capacity of gonococcal PG to consume complement in human sera, one possible mechanism for generating host mediators of inflammation.

MATERIALS AND METHODS

Complement sources. Human sera, obtained from healthy volunteers, were either freshly drawn or thawed from samples that were freshly frozen and stored at −70°C. Previously frozen samples were discarded after use. Pooled normal human serum (NHS pool) was obtained by combining equal volumes of freshly drawn sera from four volunteers who denied prior gonococcal infection. Samples of the NHS pool were frozen until used. The level of hemolytic complement (CH_{50}) in each contributor to the NHS pool was within the normal range of our laboratory (35 to 75 CH_{50}). Serum deficient in the fourth component of complement (C4) was obtained from a patient previously determined to be deficient in C4 by both hemolytic and immunochemical assays.

Complement assays. Total hemolytic complement was measured by using hemolysin-sensitized sheep red blood cells (SRBC) as described by Taliaferro (28). SRBC, preserved in Alsevers solution, were obtained from Colorado Serum Co., Denver, Colo. The extent of hemolysis was determined by using a Coleman Junior spectrophotometer (Perkin-Elmer Corp., Oak Brook, Ill.) at 530 nm. The titer (CH_{50}) was the reciprocal of the dilution giving 50% lysis.

Titers of individual complement components were determined by the procedures of Nelson et al. (16), with modifications suggested by Cordis Laboratories (Miami, Fla.). Stable cellular intermediates were prepared as described by Nelson et al. (16) and Rapp and Borsos (19). Functionally pure complement components were obtained from Cordis Laboratories. Reaction mixtures (final volume of 2.5 ml) were incubated as previously described (16) and diluted to 5 ml in cold saline, and the percent lysis was determined spectrophotometrically at 415 nm.

Consumption of C3 to C9 via alternative complement pathway (ACP) activation was determined by the method of Platts-Mills and Ishizaka (18) by using normal rabbit red blood cells (RRBC) which activate the ACP in serum containing ethylene glycol-bis(β-aminoethyl ether)-*N,N*-tetraacetic acid (EGTA) to block classical complement pathway (CCP) activation. Whole blood was collected from New Zealand white rabbits directly in heparinized tubes. The RRBC were washed three times in Veronal-buffered saline, pH 7.5, with 2 mM Mg^{2+} and 10 mM EGTA (VBS-Mg-EGTA) before use. Sera to be tested were diluted in VBS-Mg-EGTA, and 1 ml was mixed with 0.5 ml of RRBC (10^8 cells per ml) in VBS-Mg-EGTA. Percent lysis and CH_{50} titer were determined as described above.

Activation of complement by PG. Intact PG (or soluble PG fragments) in 200 μl of phosphate-buffered saline was added to 200-μl samples of undiluted normal serum, serum that had been treated with 2 mM Mg^{2+} and 10 mM EGTA, and C4-deficient serum. After incubation at 37°C for 30 and 60 min, the mixtures were assayed for total hemolytic complement or for C3 to C9 consumption as described above. Control tubes contained the designated sera and appropriate amounts of phosphate-buffered saline.

Preparation of PG. (i) Intact PG. Purified intact (insoluble) PG was prepared from *N. gonorrhoeae* strain RD_5 (nonpiliated, transparent; 27) by using a trichloroacetic acid-sodium dodecyl sulfate (SDS) extraction procedure (10, 21). The final washed SDS-insoluble preparation typically contained, by weight, approximately 1% non-PG amino acids as determined by amino acid analysis. In some experiments, washed SDS-insoluble PG (2 to 4 mg/ml) was treated for 16 h at 37°C with pronase (30 μg of pronase per mg of PG) in 0.01 M Tris-hydrochloride buffer, pH 7.4. Pronase treatment further reduced protein contamination to approximately 0.1% (wt/wt). The composition of PG (or PG fragments) isolated from strain RD_5 is typical of gram-negative bacteria (21). RD_5 PG appears to possess much fewer O-acetyl derivatives than the PG of other gonococcal strains which have been studied (1).

(ii) Sonicated PG. To obtain high-molecular-weight soluble PG fragments, we sonicated intact PG (typically 16 mg suspended in 4 ml of ice-cold, commercial, pyrogen-free water) at one-half maximal power for 12.5 min in a 15-ml glass tube (Corning no. 8441, 17 [outer diameter] by 100 mm), using a Branson 350 Cell Disrupter (Branson Ultrasonics Corp., Danbury, Conn.) equipped with a microprobe. The probe was inserted into the tube to about one-half the depth of the liquid. The cleared suspension was centrifuged (36,000 × *g*, 35 min), and the soluble PG in the supernatant was recovered. Under these standard conditions, 80 to 90% of the PG was solubilized, as determined by the distribution of [^{3}H]glucosamine that had been incorporated into the PG (25). The final product, which is referred to as sonicated PG (S-PG), was a heterogenous mixture consisting almost exclusively of fragments with an apparent molecular weight of greater than 10^6, as determined by chromatography on Sepharose 6B (Pharmacia Fine Chemicals).

Purified staphylococcal PG (kindly supplied by Brian J. Wilkinson, Normal, Ill.), prepared by the hot trichloroacetic acid method (17), and intact gonococcal PG were also subjected to treatment with a Raytheon sonic oscillator (Raytheon Co., Norwalk, Conn.). One-milliliter suspensions of PG (4 mg/ml) in 5-ml plastic tubes were placed directly in the well of the oscillator; the water level was slightly above that of the PG suspension in the tube. The two PG samples were treated simultaneously at the maximal power setting. At intervals, samples were taken and tested for complement-consuming activity. Compared with treatment with the sonic probe, treatment with the sonic oscillator is relatively mild. Sonic oscillation appeared to disaggregate but not solubilize suspensions of both peptidoglycans.

(iii) Muramidase digest of PG. Complete enzymatic digestion of intact PG or S-PG (2 to 4 mg/ml) was performed by treating samples with the muramidase hen egg white lysozyme (Boehringer Mannheim Corp.; 100 μg/ml) in 0.01 M phosphate buffer, pH 7.4, for approximately 12 h at 37°C. Complete digestion of strain RD_5 PG with muramidase yields exclusively low-molecular-weight fragments no greater in size than peptide-cross-linked disaccharide peptide tetramer, ca. 4,000 molecular weight (21).

Preparation of anti-PG sera. New Zealand white rabbits were injected with 1 mg of intact PG suspended in complete Freund adjuvant (CFA) and distributed equally between the hind footpads and the subscapular region. The animals were reinjected 2 weeks to 4 months later with 1 mg of intact PG in CFA. Sera taken at intervals after reinjection were screened by using a latex agglutination assay (12), which included the use of latex particles coated with gonococcal S-PG. Because CFA contains mycobacterial PG which conceivably could cross-react with gonococcal PG, other (control) rabbits were administered CFA alone. Agglutination titers of hyperimmune sera typically were in the range of 1:80 to 1:640. Preimmune sera and sera from animals injected with CFA alone had titers equal to or less than 1:10.

RESULTS

Consumption of complement by gonococcal PG. Gonococcal S-PG reduced the level of whole hemolytic complement activity present in the NHS pool (Table 1). To the extent tested, the amount of complement consumed was dependent on the amount of S-PG employed, and for each of several different batches of S-PG tested, the minimal dose necessary to reduce complement levels was approximately 500 μg of S-PG per ml of serum. This was similar to the minimal effective concentration of intact (insoluble) PG. Digestion of PG with lysozyme, a PG-specific hydrolase, completely destroyed the complement-consuming activity (Table 1).

Treatment of the NHS pool with S-PG (500 μg of S-PG per ml of serum) resulted in 78 and 90% reductions in the individual levels of C3 and C4, respectively. Because utilization of C4 is associated with CCP activation, these results suggested that PG-mediated complement consumption occurred, at least in part, via the CCP. These results, however, did not rule out the possibility that the ACP was also involved.

Two lines of evidence, however, did indicate that the ACP played little or no role in the depletion of hemolytic complement by S-PG. First, we employed a C4-deficient human serum (which had functional ACP activity but no detectable CCP activity) in the Platts-Mills assay for C3 to C9 consumption. S-PG did not reduce the levels of hemolytic complement in the C4-deficient serum (Table 2). Second, S-PG did not consume complement in the NHS pool that had been treated with Mg^{2+}-EGTA to specifically impair the function of CCP (Table 3). Thus, PG-mediated consumption of hemolytic complement did not occur when the complement source was genetically deficient in a component specifically required for CCP (but not ACP) activity or when the NHS pool was made functionally defective in CCP activity.

Enhancement of S-PG–mediated complement consumption by rabbit anti-PG antibody. To assess the role of anti-PG antibody in PG-mediated complement consumption, we tested mixtures containing S-PG plus heated (55°C, 30 min) hyperimmune anti-PG (and preimmune) sera from rabbits for their effects on complement levels in the NHS pool. Whereas the addition of 25 μl of normal (preimmune) rabbit serum to S-PG resulted in somewhat-increased complement-consuming activity (presumably a reflection of natural anti-PB in rabbit sera), 25 μl of hyperimmune serum considerably enhanced the consumption of complement (Table 4). It should be noted that the amount of S-PG used in these experiments was 125 μg of S-PG per ml of NHS pool, some fourfold less than the minimal amount required to show significant complement consumption in the absence of exogenous anti-PG (Table 1). Compared with mixtures of S-PG and anti-PG serum, mixtures of an equivalent amount of lysozyme-digested S-PG and anti-PG serum showed much-diminished complement-consuming activity (Table 4). This effect of lysozyme treatment was not observed if the lysozyme was heat inactivated (80°C, 30 min) before the addition of S-PG (data not shown), suggesting that the reduction in complement consumption was due to the enzymatic digestion of PG and not to some nonspecific effects of lysozyme on the assay system.

Complement consumption: gonococcal PG ver-

TABLE 1. Consumption of human complement by gonococcal PG

NHS pool +	PG concn in serum (μg/ml)	CH_{50} (% drop)[a]
PBS[b]		
S-PG	250	<5
S-PG	500	26 ± 3.0
S-PG	1,000	62 ± 1.5
LZ-S-PG[c]	500	<5
LZ-S-PG	1,000	<5

[a] Average of three independent experiments ± SD.
[b] PBS, Phosphate-buffered saline.
[c] LZ-S-PG, S-PG completely digested with lysozyme.

TABLE 2. Consumption of complement components C3 to C9 by gonococcal S-PG (500 μg of S-PG per ml of serum) in NHS pool and in C4-deficient human serum

Complement source	Treatment	C3–C9 consumption (CH_{50})	
		U	% Drop
NHS pool	Buffer	28	
NHS pool	S-PG	19	32
C4-deficient serum	Buffer	24	
C4-deficient serum	S-PG	23	4

TABLE 3. PG-mediated consumption of C3 to C9 in EGTA-treated serum[a]

Complement source	Treatment	CH_{50}	
		Expt 1	Expt 2
NHS pool	Buffer	29	29
EGTA-treated NHS pool	Buffer	28	25
EGTA-treated NHS pool	S-PG[b]	28	26

[a] Serum-PG mixtures were incubated for 60 min at 37°C before assay for C3 to C9 consumption.
[b] 500 μg of S-PG per ml of serum.

sus staphylococcal PG. The relatively high levels of gonococcal PG required to reduce complement levels in the NHS pool and the apparent inability of gonococcal PG to fix complement via the ACP seemed to contrast with the reported efficiency and mechanism of complement consumption by PG from gram-positive bacteria (2, 8, 31, 35). To rule out procedural differences, e.g., the use of concentrated versus dilute serum, or kinetic assays (29), as the reason for this observation, we felt it important to compare complement consumption by gonococcal PG directly with a reference (staphylococcal) PG under a standard set of conditions.

Treatment with the sonic oscillator appeared to increase only marginally the complement-consuming activity of intact gonococcal PG (Fig. 1). In fact, the extent of complement consumption by sonic-oscillator-treated gonococcal PG (500 μg/ml) was similar to that of the sonic-probe-treated samples which had been used throughout (Table 1). However, in agreement with the findings of Wilkinson et al. (34), we found that sonic oscillation greatly enhanced depletion of hemolytic complement by staphylococcal PG (Fig. 1). At a concentration of only 100 μg of PG per ml of serum, staphylococcal PG consumed all of the hemolytic complement after 15 min of sonic oscillation. Neither gonococcal nor staphylococcal PG appeared to be solubilized to any extent by the sonic oscillation treatment, although both suspensions appeared to undergo considerable disaggregation.

We attempted to prepare soluble staphylococcal PG by using the sonic probe, as we had used it for preparing gonococcal PG. However, treatment for 50 min, using the sonic probe under the conditions used for solubilizing gonococcal PG, failed to solubilize any of the staphylococcal PG. The same extensive (50-min) treatment of gonococcal PG with the sonic probe virtually eliminated complement-consuming activity (data not shown).

We also examined C3 to C9 consumption by unsonicated PG from staphylococci and gonococci by using serum that had been chelated with Mg^{2+}-EGTA to functionally inhibit activation of the CCP. Similar to gonococcal S-PG, intact gonococcal PG (500 μg of PG per ml of NHS pool) did not consume complement in EGTA-chelated serum. Under these conditions, intact staphylococcal PG (100 μg of PG per ml of NHS pool) reduced the complement levels by 40%. This confirms the observations of others (35) that staphylococcal PG can activate the ACP. As a positive control for ACP activity, these data also tend to underscore the relative inability of gonococcal PG to consume complement by the ACP.

Complement consumption by gonococcal S-PG in individual human sera. The above experiments utilized the NHS pool as a complement source. Because complement consumption by gonococcal PG appeared to depend, at least in part, on the presence of natural anti-PG antibody, and because different levels of these antibodies could have different biological consequences, it was of interest to examine the effect of PG on complement levels in individual normal human sera. Individual sera ($n = 34$) were found to vary markedly in the percent decrease in hemolytic complement (CH_{50}) activity after treatment with S-PG (500 μg of S-PG per ml of serum). The hemolytic complement reduction range was from a minimum of 5 to a maximum of 100%, most values being less than 50% (Fig. 2). Five of the sera (selected to cover the full range in complement consumption based on the decrease in CH_{50}) were individually treated with Mg^{2+}-EGTA and tested by using the RRBC assay for PG-mediated ACP activity. In no case was complement consumption observed.

DISCUSSION

It has become apparent over the past few years that PG and PG fragments have a propensity to influence components of the host's immune and inflammatory machineries (4, 11, 24). If provided the opportunity to interact with host

TABLE 4. Effect of rabbit sera on complement consumption by gonococcal PG[a]

NHS pool pretreated with:		CH_{50}	
PG	Heated rabbit serum (μl)	U	% Drop
None	None	42	
S-PG	None	41	2
None	Preimmune[b] (25)	35	17
S-PG	Preimmune (25)	29	31
S-PG	Anti-PG[b] (10)	25	40
S-PG	Anti-PG (20)	22	48
S-PG	Anti-PG (25)	16	62
LZ-S-PG	Anti-PG (25)	30	29

[a] 125 μg of PG per ml of human serum.
[b] Titers in the latex agglutination assay were as follows: preimmune = 1:4; Anti-PG = 1:320.

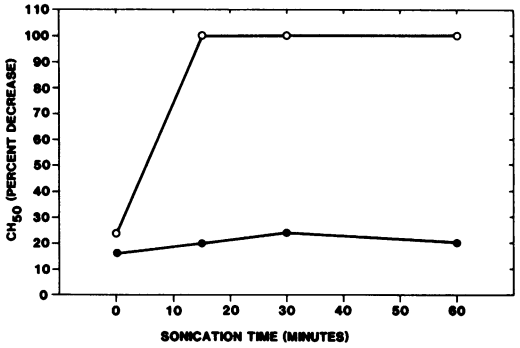

FIG. 1. Complement consumption by 500 μg of gonococcal intact PG per ml of serum (●) and 100 μg of staphylococcal PG per ml of serum (○) after sonic oscillator treatment.

cells and molecules in vivo, naturally occurring PG fragments could play a significant role in the pathophysiology of some infectious processes. Among gram-negative bacteria, *N. gonorrhoeae* is an organism for which the opportunity for direct interaction between PG and host may be uncommonly great. Indeed, based on in vitro studies, gonococci may even actively promote the interaction by shedding soluble PG fragments into the environment (20, 25).

The current studies have demonstrated that gonococcal PG caused consumption of hemolytic complement in normal human sera. This activity (i) required glycosidically linked polymers of the disaccharide peptide repeating unit of PG, (ii) occurred principally as a result of CCP activation, (iii) appeared to depend on the presence of natural anti-PG antibody, and (iv) varied widely among individual normal human sera. The dependency on PG of relatively large molecular weight is consistent with previous reports (8, 34) which indicated that complete enzymatic digestion of streptococcal or staphylococcal PG eliminates complement-consuming activity. It appears, therefore, that low-molecular-weight PG fragments are generally incapable of initiating complement activation by either ACP or CCP. It is of interest that this apparent requirement of PG-mediated complement consumption for relatively large PG fragments is similar to that of PG-mediated arthritogenicity in rats (14, 15). The relevance of this correlation remains to be determined.

The relatively low efficiency of complement consumption by gonococcal PG and its inability to initiate the ACP differed markedly from the behavior shown by staphylococcal PG. There are several factors which, collectively, may account for the different complement-consuming activities among peptidoglycans so far tested and, specifically, for differences between gonococcal and staphylococcal PGs. These include (i) intrinsic differences in the physicochemical form of gram-negative versus gram-positive PG, (ii) differences in the gross physical forms, e.g., soluble versus particulate, of the preparations used, (iii) differences in purification procedures which may contribute to differences in the structure of the final products, (iv) differences in the levels of natural antibody to a given PG, and (v) intrinsic differences among PGs from various organisms in susceptibility to PG hydrolase activity in serum.

At present, it is difficult to differentiate among these possibilities. To assess the first possibility will require more extensive comparisons of chemically defined soluble PG fragments from

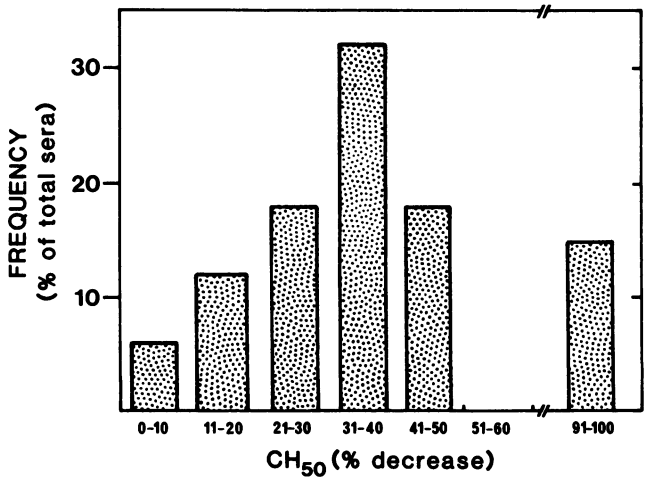

FIG. 2. Distribution of the extent of S-PG–mediated complement consumption in individual human sera (n = 34).

gram-negative and gram-positive organisms; to our knowledge this is the first report of complement consumption by a gram-negative PG. In regard to the second possibility, we originally had made the simple working hypothesis that complement activation via ACP depends more on the gross physical state of PG than on the source of PG; i.e., both soluble and particulate PGs initiate CCP in the presence of antibody, but only particulate PG can activate ACP. However, our findings that soluble and particulate gonococcal PGs consume complement with similar efficiencies and do so via CCP exclusively, whereas staphylococcal PG fixes complement via both CCP and ACP, is contrary to this idea. In regard to the third possibility, Wilkinson's group has recently provided evidence (34) that, indeed, the various chemical extraction procedures used routinely to isolate PG can influence PG-mediated complement consumption; i.e., intact PG made from formamide- and periodate-extracted staphylococci had lower complement-consuming activity than the corresponding trichloroacetic acid and NaOH extracts.

A key finding which conceivably could have implications for the expression of PG-mediated biological activities in vivo was that the extent of complement consumption by gonococcal PG varied widely among individual normal human sera. Our data are consistent with (but do not prove) the contention that the intrinsic differences among these sera were related, at least in part, to the levels of natural antibody capable of reacting with gonococcal PG. In light of the ubiquity and structural relatedness of PGs of diverse bacteria, it would not be surprising that antibody capable of cross-reacting with gonococcal PG would be found in human sera. It has been noted previously that antibody against some gram-positive PGs (differing antigenically from gram-negative PG principally in the structure of the peptide moiety) is quite common, particularly in individuals suffering from one of several inflammatory disorders (3, 13, 22). We are currently determining if there is a correlation between the level of anti-PG antibody and the extent of PG-mediated complement consumption among these human sera. We are also comparing the ability of these sera to digest gonococcal PG enzymatically and thus to degrade the macromolecular PG into fragments that are inactive in complement consumption.

A principal stimulus for this work was that soluble PG fragments, released from gonococci by the action of host or bacterial PG hydrolases, may contribute to the intense inflammatory reactions associated with gonococcal infections. We previously reported (20, 25) that the majority of PG products released by growing gonococci in vitro were unusual derivatives possessing a nonreducing anhydro-muramyl end and having an apparent molecular weight equal to or less than that of bis-disaccharide peptide dimers (ca. 2,000). If similar free PG fragments are released in vivo, it seems likely, based on present data, that their small size precludes involvement in complement activation. On the other hand, gonococci growing in vivo may release large-molecular-weight free PG fragments (some of which may possess O-acetylated derivatives resulting in resistance of PG to degradation by lysozyme) (1) or complexes containing PG associated with other cell envelope components, e.g., protein (9). These PG fragments presumably would be available to react with natural anti-PG antibody and complement in a manner that may generate active mediators of inflammation.

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