Bacteremia and one of its most serious complications, the sepsis syndrome, are a leading cause of morbidity and mortality in this country, with an estimated 500,000 new cases reported each year (35) and a mortality rate that may exceed 35% (7). The actual mediators of the sepsis syndrome are host-derived cytokines, e.g., interleukin-1, interleukin-6, and tumor necrosis factor alpha (TNF-α), whose synthesis and release are induced by bacterial lipopolysaccharides (LPS) from gram-negative bacteria and by peptidoglycan (PG) and lipoteichoic acids from gram-positive bacteria (4, 5, 9, 11, 12, 23, 32). While there is substantial experimental evidence that release are induced by bacterial lipopolysaccharides (LPS) from gram-negative bacteria and by peptidoglycan (PG) and lipoteichoic acids from gram-positive bacteria (4, 5, 9, 11, 12, 24, 32). While there is substantial experimental evidence that PG fragments do contribute to cytokine induction resulting in shock (3, 12, 13, 23), there are limited reports documenting that soluble PG fragments are released from bacteria into the systemic circulation during bacteremia. In vitro, for example, PG fragments have been detected in cultures of *Staphylococcus* spp. grown in the presence of antibiotics (20, 37) and in culture filtrates from *Streptococcus pyogenes*, *Staphylococcus epidermidis*, and *Staphylococcus aureus* (6). In vivo, PG has been detected in the spleens (10, 30) and leukocytes (18, 19) of healthy humans, in the cerebrospinal fluid of patients with pneumococcal meningitis (17), and in the urine of patients treated with antibiotics (25, 38). Using a silkworm larva plasma test that reacts to both PG and fungal cell wall glucans, Kobayashi et al. (14) recently reported an absence of PG in the blood of healthy individuals and presented evidence that PG was present in the blood of >80% of tested patients with serious bacterial infections. Those authors suggested that their assay for PG could be developed into a diagnostic test for bacterial infection.

To determine if PG actually does occur in the blood of patients with bacteremia, we used the rationale that a more specific, and perhaps more sensitive, method of detection would utilize a monoclonal antibody (MAb) that specifically recognized PGs from both gram-positive and gram-negative bacteria. Should the presence of PG in the blood of patients with bacteremia be confirmed, the highly specific MAb might then be used in the development of a rapid diagnostic test for this potentially life-threatening infection. Here we report on the characterization of such an anti-PG MAb that was produced by immunizing mice with purified soluble PG derived from *Neisseria gonorrhoeae*. Purified soluble O-acetylated and non-O-acetylated PG fragments (O-PG and non-O-PG, respectively) from gram-negative bacteria were provided by Raoul Rosenthal (Indiana University School of Medicine, Indianapolis). Soluble PG from *S. aureus* was provided by Roman Dziarski (Indiana University School of Medicine, Gary). The PGs supplied by these labs were purified and characterized by them as described previously (29). O-PG and non-O-PG were hydrolyzed to un-cross-linked peptide monomers and peptide-cross-linked dimers, trimers, and tetramers by overnight incubation at 37°C with *N*-acetylglucosaminidase SG (hereafter referred to as muramidase) (also provided by Raoul Rosenthal) at a final concentration of 0.5 μg/ml as previously described (28, 33). Non-O-PG was digested overnight at 37°C with chicken egg white lysozyme (Sigma Chemical Co., St. Louis, Mo.) at a final concentration of 5 μg/ml in 0.05 M phosphate buffer, pH 7.2. Enzymatic activity was destroyed by heating the solutions at 70°C for 30 min before use in the enzyme-linked immunosorbent assay (ELISA) described below.

The procedures used for hybridoma development, maintenance, and MAb preparation were modified from those described previously (21, 22). BALB/c mice were administered four intraperitoneal injections of soluble O-PG derived from *N. gonorrhoeae* at 2-week intervals. The first injection contained 50 μg of O-PG in 0.2 ml of H2O derived with an equal volume of Freund’s complete adjuvant. The second injection contained 50 μg of O-PG in 0.2 ml of H2O mixed with an equal volume of Freund’s incomplete adjuvant. The final two injections each contained 25 μg of O-PG in phosphate-buffered saline without adjuvant. Hybridomas were produced by standard procedures modified from those described by Kohler (15). Culture supernatants were screened for the presence of
anti-PG antibody by the ELISA described below. Cells from wells yielding positive anti-PG reactions were cloned and expanded, and culture supernatants were retained for antibody collection. The isotype of the MAb described here (designated B10.G6) was determined to be immunoglobulin M (IgM) with a mouse antibody typing kit (The Binding Site, San Diego, Calif.). Concentrations of the IgM MAb were measured with a mouse radial immunodiffusion kit (The Binding Site).

The inhibition ELISA used here was modified from that described in our previous publication (22). ELISA plates (Costar, Corning, N.Y.) were coated overnight at 5°C with 0.5 μg of O-PG or non-O-PG per well in 0.1 ml of 50 mM carbonate buffer (pH 9.7). The wells were blocked with 20 mM TBS (Tris HCl, 500 mM NaCl [pH 7.5]), containing 1% bovine serum albumin. The MAb (1 to 3 μg per well in 0.1 ml of TBS containing 0.05% Tween 20 [TTBS]), with or without inhibitor PG, was then added and incubated at room temperature for 45 min. After the wells were washed with TTBS, binding of the MAb to the coating PG was detected by the addition of polyclonal antimouse antibody conjugated to peroxidase (diluted 1:2,000 in TTBS) (Sigma) and chromogen (0.5 mg of clonal antimouse antibody conjugated to peroxidase (diluted 1:2,000 in TTBS) (Sigma) and chromogen (0.5 mg of O-phenylenediamine per ml in 0.05 M Na-citrate buffer [pH 5.0] and H₂O₂ added to 0.005%). The reaction was stopped by the addition of 0.1 ml of 1 M H₂SO₄, and the absorbance measured at 490 nm in a multiwell plate reader. The Student t test (unpaired) was performed to assess statistical significance, and P values of <0.05 were considered to represent significant differences between values. All experiments were repeated on different days and yielded results comparable to those reported here.

In dose-response curves, determined in the absence of inhibiting PG, where the amount of anti-PG MAb increased and the amount of coating PG remained constant, maximum absorbance (~2.5 at 490 nm) occurred at a MAb concentration of 10 to 12 μg per well. The sensitivity of the inhibition ELISA was greatest, however, when a MAb concentration that yielded an absorbance of 0.4 to 1.0 was used. To achieve this, 2 to 3 μg of MAb per well was used in all experiments. Since some variability in the binding of the MAb to the coating PG occurred between different ELISA plates, direct comparisons of affinities for different inhibiting PGs were always conducted in the same plate.

Figure 1A shows a typical inhibition ELISA, where increasing concentrations of free O-PG and non-O-PG inhibited the binding of the MAb to O-PG-coated wells. At the lowest concentration shown (0.001 μg/well), there was significant inhibition of MAb binding to the coating PG (P < 0.01 for both O- and non-O-PG). There was no significant difference between inhibition by the O- or non-O-PG (P = 0.698 at 0.001 μg/well). As described previously (2, 26, 27, 33), O acetylation prevents lysozyme from degrading the glycan backbone. This is demonstrated in Fig. 1A, where at a PG concentration of 0.001 μg/well there was no significant difference in inhibition by the lysozyme-treated and untreated O-PG (P = 0.121), indicating that the PG remained intact. As shown in Fig. 1B, however, digestion of non-O-PG with lysozyme and digestion of both O-PG and non-O-PG with muramidase resulted in a significant reduction in PG binding to the MAb. This was apparent, for example, in that there was no significant inhibition of MAb binding to the coating PG by the digested PG at 0.01 μg/well (P > 0.200 compared to controls without inhibitor). As shown in Fig. 1A, that same concentration of intact PG resulted in complete inhibition of MAb binding to the coating PG. Higher concentrations of the muramidase- and lysozyme-digested non-O-PG did result in approximately 50% inhibition, indicating some low-affinity interaction of this digested PG with the MAb. Similar results were obtained when the ELISA wells were coated with non-O-PG (data not shown). These data indicated that an intact glycan chain was necessary for optimum binding of the MAb and that MAb binding to its epitope was not affected by O acetylation. The latter observation may indicate that the epitope recognized by this MAb is actually conformational. Gyorffy and Clarke (8) reported recently that a MAb that they developed to O-acetylated PG from Proteus mirabilis also bound to an epitope on the glycan backbone, but it bound poorly to de-O-acetylated PG. Our MAb appears to be unique in that both non-O-PG and O-PG are bound with apparent equal affinity.

We tested the ability of other microbial products to inhibit the binding of the anti-PG MAb to the coating PG. Figure 2A shows that neither LPS from Escherichia coli, LPS from Salmonella enterica serovar Minnesota, nor lipid A from S. enterica serovar Minnesota (all from Sigma) significantly inhibited the binding of the anti-PG MAb to the coating PG at any of the concentrations tested (P > 0.1 for all tested, compared to the control without inhibitor). Additionally, Fig. 2B shows
that fungal glucan (Sigma) also had no significant effect on MAb binding to the coating PG (P < 0.168 and 0.063 for 0.1 and 5.0 µg/well, respectively, compared to controls with no inhibitor). These data further support the contention that this MAb was specific for an epitope only on the PG molecule.

Figure 3 shows that this MAb reacted to a common epitope found on PGs from a diverse group of bacteria. At PG concentrations of 0.0005 µg/well, purified *N. gonorrhoeae* O-PG, *Bacteroides fragilis* PG, *Bordetella pertussis* PG, and *S. aureus* PG all significantly inhibited binding of the anti-PG MAb to the coating PG (P < 0.002 for all compared to the controls with no inhibitor). At this concentration of inhibiting antigen, *S. aureus* PG was significantly better than all of the other PGs at inhibiting MAb binding (P < 0.0005). This could have indicated the occurrence of more MAb-reactive epitopes per microgram of *S. aureus* PG than found on the PGs from the gram-negative bacteria.

These data collectively showed that our anti-PG MAb was unique compared to other anti-PG MAbs described in the literature. For example, Bahr et al. (1) reported on a MAb to synthetic muramyl dipeptide; however, whether the MAb recognized this epitope on intact soluble PG was not addressed. The MAb described by Gyorffy and Clarke (8) was reactive only to O-acetylated PG and not to de-O-acetylated PG from *P. mirabilis*. Those authors also reported that 20 to 40 µg of PG per ml was needed to yield 80 to 90% inhibition in their ELISA. That concentration was substantially higher than what was required in our assay to yield equivalent inhibition. The MAb described by Wergeland et al. (36) was very specific in that it reacted with the pentaglycines from only *S. aureus* and *S. epidermidis*. Likewise, Ziola et al. (39) have recently reported on the production of MAbs to the PGs from anaerobic gram-negative beer spoilage bacteria. These MAbs were again very specific for the PG associated with this group of bacteria. Additionally, Kool et al. (16) reported on the production of a MAb directed at the PG-polysaccharide complexes of intestinal bacteria. The specificity of the MAb and the nature of the epitope bound by this antibody, however, were not discussed. Finally, Shockman et al. (31) have patents on eight hybridomas that reportedly secrete MAbs to PG. There was, however, limited information about these antibodies available from the U.S. Patent and Trademark Office, and to our knowledge there have been no reports involving utilization of these MAbs published in the scientific literature.

Figure 4 shows that the anti-PG MAb recognized presumed PG fragments released after growth in minimal medium. For these experiments, the following bacteria (obtained from the American Type Culture Collection, Manassas, Va.) were grown 48 h at 37°C in medium composed of phosphate-buffered saline (pH 7.2) supplemented with (all wt/vol) 0.5% glucose, 0.1% (NH₄)₂SO₄, 0.01% MgSO₄·7H₂O, and 0.5% yeast extract (Difco Laboratories, Detroit, Mich.): *E. coli* (ATCC 4157), *Shigella sonnei* (ATCC 9290), *S. enterica* serovar Typhi-murium (ATCC 14028), *S. epidermidis* (ATCC 35984), *Klebsiella pneumoniae* (ATCC 13883), *Streptococcus agalactiae* (ATCC 13813), *P. mirabilis* (ATCC 7002), and *Enterococcus faecalis* (ATCC 29212). After growth for 48 h, the bacteria
were removed by centrifugation, and the resulting supernatants were filter sterilized using 0.45-μm-pore-size filters. A portion of each culture filtrate was heated at 70°C for 30 min to destroy enzymatic activity. As shown in Fig. 4, all of the culture filtrates significantly inhibited MAb binding to the coating non-O-PG (P ≤ 0.002 when all were compared to the no-inhibitor control). There was no significant inhibition by the minimal medium that had not been used to grow any bacteria (P = 0.461). There was also no significant reduction in the amount of inhibition by the culture filtrates that had been heated at 70°C (data not shown), indicating that any enzymatic activity contained in the filtrates was not affecting the results. It is interesting that culture filtrates from the gram-positive bacteria were more inhibitory than those from the gram-negative bacteria. This may have reflected a difference in the amounts of PG secreted into the medium. While we have not unequivocally shown that PG was the inhibiting material in the culture filtrates, the highly specific nature of our anti-PG MAb locally shown that PG was the inhibiting material in the culture of PG secreted into the medium. While we have not unequivocally shown that PG was the inhibiting material in the culture filtrates, the highly specific nature of our anti-PG MAb locally shown that PG was the inhibiting material in the culture of PG secreted into the medium. We are currently engaged in testing this hypothesis.

In conclusion, we have presented evidence that our MAb binds to an epitope on the PG backbone that is common to many gram-negative and gram-positive bacteria. From the studies with enzymatically digested PG it appears that the epitope is a macromolecular segment of the glycan chain that is unaffected by O acetylation. Since it is assumed that PG is released into the blood during bacterial infections (reviewed in reference 32), our anti-PG MAb may facilitate its detection and thus may be developed as an aid in the diagnosis of bacteremia. We thank Raoul Rosenthal for encouraging us to undertake this project and for his many helpful suggestions while we were conducting the experiments reported here.

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