Release of Gram-Negative Outer-Membrane Proteins into Human Serum and Septic Rat Blood and Their Interactions with Immunoglobulin in Antiserum to Escherichia coli J5

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Prior studies indicate that 3 bacterial outer-membrane proteins (OMPs) are released into serum associated with lipopolysaccharide (LPS) and are bound by IgG in antiserum to Escherichia coli J5 (anti-J5 IgG). The present studies analyzed the interaction of the OMPs with anti-J5 IgG and evaluated their release in an infected burn model of gram-negative sepsis. Affinity purification studies were performed on filtrates of bacteria incubated in human serum and plasma from rats with sepsis by use of O chain–specific anti-LPS IgG and anti-J5 IgG. All 3 OMPs were captured from septic rat blood by anti-LPS IgG. Release of OMPs into serum was highest for immature bacterial cultures and was increased by antibiotics in vitro and in vivo. Anti-J5 IgG selectively captured an 18-kDa OMP released into serum and into plasma from septic rats. The results raise the possibility that anti-J5 IgG may, in part, protect via anti-OMP antibodies.

The notion of treating gram-negative sepsis with antibody directed to conserved cell-wall components has been supported by many studies over the last 30 years that show that administration of polyclonal antiserum raised to rough mutant bacteria protects in gram-negative sepsis caused by heterologous gram-negative bacteria [1–6]. Although it has generally been assumed that immunoglobulins in antiserum to rough mutant strains such as Escherichia coli J5 and Salmonella minnesota Re595 protect by binding to lipopolysaccharide (LPS), we have found that IgG in these antiserum binds only weakly to LPS from heterologous gram-negative strains [7, 8]. The clinical failure of anti-LPS IgG in these antisera to protect in clinical studies has resulted in decreased interest in this approach.

We recently found that IgG in polyclonal antiserum raised to heat-killed E. coli J5 (anti-J5 IgG) binds to 3 conserved gram-negative bacterial outer-membrane proteins (OMPs). These OMPs are exposed on the surface of bacteria incubated in human serum and are released into human serum in complexes that also contain LPS [9]. This work suggested that some of the protection offered by this antiserum could be conferred by anti-OMP IgG.

Most prior work has focused on LPS as the dominant component of the cell wall that induces inflammation. The role of OMPs in gram-negative sepsis has not been extensively studied. The present studies were designed to further characterize the interaction of anti-J5 IgG with released bacterial OMPs; to study the effects of growth phase, bacterial capsule, and antibiotics on the release of the OMPs into human serum and exposure of the OMPs on the surface of serum-exposed bacteria; and to determine whether OMPs are released into the bloodstream in experimental gram-negative sepsis.

Materials and Methods

Bacteria. Bacterial strains included E. coli J5 (gift of J. C. Sadoff, Walter Reed Army Institute of Research, Washington, DC) and E. coli O4:K54:H7 (CP9), E. coli O6:K5:H1, E. coli O18:K1:H7 (designated E. coli O18K1:H7), E. coli O18K1–:G2A (a nonencapsulated derivative of O18K1:H7, designated E. coli O18K1–), and E. coli O25:K5:H1 (gifts of A. Cross, University of Maryland Cancer Center, Baltimore); Salmonella typhimurium (ATCC 14028); and Klebsiella pneumoniae (ATCC 10031). All bacteria were cultured in trypticase soy broth (TSB; Difco, Detroit) from colonies stored on trypticase soy agar (Difco). Bacteria were cultured at 37°C with vigorous agitation to the desired growth phase, harvested, and washed by low-speed centrifugation in sterile normal saline (5000–8000 g, 8–10 min, 4°C).

MAbs. MAbs to O-polysaccharide of E. coli O18 LPS was
produced by a mouse hybridoma (gift of A. Cross) [10]. This antibody does not cross-react with LPS from other organisms, with OMPs, or with components of normal human serum by immunoblotting. MAbs directed to the 3 OMPs bound by anti-J5 IgG were generated by immunizing BALB/c mice (Charles River Laboratories, Wilmington, MA) with a heat-killed, lyophilized E. coli J5 vaccine. The vaccine was resuspended in sterile normal saline (1 mg/mL), and increasing doses (0.1, 0.2, and 0.3 mg) were injected intraperitoneally 3 times a week for 3 weeks. Booster injections were given monthly for 1–3 months, with the final booster 3 days before spleen harvest. Splenocytes were harvested and fused with myeloma cells by standard laboratory protocols [11, 12]. Fused cells were cultured in Dulbecco’s modified Eagle medium (Cellgro Mediatech, Herndon, VA) supplemented with glucose (4.5 g/L), t-glutamine, 20% heat-inactivated fetal calf serum (Cellgro Mediatech), penicillin (100 U/mL), and streptomycin (100 µg/mL).

Antibodies were initially screened by bacterial ELISA with heterologous smooth gram-negative bacteria as the coating antigen and hybridoma culture supernatants as the primary antibody. Nine clones were selected for amplification in these studies on the basis of titers against heterologous gram-negative bacteria. The IgGs secreted by the clones were analyzed for binding to the 3 OMPs by immunoblotting using E. coli O25 bacterial lysates as antigen, as described elsewhere [9]. A membrane processor (MilliBlot-MP; Millipore, Bedford, MA) was used for application of primary antibody. Supernatants from fusions were used as primary antibody. Immunoblots were developed as described below, using avidin-biotin-peroxidase augmentation. Polyclonal mouse anti-J5 IgG was used as a positive control, and preimmune serum served as the negative control. Antibodies against the OMPs were selected on the basis of binding to bands in bacterial lysates that were the same molecular masses as the 3 OMPs bound by anti-J5 IgG [9]. To confirm that the MAbs were binding OMPs, we made immunoblots using outer membranes that were incubated with or without proteinase K before electrophoresis, as described elsewhere [9].

Retired BALB/c breeders were used for ascites production. Ten days after intraperitoneal instillation of 0.5 mL of Pristane (Sigma, St. Louis), 5–10 × 10⁶ hybridoma cells were collected and injected intraperitoneally after 2 washes in Hank’s balanced salt solution (Cellgro Mediatech). Ascites was then collected by aspiration every 2 or 3 days [12]. Anti-OMP antibodies showed no cross-reactivity with LPS or with components of normal human serum by immunoblotting.

Polyclonal antibodies. Sera and antisera were prepared from the blood of 2–3-kg New Zealand White rabbits (AR1 Breeding Laboratories, East Bridgewater, MA) and from the blood of BALB/c mice. Rabbit antisera to the vaccine of heat-killed E. coli J5 (J5 antiserum) was prepared from pooled sera from 10 rabbits as described elsewhere [7]. Blood was drawn 9 days after the last of 11 intravenous injections of increasing dosage over 4 weeks (0.1–1.6 mg/rabbit) [7, 9]. The specific anti-J5 LPS titer in rabbit J5 antiserum was 27.5 µg/mL. Mouse J5 antiserum was prepared from blood collected from mice immunized with E. coli J5 vaccine, as described above. The vaccine of E. coli O18 O-polysaccharide consists of the O-polysaccharide conjugated to Pseudomonas aeruginosa toxin A (gift of A. Cross) [13]. Antiserum to the O-polysaccharide of E. coli O18 LPS was prepared using 10 µg of vaccine per inoculation, as described elsewhere [9]. All sera were immediately prepared, aliquotted, and frozen (−80°C) until use. IgG in polyclonal rabbit antisera to the O-polysaccharide of E. coli O18 LPS does not cross-react with LPS from heterologous bacteria, OMPs, or components of normal human serum by ELISA or immunoblotting. IgG in polyclonal antisera to heat-killed E. coli J5 does not cross-react with purified E. coli O18 LPS [9] or with components of normal human serum by immunoblotting.

Preparation of serum. Sera from animals and healthy human volunteers were prepared from blood collected into sterile polypropylene tubes containing sterile glass beads to initiate clotting. After 2 h at room temperature, the clot was gently tapped off the side of the tubes, and the tubes were incubated another 1–2 h at 4°C. Tubes were centrifuged (1000 g, 10 min, 4°C), and the upper serum layer was removed to another tube. Sera were handled for preservation of complement and were either used immediately or stored at −80°C and thawed just prior to use.

Purification of IgG. IgG was purified from ascites after ammonium sulfate precipitation [12] and from hyperimmune serum [14, 15]. In brief, affinity chromatography was performed by passage over a protein G-Sepharose 4 fast-flow column (Pharmacia, Piscataway, NJ). Bound IgG was eluted from the column with 0.1 M glycine (pH 2.7) and was immediately neutralized with 1 M Tris buffer (pH 9.0). Purified IgG was dialyzed against PBS (pH 7.2) and stored at −80°C. Protein concentration was determined by ELISA [16] and by absorption at 280 nm.

Preparation of antibody-conjugated magnetic beads. The following IgGs were covalently conjugated to magnetic beads (BioMag amine-terminated 8-4100; Per Septive Diagnostics, Cambridge, MA) according to the manufacturer’s instructions and as described elsewhere [9]: murine monoclonal IgG directed against the O-polysaccharide of E. coli O18 LPS and an unrelated murine IgG1 (ATCC, Rockville, MD). IgG from rabbit antisera to E. coli O18 O-polysaccharide vaccine and to heat-killed E. coli J5, and IgG from normal rabbit serum (normal rabbit IgG). In brief, magnetic beads were activated by incubation in 5% glutaraldehyde, washed, and incubated with dialyzed IgG at 5 mg/mL IgG. Some 85%–95% of IgG covalently coupled to the beads [9].

Bacterial ELISAs. Bacterial ELISAs were performed as described elsewhere [9]. Bacteria were grown to the desired phase as determined by optical density (OD) at 550 nm (A₅₅₀), washed in sterile saline, suspended in serum or saline to an A₅₅₀ of 1.0, and incubated at 37°C for the specified time (10 min–1 h). The bacteria were washed by centrifugation (5000–8000 g, 8–10 min, 4°C) 3 times in sterile normal saline and resuspended in an equal volume of carbonate buffer, pH 9.6 (50 mM sodium carbonate; EM Science, Cherry Hill, NJ). Polyvinyl microtitrat plates (Dynatech Laboratories, Chantilly, VA) were coated with bacteria (10/µL) and incubated overnight at 4°C. The microtiter plates were then washed 3 times (PBS, 1 mg/mL Tween 20, 1 mg/mL bovine serum albumin [BSA], 2 mg/mL MgCl₂) blocked overnight at 4°C with PBS containing BSA (1 mg/mL), and washed again. Dilutions of either normal rabbit serum or rabbit antiserum to E. coli J5 were added, and the plates were incubated (2 h, 37°C). After 3 additional washes, horseradish peroxidase–conjugated anti-rabbit IgG (Cappel, Durham, NC) was added, and the plates were incubated (2 h, 37°C) and washed. Peroxidase substrate (1 mg/mL H₂O₂ in ABTS, citric acid, Na₃HPO₄) was added, plates were incubated at room temperature for 30 min, and the A₄₅₀ of the microtiter wells was
read (ELISA reader EAR-400; SLT Lab Instruments, Hillsborough, NC). Titers were determined by use of a standard curve as described by Zollinger and Boslego [16]. Known concentrations of rabbit IgG (Cappel) were used to generate standard curves. All assays were performed in duplicate; mean values are given.

**Immunoblotting.** Immunoblotting was used to detect binding of antisera and MAbs to washed bacteria (10^5/well) and bacterial antigens that were affinity-purified from filtrates of serum-exposed bacteria. All samples were prepared in sample buffer (2.5% SDS, 22% glycerol, 0.5% β-mercaptoethanol, and trace bromphenol blue in Tris base). Samples were electrophoresed on 16% SDS-polyacrylamide gels and transferred to nitrocellulose (Bio-Rad Laboratories, Hercules, CA) by applying 200 mA of constant current at 4°C for 1 h (Hoefer Scientific Instruments, San Francisco). For most experiments, the nitrocellulose was blocked (1 h at room temperature or overnight at 4°C) with 1% powdered skim milk in TTBS (150 mM NaCl, 50 mM Tris, 0.1% Tween-20, pH 7.5), washed for 10–15 min with TTBS, washed, and incubated with primary antibodies, and washed 3 times. Primary antibodies included IgG in rabbit antisera to heat-killed *E. coli* 015 and *E. coli* O18 O-polysaccharide (both diluted 1:500 in TTBS), IgG in mouse antisera to heat-killed *E. coli* 015, and murine MAbs directed to each of the 3 OMPs (at 1 μg/mL). Blots were then incubated for 30 min with biotin-conjugated anti-rabbit or anti-mouse IgG antibody (Vectastain; Vector Laboratories, Burlingame, CA) diluted 1:240 in TTBS, washed, and incubated for 30 min in a mixture of avidin and biotinylated horseradish peroxidase complex, as described in the manufacturer’s instructions (Vectastain). After a final wash with PBS, peroxidase substrate was added (2 mL of 3 mg/mL 4-chloro-1-naphthol, 8 μL of PBS, 10 μL of 30% H_2O_2). The reaction was stopped after 30 min by repeated rinsing with distilled water.

To increase sensitivity, we used a chemiluminescence method to develop immunoblots of samples prepared from plasma from rats with sepsis. Nitrocellulose was blocked with 5% powdered skim milk in TTBS, washed, and incubated with primary and secondary antibodies and with avidin-biotin-peroxidase as described above. Blots were then rinsed 3 times with TTBS and developed by use of equal volumes (1–2 mL each) of enhanced luminol and oxidizing reagents (Renaissance chemiluminescence reagents; NEN Life Sciences Products, Boston). Film (reflection autoradiography; NEN Life Sciences Products) was exposed for 30 s to 1 min. Molecular masses (kilodaltons) indicated in the figures are estimates on the basis of the migration of prestained markers and unstrained proteins of known molecular mass.

**Effects of antibiotics on release of OMP-containing bacterial components into serum.** *E. coli* O18K+ bacteria were washed and suspended in human serum or saline with or without the cell-wall–active β-lactams, ampicillin, cefazidime, and imipenem, each at a concentration of 200 μg/mL. Samples were incubated with end-over-end rotation (2 h, 37°C), and filtered through 0.45-μm filters (Acrodisc; Gelman Sciences, Ann Arbor, MI) to remove intact bacteria. Filtrates were then incubated with anti-LPS (O-polysaccharide chain specific) and anti-J5 IgG-conjugated magnetic beads and processed as described below.

**Effects of bacterial growth phase.** ELISA and affinity purification experiments were performed to study the effects of bacterial growth phase on release of OMP-containing bacterial components into serum and exposure of previously inaccessible bacterial surface components by human serum. Overnight cultures of bacteria were diluted 50-fold into TSB, which resulted in a starting A_{550} of 0.1. The A_{550} of the culture was measured every 20–30 min, and samples were withdrawn when the culture reached the specified ODs. After a wash in sterile normal saline, the A_{550} of each sample was adjusted to 1.0 (bacterial ELISA) or 0.8 (affinity purification experiments) to ensure similar bacterial concentrations at the beginning of serum exposure. Bacteria were pelleted, resuspended in human serum (the same volume used to adjust the A_{550} to 0.8 or 1.0), and incubated at 37°C with gentle agitation for the specified period of time. Bacterial ELISA samples were processed as described above. For affinity purification experiments, samples were filtered to remove intact bacteria, and filtrates were incubated with anti-LPS (O-polysaccharide chain specific) and anti-J5 IgG-conjugated magnetic beads and processed as described below. In some experiments, quantitative bacterial cultures were performed on samples before and after adjustment of A_{550} and after serum exposure. Quantitative cultures for colony-forming units per milliliter (cfu/mL) were obtained by serial 10-fold dilutions in sterile normal saline. We spread 100 μL of each dilution on TSB plates, which were incubated overnight at 37°C. Bacterial colonies were counted the next day.

**Affinity purification from sterile filtrates of serum-exposed bacteria.** Affinity purification studies were performed on filtrates of serum-exposed bacteria that were prepared as described above. Antibodies for affinity purification studies included anti-J5 IgG, normal rabbit IgG, rabbit and mouse IgG against the O-polysaccharide side chain of *E. coli* O18 LPS (O-chain–specific IgG), and an unrelated control mouse IgG1. Each sample (100 μL) was diluted 5-fold into antibody-conjugated beads that had previously been washed and resuspended in 400 μL of PBS (final IgG concentration, 100 μg/mL). Reaction mixtures were then incubated for 16–20 h at 4°C with end-over-end mixing. The antibody-conjugated beads with attached antigens were then separated from the serum by placing the tubes in a strong magnetic field, and the beads were washed 3 times with PBS. Antigen was eluted by heating the beads (5 min, 100°C) in 100 μL of SDS-PAGE sample buffer (2.5% SDS, 22% glycerol, in Tris base). After supernatants were carefully separated from the beads, β-mercaptoethanol (0.5%) and trace bromphenol blue were added. Each sample (20 μL) was electrophoresed on 16% SDS–polyacrylamide gels, transferred to nitrocellulose, and analyzed by immunoblotting. Primary antibodies for immunoblots included rabbit anti-J5 IgG, rabbit anti-O chain–specific IgG, or a mixture of murine MAbs directed against each of the 3 OMPs.

**Model of gram-negative sepsis in burned rats.** Release of OMPs was studied in an infected rat burn model adapted from a murine sepsis model [17]. Male Sprague-Dawley rats weighing 225–250 g were anesthetized with ether (Sigma) and subjected to a 15% total body surface area full-thickness burn by application of heated brass bars (100°C, 15 s). Rats were then inoculated by subcutaneous injection of *E. coli* O18K+ (10–100 cfu) into the burned area. Control rats received the burn but were inoculated with saline instead of bacteria. At 72 h, all rats that were inoculated with bacteria were bacteremic. Blood cultures from saline-inoculated rats were sterile. All rats were given an intravenous dose of cefazidime (80 mg/kg) at 72 h via the tail vein. Blood was collected into 5 mM EDTA (to prevent coagulation) by cardiac puncture 3 h later and diluted 4-fold with PBS. Plasma was prepared by centrifugation.
(200 g, 5 min, 4°C) and then filtered (0.45 μm) to remove intact bacteria.

**Affinity purification from sterile filtrates of septic rat plasma.** Filtered plasma from rats with sepsis was incubated with magnetic beads covalently conjugated with normal rabbit, anti-J5 IgG and anti-O chain–specific IgG. Antibody-conjugated beads were washed and resuspended in 500 μL of filtered rat plasma (final IgG concentration, 100 μg/mL), incubated overnight, and washed with PBS. Antigen was eluted by heating beads in 50 μL of SDS-PAGE sample buffer, and samples were further processed as described above. After 20 μL of each sample was electrophoresed on 16% gels and transferred to nitrocellulose, captured bacterial antigens were assessed for the 3 OMPs by immunoblotting, using a mixture of murine MAbs directed against each of the 3 OMPs as the primary antibody.

**Results**

**Production of anti-OMP MAbs from mice immunized with E. coli J5 vaccine.** Our prior studies indicated that anti-J5 IgG binds 3 OMPs of 35, 18 (previously estimated as 37 and 24 kDa, respectively [9]), and 5–9 kDa that are present on the bacterial surface and are released into human serum as OMP-LPS complexes. Hybridoma cell lines producing MAbs against each of the 3 OMPs were developed by fusing lymphocytes from mice immunized with E. coli J5 vaccine. Of the 10 splenic fusions, 9 IgG antibodies were identified that bound to the surface of heterologous serum-exposed bacteria by ELISA (data not shown). Immunoblot analysis was then done to identify the epitopes bound by these antibodies. Lysates of E. coli O25 or O6 bacteria were used as antigen. Immunoblots were stained with mouse anti-J5 IgG and the monoclonal IgGs. Seven of the 9 IgGs bound to 1 of 3 bands of 35, 18, and 5–9 kDa. Three of these MAbs (2D3, 6D7, and 1C7) were selected for increased production, each with specificity for 1 of the 3 bands. Figure 1 is a representative immunoblot of lysates of E. coli O6 stained with the 3 monoclonal and polyclonal anti-J5 IgGs.

The pattern of staining of the 5–9-kDa OMP by 1C7 and IgG in J5 antiserum raised the question of whether the antibodies were directed to the core region of LPS. No bands were detected on immunoblots when purified, protein-free E. coli O18 LPS was used as antigen and anti-J5 IgG or 1C7 was used as staining antibody (data not shown). In addition, the 5–9-kDa band disappeared when purified outer membranes were digested with proteinase K before electrophoresis and staining with anti-J5 IgG [9] or with 1C7 (data not shown). These results indicate that protein is required for binding of these IgGs to the 5–9-kDa band. Given the tight association between LPS and some OMPs, it is possible that the 5–9-kDa band also contains LPS [18].

**Anti-J5 IgG affinity-purifies an 18-kDa OMP from filtrates of serum-exposed bacteria.** To further characterize the interaction of anti-J5 IgG with bacterial components released into human serum, filtrates of heterologous smooth strains of serum-exposed bacteria were incubated with anti-J5 IgG and normal rabbit IgG that were covalently bound to magnetic beads. Bound antigens were analyzed for the 3 OMPs by use of a mixture of the 3 MAbs against the OMPs. There was heavy staining of the 18-kDa OMP on immunoblots prepared from E. coli O6 as antigen. Primary antibodies for immunoblots included polyclonal mouse anti-J5 IgG (lane 1) and 3 separate MAbs, 2D3, 6D7, and 1C7 (lanes 2–4), derived from mice immunized with E. coli J5 vaccine. Left, estimated molecular mass markers.
Effects of antibiotics on release of LPS-associated OMPs in vitro. We previously determined that complexes containing LPS and the 3 OMPs bound by anti-J5 IgG (OMP-LPS complexes) are released by *E. coli* O18 into human serum and are affinity-purified by using IgG directed against the O-polysaccharide side chain of *E. coli* O18. To study the effects of antibiotics on release of the 3 OMPs, *E. coli* O18K was incubated in human serum with and without ampicillin, ceftazidime, or imipenem and filtered and incubated with anti-O chain-specific IgG and anti-J5 IgG conjugated to magnetic beads. Captured antigens were assayed by immunoblotting. Release of OMP-LPS complexes was enhanced by antibiotics, as indicated by the presence of the 3 OMP bands on immunoblots of samples captured by anti-O chain-specific IgG (figure 4). Similar antibiotic-enhanced release was not as apparent for the 18-kDa OMP captured by anti-J5 IgG, although there was light staining of the 5-9- and 35-kDa OMPs in samples treated with imipenem (figure 3). In addition, capture of the 18-kDa OMP was markedly reduced in samples incubated in saline instead of serum prior to affinity purification, even in the presence of antibiotics (figure 3).

Effects of bacterial growth phase on release of OMP- and LPS-containing bacterial fragments into human serum. Affinity purification studies were performed to determine whether the growth phase of the bacterial culture affects release of the OMP-LPS complexes and separate release of the 18-kDa OMP. *E. coli* O18 were grown to different ODS, incubated in human serum, and filtered. Filtrates were then incubated with monoclonal anti-O chain–specific IgG and anti-J5 IgG. A representative immunoblot using anti-O chain–specific IgG as the antibody for affinity purification is shown in figure 5. For the encapsulated strain, *E. coli* O18K, capture of OMP-LPS complexes by anti-O chain–specific IgG was greatest during early to midlogarithmic growth. There was much less staining of both LPS and the 3 OMPs on immunoblots of samples prepared from filtrates of serum-exposed bacteria at an A500 >0.6. An effect of growth phase on release of LPS-OMP complexes was not apparent for the nonencapsulated strain, in which there was heavy staining of the OMPs and LPS in samples prepared from filtrates of serum-exposed *E. coli* O18K bacteria at all ODS (data not shown). The results suggest that capsule may also play a role in release of complexes into serum. Immunoblots of antigens captured by anti-J5 IgG indicate that release of the 18-kDa OMP is also influenced by the growth phase of the bacterial culture. A similar cutoff was observed at A500 0.4-0.8, with heavier staining of the 18-kDa OMP at lower ODS (data not shown). Culture data indicate that the differences in release of LPS-OMP fragments into serum by the encapsulated strain cannot be explained by either greater proliferation of bacteria or increased death of more immature cultures (figure 5).

Effect of bacterial growth phase and duration of serum exposure on binding of anti-J5 IgG to serum-exposed bacteria. We previously reported that anti-J5 IgG titers were higher against bacteria incubated in serum than against bacteria incubated in saline [9]. We examined by ELISA the effects of growth phase, capsule, and duration of serum exposure on binding of anti-J5 IgG to serum-exposed *E. coli* O18 bacteria. To ensure equivalent starting bacterial concentrations in experiments examining growth phase, all samples were adjusted to the same OD prior to serum exposure. For the encapsulated strain, anti-J5 IgG bound better to bacteria grown to a lower initial OD. The effect of growth phase was evident after 10 and 20 hours of growth.
of bands. were not detected in filtrates incubated with control beads conjugated or stationary growth phases. A similar effect of growth phase immature cultures compared with bacteria in late logarithmic 60 min of serum exposure, with higher binding to bacteria from serum exposure of buried outer membrane epitopes (data not shown). Perhaps the strongest evidence that anti-LPS antibody might 60 min of serum exposure, with higher binding to bacteria from immature cultures compared with bacteria in late logarithmic or stationary growth phases. A similar effect of growth phase was observed for the nonencapsulated mutant, E. coli O18K, at short serum exposure times. However, by 60 min of serum exposure, anti-J5 IgG titers were equivalent for immature and mature bacterial cultures. Titers were consistently higher against the nonencapsulated than against the encapsulated strain, suggesting that the capsule may confer some resistance to serum exposure of buried outer membrane epitopes (data not shown).

Affinity purification of OMPs from septic rat plasma. A burn model of infection was used to study release of OMPs in experimental gram-negative sepsis in rats. This model was adapted from a murine infected burn model [17]. Plasma was obtained from burned rats with E. coli O18K sepsis before and 3 h after administration of ceftazidime. Plasma was filtered and affinity-purified on anti-J5 or anti-O chain–specific IgG, using normal rabbit IgG as the negative control as above. Captured antigens were analyzed by immunoblotting, using anti-OMP MAbs as the primary antibodies (figure 6). All 3 OMPs were detected in samples that were affinity-purified by anti-O chain–specific IgG in 5 of 11 rats, and at least 1 or 2 bands were present in 8 of 11 rats. The 18-kDa OMP was present in samples affinity-purified with anti-J5 IgG in 9 of 11 rats. In 3 of 11 rats, there was also some capture of the 5–9- and 35-kDa OMP by anti-J5 IgG. The OMPs were not detected in samples that were affinity-purified from filtered plasmas from control rats that were burned and inoculated with saline instead of bacterium (data not shown). Immunoblots using plasma from selected septic rats verified that LPS was present in samples that were affinity-purified by use of anti-O chain–specific IgG (data not shown).

Discussion

In 1968, Chedid et al. [1] reported that gram-negative bacteria share common outer membrane antigens that are exposed by incubation in serum and are bound by antisera raised to rough mutant bacteria. Subsequently, antisera raised to heat-killed rough mutant bacteria (e.g., E. coli J5, S. minnesota Re595) were shown to protect in animal models and clinical studies of sepsis [1–6]. These antisera were hypothesized to protect by antibody binding to conserved core components of LPS (lipid A and core oligosaccharide). However, there has been no direct evidence that anticore MAbs protect, except for MAb WN1 222-5, which binds core structures of LPS from heterologous enteric gram-negative bacteria and protects against endotoxin challenge in rabbits and mice [19]. In addition, it has been difficult to directly demonstrate substantial increased binding to LPS from heterologous gram-negative bacteria by the immunoglobulins in polyclonal antiserum to E. coli J5 [7, 8]. Perhaps the strongest evidence that anti-LPS antibody might be involved comes from experiments in which passive immunization with immunoglobulin directed against a vaccine consisting of decacylated E. coli J5 LPS protected in a neutropenic model of sepsis [20]. Nonetheless, although antisera raised to heat-killed rough strains have been reported to protect, the exact mechanism by which this protection occurs remains elusive [21].

Our prior studies indicate that antisera raised to heat-killed E. coli J5 bacteria contains high titers of IgGs that bind 3 conserved OMPs (5–9, 18, and 35 kDa) that are released from bacteria incubated in human serum. These data raised the question of whether anti-OMP antibodies in J5 antiserum could be responsible for some of the protection that has been ascribed to antibodies directed to the LPS core. The present studies indicate that the 18-kDa OMP is selectively affinity-purified by anti-J5 IgG from filtrates of heterologous gram-negative bacteria incubated in human serum and from the blood of burned rats with E. coli O18K sepsis.

The growth phase of the majority of bacteria and the structure of bacterial cell-wall components in tissues in an evolving infection are not known. We found that the growth phase of the bacterial culture of E. coli O18K influences subsequent serum exposure of bacterial surface components and release of the OMPs into human serum. Binding of anti-J5 IgG to serum-exposed E. coli O18K in early logarithmic growth was higher

Figure 4. Immunoblot analysis of serum-released outer-membrane protein (OMP)–lipopolysaccharide (LPS) fragments. E. coli O18K were incubated in human serum without antibiotics (lane 1) or with ampicillin (lane 2), ceftazidime (lane 3), or imipenem/cilastatin (lane 4) and filtered. LPS was then affinity-purified from filtrates by use of magnetic O-polysaccharide–specific anti-E. coli O18 LPS IgG conjugated to magnetic beads. Antigens eluted from beads were immunoblotted, using mixture of murine monoclonal IgGs (2D3, 6D7, and 1C7) directed against each of the 3 OMPs as staining antibodies. Bands were not detected in filtrates incubated with control beads conjugated with normal rabbit IgG (not shown). Left, estimated molecular masses of bands.
than binding to late logarithmic growth or in stationary phase bacteria, as assessed by ELISA. Similarly, release of OMP-LPS complexes and the separate release of the 18-kDa OMP from *E. coli* O18K+ was greater for bacteria in early logarithmic than in late logarithmic growth. These data are consistent with prior reports of higher anti-J5 IgG titers to bacteria at earlier growth phases [22]. The findings parallel reported maximal release of LPS (as opposed to OMPs) into serum from bacteria harvested from logarithmic cultures as opposed to stationary-phase bacteria [23]. The presence of capsule also appeared to influence binding of anti-J5 IgG to serum-exposed bacteria and release of the OMPs in our in vitro studies. For the nonencapsulated strain, *E. coli* O18K+, maximum anti-J5 IgG titers were higher and growth phase did not appear to influence anti-J5 IgG titers to whole serum-exposed bacteria or release of OMPs into serum.

Our data indicate that antibiotics increase the release of the 18-kDa OMP into human serum in the OMP-LPS complex form. Separate release of the 18-kDa OMP that is affinity-purified by anti-J5 IgG appears to be enhanced by antibiotic administration in the infected burn model. Although there are multiple reports of antibiotic-induced release of LPS in vitro and in experimental gram-negative sepsis [24–29], we do not believe that the effects of antibiotics on release of OMPs have been
studied. The 18-kDa OMP was barely detectable in filtrates of bacteria incubated in saline, indicating that serum enhances release of this OMP.

It is not clear whether the OMPs are released by dying bacteria, shed from the surface of proliferating bacteria, or both. Release from dying bacteria is suggested by our data indicating that antibiotics enhance release of the OMPs, whereas the growth phase data suggest that OMPs may be shed during logarithmic growth. Other investigators reported that bacteria in logarithmic growth in culture media release blebs containing LPS and protein into the culture supernatant, supporting the notion that OMP-LPS complexes may be released by proliferating bacteria [30, 31]. It is unclear whether our serum-released OMP-LPS complexes are structurally and compositionally similar to blebs released into bacterial culture supernatants.

Circulating bacterial toxins are believed to be important in the pathogenesis of gram-negative sepsis, but little is known about the composition of released bacterial components. Most studies have focused on release of LPS [32–41], and it has been assumed that LPS is released in membrane blebs that then disaggregate into LPS monomers [42]. Prior studies showed that live bacteria incubated in human serum release fragments containing OMPs and LPS (OMP-LPS complexes) that can be affinity-purified with antibodies directed to the O-polysaccharide side chain of LPS [9, 43]. Freudenberg et al. [43] reported that samples that were affinity-purified from filtrates of serum-exposed Salmonella abortus equi by use of anti-LPS IgG also contained OmpA and a second 17-kDa protein that was not identified. The current studies extend these findings and indicate that OMP-LPS complexes that contain at least 3 OMPs are released in vivo into the bloodstream in an infected burn model of gram-negative sepsis. The 18-kDa OMP is also released into septic rat blood in a form separate from the OMP-LPS complex and is selectively affinity-purified by IgG in antisera raised to heat-killed Escherichia coli J5.

Although many studies report that proteins that are tightly associated with LPS are biologically active [44–61], the role of OMPs in the pathogenesis of sepsis has not been defined. Given the results of these studies and the previously described protective efficacy of J5 antisera, our data raise the question as to whether released OMPs, such as the 18-kDa OMP, could play a role in the pathogenesis of gram-negative sepsis.

References


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