Outer Membrane Protein A, Peptidoglycan-Associated Lipoprotein, and Murein Lipoprotein Are Released by *Escherichia coli* Bacteria into Serum

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Complexes containing lipopolysaccharide (LPS) and three outer membrane proteins (OMPs) are released by gram-negative bacteria incubated in human serum and into the circulation in an experimental model of sepsis. The same OMPs are bound by immunoglobulin G (IgG) in the cross-protective antiserum raised to *Escherichia coli* J5 (anti-J5 IgG). This study was performed to identify the three OMPs. The 35-kDa OMP was identified as outer membrane protein A (OmpA) by immunoblotting studies using OmpA-deficient bacteria and recombinant OmpA protein. The 18-kDa OMP was identified as peptidoglycan-associated lipoprotein (PAL) based on peptide sequences from the purified protein and immunoblotting studies using PAL-deficient bacteria. The 5- to 9-kDa OMP was identified as murein lipoprotein (MLP) based on immunoblotting studies using MLP-deficient bacteria. The studies identify the OMPs released into human serum and into the circulation in an experimental model of sepsis as OmpA, PAL, and MLP.

Bacterial cell wall components released into the bloodstream are believed to be important in the pathogenesis of gram-negative sepsis. Although prior investigators have reported that bacteria release lipopolysaccharide (LPS) into serum and into the circulation (4, 18, 56, 66), the full composition of released bacterial products has not been established. Very little is known about release of non-LPS gram-negative outer membrane components such as outer membrane proteins (OMPs) in sepsis. Fragments containing LPS, OmpA, and another faintly staining protein, of 17 kDa, were affinity purified from filtrates of human serum incubated with *Salmonella enterica* serovar Abortus equi bacteria using O-chain-specific anti-LPS immunoglobulin G (IgG) (20). Similarly, we have affinity purified complexes containing LPS and at least three OMPs, with estimated molecular masses of 35, 18, and 5 to 9 kDa, from filtrates of normal human serum incubated with *Escherichia coli* bacteria, using O-chain-specific anti-LPS IgG (29, 30).

Previous studies indicated that passive and active immunity directed to rough mutant bacteria such as *S. enterica* serovar Minnesota Re595 and *E. coli* J5 protect in experimental and clinical gram-negative sepsis (1, 5, 11, 42, 43, 68). Protection has been attributed to antibodies directed to conserved core components of LPS (lipid A and core oligosaccharide). However, it has been difficult to prove that antisera to rough strains of bacteria contain cross-reactive anti-lipid A or anti-core oligosaccharide IgGs (15, 57), and the exact mechanism of protection remains unclear and controversial.

We have demonstrated that IgG in antiserum raised to heat-killed *E. coli* J5 (J5 antiserum) binds to the same three gram-negative bacterial OMPs that are released into serum in the OMP-LPS complexes described above (30). OMP-LPS complexes are also released into the bloodstream of burned rats with *E. coli* O18K7 sepsis (29). In addition, at least one OMP, with an estimated molecular mass of 18 kDa, is released from bacteria separately from the OMP-LPS complexes and in a form that is selectively affinity purified from human serum and septic rat plasma by IgG in J5 antiserum (29).

This study was performed to identify the 35-, 18-, and 5- to 9-kDa OMPs that are released in vitro into human serum (30) and in vivo into the circulation in experimental gram-negative sepsis (29) and are bound by IgG in J5 antiserum.

**MATERIALS AND METHODS**

**Bacterial strains, media, and growth conditions.** *E. coli* J5 was a gift from J. C. Salsford (Walter Reed Army Institute of Research, Washington, D.C.); *E. coli* O18K1:H7 (designated *E. coli* O18K1’), *E. coli* O18K1:G2A (a nonencapsulated derivative of O18K1:H7, designated *E. coli* O18K1’), *E. coli* O8K45:H1, *E. coli* O18K1:H46, and *E. coli* O25K5:H1 were gifts from A. Cross (University of Maryland Cancer Center, Baltimore). OMP-deficient *E. coli* K12 and *E. coli* O18 mutants and closely related OMP-containing bacteria were used for immunoblotting studies. *E. coli* O18 E91 (OMP-deficient derivative of *E. coli* O18: K1:H7) and E69 (OMP-restored derivative of *E. coli* O18:K1:H7) were generated as previously described (52). *E. coli* K12 1292 (39), JC7752 (peptidoglycan-associated lipoprotein [PAL]-deficient derivative of 1292), and 7752(p417) (PAL-restored mutant of JC7752) were kindly provided by J-C. Lazzaroni (Université Claude Bernard, Lyon, France). *E. coli* K-12(p400), CH202 (PAL-deficient mutant of *E. coli* K-12(p400)), and CH202(pRC2) (PAL-restored derivative of CH202) were kindly provided by U. Henning (Max-Planck-Institut für Biologie, Tübingen, Germany) (12). The *E. coli* K-12 mutant that lacks murein lipoprotein (MLP; Braun’s lipoprotein) due to the deletion of the *lpo* gene, JES505 (F’ lpo his proA argE thl gal lac xyl mtl tet), and its otherwise identical *lpo*-positive partner that contains MLP, JES506 (F’ lpo his proA argE thl gal lac xyl mtl tet), were kindly provided by H. Nikaido (University of California, Berkeley) (32). Bacteria were cultured in trypticase soy broth (Difco, Detroit, Mich.) from colonies stored on Trypticase soy agar (Difco). Media were supplemented with kanamycin (50 mg/ml) for *E. coli* K-12 CH202(pRC2) and ampicillin (100 mg/ml) for *E. coli* K-12 JC7752(p417) to maintain the plasmids. 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 (5,000 to 8,000 × g, 8 to 10 min, 4°C).

**Monoclonal antibodies.** Monoclonal antibodies were prepared against each of the three OMPs bound by IgG in J5 antiserum and against the O-polysaccharide of *E. coli* O18K7 LPS. For production of anti-OMP monoclonal antibodies, BALB/c mice (Charles River Laboratories, Wilmington, Mass.) were immunized with heat-killed, glyophilized *E. coli* J5 vaccine prepared as described elsewhere (57).
Vaccine was resuspended in sterile normal saline (1 mg/ml). Increasing doses (0.1, 0.2, and 0.3 mg) were injected intraperitoneally three times per week for 3 weeks. Booster injections were given monthly for 1 to 3 months, with the final boost given 10 min, 4°C). Sera were either used immediately or frozen (−80°C) until use. IgG in polyclonal rabbit antisera to heat-killed E. coli 35 (J5 antiseraum) was prepared from pooled blood from 10 rabbits as previously described (38, 57). Murine antisera against E. coli 35 cells was prepared from animals immunized with 10 mg of O-polysaccharide consisting of the purified O-polysaccharide conjugated to toxin A of E. coli collected from mice immunized as described above. Monoclonal antibodies against the 18-kDa OMP were covalently conjugated to cyanogen bromide-activated Sepharose 4B beads (Pharmacia) according to the manufacturer’s instructions.

Immunoblotting. Immunoblotting was used to detect binding of antisera and monoclonal antibodies to lysates of bacteria (106/well), samples collected during the purification of the 18-kDa OMP, and bacterial antigens that were affinity purified from filtrates of human serum incubated with bacteria. All samples were prepared in sample buffer (2.5% sodium dodecyl sulfate [SDS], 2.0% 4-(2-aminoethyl)benzenesulfonic acid, and trace bromophenol blue in Tris base), electrophoresed on SDS–15% polyacrylamide gels, and transferred to nitrocellulose (Bio-Rad Laboratories, Hercules, Calif.) by applying 200 mA of constant current at 4°C for 1 h (Hoefer Scientific Instruments, San Francisco, Calif.). The nitrocellulose was blocked with 1% milk in TBS, incubated with primary antibodies in TBS, and washed. Primary antibodies included rabbit or mouse antisera (diluted 500- to 1000-fold) and murine monoclonal antibodies to each of the three OMPs at a concentration of 1 mg/ml, or polyclonal anti-O18 IgG (diluted 500-fold). Blots were then incubated for 30 min with biotin-conjugated anti-rabbit or anti-mouse IgG antibody ( Vectastain; Vector Laboratories, Burlingame, Calif.) diluted 1:200 in TBSB, washed, and then incubated for 30 min in a mixture of avidin and biotinylated horseradish peroxidase complex, as specified by the manufacturer (Vectastain). After a final wash with PBS, peroxidase substrate (2 ml of 4-chloro-1-naphthol, 3 mg/ml), 8 ml of PBS, 10 mM of 30% H2O2 was added. The reaction was stopped after 20 to 30 min.

Purification of the 18-kDa OMP. The final purification procedure for the 18-kDa OMP consisted of (i) preparation of total bacterial membranes, (ii) Triton X-100 extraction of bacterial membranes, (iii) affinity chromatography using Sepharose beads conjugated with 6×7 (the anti-18-kDa OMP monoclonal antibody), and (iv) reverse-phase high-performance liquid chromatography (HPLC) separation. Details of the purification steps are described below.

Total bacterial membranes were prepared from mid-late-log-phases cultures of E. coli O18K as essentially described as previously indicated (30, 49). Unless otherwise indicated, all steps were performed at 4 to 6°C. Two-liter cultures of bacteria were harvested by centrifugation, and the resultant pellets were resuspended in a total of 60 ml of prechilled 10 mM HEPES buffer (pH 7.4) with 25% sucrose (wt/vol) and 0.2 mM dithiothreitol (Fisher Biochemicals, Fair Lawn, N.J.). Lysate was prepared by addition of 0.5 ml of a prechilled 60 mM NaCl solution to each added to a final concentration of 4 µg/ml. Cells were disrupted by sonicating the suspension on ice (microtip, 30- to 60-s bursts separated by 60 to 90 s, total sonication time of 4 min). Unbroken bacteria and other debris were removed by centrifugation (10,000 × g, 40 min). The supernatant (0.1 ml) was added to each of 2-cm diameter wells containing EDTA (25 mM), and dithiothreitol (0.2 mM) was added to the 60 ml to adjust the concentration of sucrose to 20% (wt/vol) and the concentration of EDTA to 5 mM. Samples were layered onto a 60% sucrose cushion at 7.5 g/ml per 4.5 ml of cushion, and ultracentrifuged (100,000 × g, 2 h, 4°C). Bacterial membranes present in the hazy yellow/brown band at the interface were collected by puncturing the side of the tube with a 20-gauge needle and aspirating with a 1-ml syringe (approximately 0.5 ml/tube, final volume, 5 ml). Total bacterial membranes were then analyzed and ultracentrifuged in a 60-mM Tris-HCl (pH 7.5) buffer and the final volume of dialyzed material was approximately 15 ml per 2 liters of the starting bacterial culture.

Sixty milliliters of dialyzed total membranes representing liters of the starting bacterial culture was concentrated to 36 ml using a nitrogen pressurized system and a Diaflow ultrafiltration membrane, YM30 filter (Millipore Company, Danvers, Mass.), and extracted with Triton X-100. Twelve milliliters of 10% Triton X-100 in Tris-HCl (20 mM, pH 8.4) with the protease inhibitor 4-(2-aminoethyl)-benzenesulfonyl fluoride (0.2 mM) was added to the 60 ml containing EDTA (25 mM) and dithiothreitol (0.2 mM) was added to the 60 ml to adjust the concentration of sucrose to 20% (wt/vol) and the concentration of EDTA to 5 mM. The sample was incubated at room temperature for 30 min and then ultracentrifuged (TH6411 swinging-bucket rotor, 100,000 × g, 2 h, 4°C). The resultant supernatant (48 ml) was circulated overnight at 9 to 10 ml/h through a 5.5-ml column of mouse monoclonal IgG directed against the 18-kDa OMP covalently conjugated to cytochrome c-activated Sepharose 4B beads (4°C). The column was washed (36 ml, 2.5% Triton X-100 in 200 mM sodium phosphate, 0.5 M NaCl [pH 6.8]), and then bound antigen was eluted in 0.5 and 1% SDS (in 200 mM phosphate, 0.5 M NaCl [pH 6.8]). Eluted material was concentrated to 4 ml by centrifugation in a Centricon Plus-20 centrifugal filter device (10-kDa cutoff; Biomax-8 series; Millipore). Three milliliters of the concentrated affinity-purified sample was applied to an analytical C4 reverse-phase HPLC column (Vydac, Hesperia, Calif.) and eluted using a linear gradient of 5 to 95% acetonitrile–0.1% trifluoroacetic acid–H2O at a flow rate of 1 ml/min. Fractions were collected at 1-min intervals into 20 µl of two-fold-concentrated SDs-polycrylamide gel electrophoresis (PAGE) sample buffer (5% SDS and 44% glycerol in Tris base) and lyophilized. Lyophilized samples were resuspended in 40 µl of water with β-mercaptoethanol (0.5%) and trace bromophenol blue and heated (100°C, 5 to 10 min). Fractions were electrophoresed on an SDS–15% polyacrylamide gel and visualized by staining with a Coomassie brilliant blue R-250 stain. The gel was rinsed twice with the gel, washed twice (50% acetonitrile, 0.5 ml, 3 min), and frozen. Sequence analysis of two peptides of a trypsin digestion of the protein in the gel was
performed at the Harvard Microchemistry Facility by tandem mass spectrometry on a Finnigan LCQ quadrupole ion trap mass spectrometer.

Recombinant OmpA. The coding region of the 325-amino-acid mature OmpA protein, excluding the 21-amino-acid signal sequence (GenBank accession no. V00307), was generated by PCR amplification of DNA from an extract of E. coli O18:K1:H7. OmpA-specific PCR primers ([GACGACGACAAG GCTCGAAGAGATAAACCTGG] and OmpAbaac2 [GGGAGGAAACG CCCTGTAAGGGGCCGAGTAAC]) contained 5' extensions for cloning into the transfer plasmid pBACgus-2cp (Novagen, Madison, Wis.). The transfer plasmid containing the OmpA coding sequence, OmpA/pBACgus-2cp, was then transfected into the BacVector-200 Triple Cut Baculovirus DNA in Sf9 cells as instructed by the manufacturer (Novagen, Madison, Wis.). Positive recombinants were expanded, and high-titer virus was produced, to give multiplicities of infection in the range of 10 to 20 for maximal protein expression in Sf9 cells. The final baculovirus construct contains the OmpA coding sequence, with an in-frame amino-terminal extension (fusion sequences were encoded by the pBACgus-2cp transfer plasmid) containing an enterokinase recognition sequence, an S-protein binding site, and a polyhistidine tail. The 36.5-kDa (calculated molecular mass) OmpA fusion protein was purified from baculovirus-infected Sf9 cell lysates by polyhistidine affinity chromatography over a Talon cobalt metal affinity resin as specified by the manufacturer (Clontech, Palo Alto, Calif.).

Affinity purification of OMPs from sterile filtrates of serum-exposed bacteria. E. coli O18K1:H7 was grown to mid-log phase, harvested, and washed. The resultant bacterial pellet was resuspended in an equal volume of normal human serum (100 bacteria/ml) with ampicillin (200 μg/ml) and incubated for 2 h at 37°C on a rotating drum. The serum was filtered through a 0.45-μm pore-size filter to remove intact bacteria. The serum filtrate was then incubated with antibody-conjugated magnetic beads. Antibodies used for these affinity purification studies included polyclonal anti-O18 IgG, IgG from J5 antisera, and IgG from normal rabbit serum. Two hundred microliters of each sample was incubated with IgG-conjugated beads that had previously been washed and resuspended in 800 μl of PBS. The final concentration of IgG was 100 μg/ml. The final concentration of filtered serum was 20%. Reaction mixtures were incubated for 16 to 20 h at 4°C, with end-over-end mixing. The antibody-conjugated beads with attached antigen were then separated from the 20% filtered serum by polyhistidine affinity chromatography over a Talon cobalt metal affinity resin as specified by the manufacturer (Clontech, Palo Alto, Calif.).

RESULTS

The 35-kDa OMP is OmpA. We hypothesized that the 35-kDa protein was OmpA based on the apparent molecular weight and the fact that the electrophoretic mobility of the band was altered by boiling (data not shown) (31). Immunoblotting studies were performed to identify this protein. Iso-

lates of E. coli O18 bacteria in which the OmpA gene had been deleted and then reinserted into the strain (52) and recombinant OmpA were electrophoresed on SDS–16% polyacrylamide gels and transferred to nitrocellulose. Primary antibodies included polyclonal mouse anti-J5 IgG (left) and the monoclonal antibody directed against the 35-kDa OMP (2D3; right).

FIG. 1. Immunoblot of OmpA-deficient bacteria. Bacteria were grown to mid-log phase and then boiled in SDS-PAGE sample buffer. Bacterial lysates were then electrophoresed on SDS–16% polyacrylamide gels and transferred to nitrocellulose. Staining antibodies included polyclonal rabbit anti-J5 IgG (left) and a monoclonal antibody directed to the 35-kDa OMP (2D3; right). Bacterial strains: wild-type OmpA+ E. coli O18:K1:H7 (lane 1); E91, an OmpA-deleted mutant of E. coli O18:K1:H7 (lane 2); E69, an OmpA-restored mutant of E. coli O18:K1:H7 (lane 3). Positions of molecular weight markers (in kilodaltons) are shown at the left.

FIG. 2. Immunoblot of recombinant OmpA. Recombinant OmpA (lane 1 of each panel) and lysates of E. coli O18:K1:H7 bacteria (lane 2 of each panel) were electrophoresed on an SDS–16% polyacrylamide gel and transferred to nitrocellulose. Primary antibodies included polyclonal mouse anti-J5 IgG (left) and the monoclonal antibody directed against the 35-kDa OMP (2D3; right).

Sequence 1 - VTVEGHADER

Sequence 2 - [G] [V] SADQ* I* VSYGK*

FIG. 3. Peptide sequences of purified 18-kDa protein. Protein was purified as indicated in Materials and Methods. The lightly staining band on an SDS-polyacrylamide gel was excised and sequenced by mass tandem spectrometry. After trypsin digestion, two peptide sequences were obtained. Brackets indicate that the amino acid is isobaric and cannot be unambiguously differentiated by mass spectrometric sequencing. All other amino acids are assigned with the highest confidence.
ies. Lysates of \( E. coli \) K-12 in which the PAL gene (excC) was deleted, or was deleted and then replaced, were immunoblotted with anti-J5 IgG or 6D7 as primary antibody. Anti-J5 IgG and 6D7 did not react with the 18-kDa band in lysates of PAL-deficient bacteria but did react with an 18-kDa band in the wild-type strain and the strain with the gene reinterted (Fig. 4). These results indicate that the 18-kDa OMP is PAL and that 6D7 is a monoclonal anti-PAL antibody.

The 5- to 9-kDa OMP is MLP. We hypothesized that the 5- to 9-kDa OMP was MLP based on its low molecular weight and size heterogeneity (26). Accordingly, a mutant of \( E. coli \) K-12 lacking MLP (JE5505) and its MLP-positive, otherwise identical partner (JE5506) were used as antigens on immunoblots (Fig. 5). One blot was developed with anti-J5 IgG, and the other blot was developed with our monoclonal antibody, 1C7. Anti-J5 IgG and 1C7 IgG did not react with the 5- to 9-kDa band in bacterial lysates of the MLP-deficient strain. These results indicate that the lower cross-reactive OMP is MLP and that 1C7 is an anti-MLP IgG.

Identification of the OMPs released by bacteria incubated in human serum. Our prior studies (30) and work by others (20) have demonstrated that \( E. coli \) and \( Salmonella \) bacteria incubated in human serum release complexes of OMPs and LPS that can be affinity purified using O-chain specific anti-LPS IgG. To test the hypothesis that OmpA, PAL, and MLP are present in OMP-LPS complexes released by bacteria into human serum, polyclonal anti-O18 IgG was used to affinity purify LPS from sterile filtrates of human serum incubated in human serum, polyclonal anti-O18 IgG was used to affinity purify LPS from sterile filtrates of human serum incubated

FIG. 4. Immunoblot of PAL-deficient bacteria. Overnight cultures of bacteria were boiled in SDS-PAGE sample buffer. Bacterial lysates were then electrophoresed on SDS–16% polyacrylamide gels and transferred to nitrocellulose. Staining antibodies included polyclonal rabbit anti-J5 IgG (left) and a monoclonal antibody directed against the 18-kDa OMP (6D7, right). Bacterial strains: \( E. coli \) K-12 p400 containing PAL (lane 1); CH202, a PAL-deficient mutant of \( E. coli \) K-12(p400) (lane 2); CH202(pRC2), a PAL-restored mutant of CH202 (lane 3); \( E. coli \) K-12 1292 containing PAL (lane 4); JC7752, a PAL-deficient mutant of 1292 (lane 5); and JC7752(p417), a PAL-restored mutant of JC7752 (lane 6). Positions of molecular weight markers (in kilodaltons) are on the left.

DISCUSSION

This study identifies three outer membrane proteins that are released by \( E. coli \) bacteria into human serum in vitro (30) and in an animal model of gram-negative sepsis (29) as OmpA, PAL, and MLP. OmpA, PAL, and MLP are structural outer membrane proteins (5, 10, 39, 54, 58, 61) that are highly conserved among different enteric gram-negative bacteria (2, 8, 33, 47). Proteins similar to each exist in nonenteric gram-negative bacteria (46, 48, 59). MLP and PAL are lipoproteins with covalently attached lipids. The OMPs are tightly associated with LPS (37, 53). OmpA, PAL, and MLP have not been studied extensively in the context of gram-negative sepsis, although it has been known for several decades that many proteins associated with LPS are biologically active (14, 19, 21, 22, 24, 25, 28, 38, 40, 41, 44, 51, 67).

OmpA, initially described by Henning and colleagues (23, 31), has 325 amino acid residues (13) and exhibits heat-modifiable electrophoretic mobility on SDS-PAGE (13, 50). The
N-terminal domain of OmpA is comprised of 177 amino acids and is believed to traverse the outer membrane eight times (35). The C-terminal domain is believed to protrude into the periplasmic space. OmpA is involved in maintaining the shape of bacteria (58), serves as a phage receptor and a receptor for F-mediated conjugation, and may have pore-forming properties (60). OmpA enhances uptake of LPS into macrophages (38) and has been reported to be involved in E. coli invasion of the central nervous system (52). An OmpA-deficient mutant of the virulent strain E. coli O18K1 was shown to be less virulent than its OmpA+ parent strain in neonatal rat and embryonated chick egg models of sepsis (65).

PAL was initially described and characterized by Mizuno (47). It has 173 amino acid residues and is closely, but not covalently, associated with the peptidoglycan layer (39, 46, 47). PAL has a hydrophobic region of 22 amino acids at the N-terminal domain that interacts with the outer membrane (39). The C-terminal domain is involved in interactions with the peptidoglycan layer (39).

MLP, first described and characterized by Braun and colleagues (7, 10, 26), is the most abundant outer membrane protein (10). MLP has 58 amino acid residues and exists in two forms, a free form and a form that is covalently linked to peptidoglycan by the C-terminal domain (6, 7). Recently Zhang reported that MLP induces lethal shock in a strain of mouse (C3H/HeJ) that is genetically hyporesponsive to LPS (67). Furthermore, they found that MLP was synergistic with LPS for lethal toxicity.

We have previously shown that epitopes of all three OMPs are exposed on the surface of bacteria that have been incubated in human serum and that antisera raised to a rough (67). Furthermore, they found that MLP was synergistic with LPS for lethal toxicity.

We have previously shown that epitopes of all three OMPs are exposed on the surface of bacteria that have been incubated in human serum and that antisera raised to a rough mutant vaccine of E. coli J5 results in high titers of antibodies that bind to the same three OMPs on the bacterial surface (30). The identity of two of these proteins as PAL and MLP is surprising, as both proteins are situated in the deep periplasmic space and only short N-terminal segments are believed to interact with the outer membrane (9, 39). Therefore, the increased clearance of heterologous smooth bacterial strains by infusion of antisera to E. coli J5 (55) may be mediated through binding of immunoglobulin in this antisera to epitopes of OmpA, PAL, and MLP on the bacterial surface.

Previous investigators have focused on LPS as the primary bacterial toxin released in gram-negative sepsis. Release of bacterial membrane components other than LPS, either alone or in conjunction with LPS, has not been studied extensively. Our studies indicate that bacteria incubated in human serum release at least three OMPs in addition to LPS. Although the present studies focused on the three predominant proteins that we have demonstrated are bound by anti-J5 IgG, it seems likely that other bacterial components, including additional OMPs, may also be present in the OMP-containing fragments that are released into serum.

More recent studies suggest that complexes containing these three OMPs and LPS are released by E. coli bacteria into human serum (30) and into septic rat blood (29). To our knowledge, release of OmpA, PAL, or MLP into the bloodstream in sepsis has not been previously described. A pathogenic role for at least one of these OMPs is suggested by studies indicating that MLP causes lethal shock in C3H/HeJ mice (67). More study will be needed to test the hypothesis that these three OMPs may contribute to the pathogenesis of gram-negative infection.

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