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Effect of Anticoagulants on Binding and Neutralization of Lipopolysaccharide by the Peptide Immunoglobulin Conjugate $CAP18_{106\text{-}138}$–Immunoglobulin G in Whole Blood

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The 18-kDa cationic protein CAP18 is an antimicrobial protein isolated from rabbit granulocytes that binds lipopolysaccharide (LPS) and inhibits many of its biological activities. We covalently coupled a synthetic peptide representing amino acids 106 to 138 of CAP18 to human immunoglobulin G (IgG) by using the heterobifunctional linker *N*-succinimidyl-3-(2-pyridyldithio)propionate. The ability of $CAP18_{106\text{-}138}$–IgG to bind and neutralize LPS in whole blood in the presence and absence of anticoagulants was studied. Both $CAP18_{106\text{-}138}$ and $CAP18_{106\text{-}138}$–IgG significantly suppressed LPS-induced tumor necrosis factor (TNF) production in whole blood in the absence of anticoagulants. EDTA potentiated the ability of $CAP18_{106\text{-}138}$ and $CAP18_{106\text{-}138}$–IgG to decrease LPS-induced TNF production in a dose-dependent manner. In contrast, heparin inhibited the ability of $CAP18_{106\text{-}138}$ and $CAP18_{106\text{-}138}$–IgG to suppress LPS-induced TNF production. EDTA also enhanced LPS capture in a fluid-phase binding assay that utilizes magnetic anti-IgG beads to capture $CAP18_{106\text{-}138}$–IgG (and bound [^{3}H]LPS) in whole blood. In contrast, heparin inhibited the binding dose dependently. We conclude that $CAP18_{106\text{-}138}$–IgG binds to and neutralizes LPS in whole blood in the absence of anticoagulants. Further studies of its protective efficacy in animal models are warranted. Caution should be used in interpreting assays that measure the binding and neutralization of LPS in whole blood in the presence of calcium-binding anticoagulants or heparin.

Lipopolysaccharide (LPS), an endotoxin that is released from the outer surface membrane of gram-negative bacteria, is believed to be a major toxic mediator that contributes to the septic syndrome in infections caused by these bacteria. It has been known since the 1950s that LPS interacts with plasma proteins and lipoproteins and that these interactions affect the biological and physicochemical nature of LPS (33). These and subsequent studies revealed that LPS binds to high-density lipoprotein (HDL) and that the resulting LPS-HDL complexes are markedly less active than native, unbound LPS (39, 40). The more recent discovery of LPS-binding protein (LBP) led to a new awareness of the role of plasma proteins in endotoxin-induced cellular responses (31, 36). Binding of LPS-LBP to CD14 on macrophage-monocytes triggers and enhances the production of inflammatory mediators, including eicosanoids, reactive oxygen intermediates, and cytokines, that are believed to play an important role in the development of septic shock and multiple organ failure (12, 35, 38, 48). It has recently been proposed that LBP may also mediate the binding of LPS to HDL (49), providing a mechanism by which the biological activities of LPS are first increased and then decreased in the circulation. Considerable effort has been spent over the last several years in attempting to develop drugs that are designed to treat sepsis by binding and neutralizing LPS. Any such agent will need to function in blood in the presence of these complex LPS-protein interactions.

Granulocytes contain several antimicrobial proteins. Two such proteins, bactericidal/permeability-increasing protein (BPI) (8, 45) and the 18-kDa cationic antibacterial protein CAP18 (14, 19), bind LPS with high affinity and neutralize many of the biological activities of LPS in vitro and in vivo. Smaller portions of each protein have been described that contain antimicrobial and neutralizing properties (19, 22, 25, 37). Recombinant BPI (rBBI) fragments (such as $rBPI_{23}$) are antibacterial for gram-negative bacteria, neutralize LPS in vitro and in vivo, protect against live bacterial challenge in animal models, and are being developed as adjunctive therapy for gram-negative sepsis (2, 3, 18, 42, 45). Recombinant or synthetic peptides mimicking and including all or some of the amino acids in positions 104 to 142 of CAP18 have recently been described that are antimicrobial (19, 20), bind and neutralize LPS (14, 19, 21), and protect against LPS challenge in mice (21) and pigs (41). Accordingly, these peptides are also candidate agents for the therapy of sepsis.

Potential problems with the use of CAP18 peptides and BPI include possible toxicity and probable rapid clearance from the circulation. Although there are no data available regarding the circulating half-life of CAP18 peptides in the bloodstream, their small size makes them likely to have a very short half-life. rBPI has been described to be cleared with an α half-life of 7.8 min and a β half-life of 32 min (42). Advantages of a peptide-immunoglobulin G (IgG) conjugate would be that the peptide should not be cleared to the renal tubules and thus should be retained in the circulation for a longer time. Ideally, such a peptide-IgG conjugate would take advantage of the endotoxin-binding domain of the peptide while using the Fc portion of the

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immunoglobulin to facilitate opsonization of LPS or bacteria via Fc receptors on phagocytes. We created a peptide-IgG conjugate (7, 10) consisting of $CAP18_{106-138}$ covalently coupled to human IgG by using the heterobifunctional linker *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP). We have found that $CAP18_{106-138}$ in such a conjugate ($CAP18_{106-138}$–IgG) retains its bactericidal activity, as well as its ability to bind to and neutralize LPS, in aqueous buffers (11).

Most peptides and proteins that bind and neutralize LPS in buffer are less active in serum or blood. Accordingly, many investigators have recently sought to measure LPS neutralization in whole blood (3, 43, 45, 47). Here we describe the ability of $CAP18_{106-138}$–IgG to neutralize LPS-induced tumor necrosis factor (TNF) and bind to [^{3}H]LPS in 20% whole blood. These experiments required the development of a new binding assay that would function with whole blood. In the course of the studies, it became apparent that different anticoagulants markedly altered the binding and neutralization of LPS in blood by $CAP18_{106-138}$–IgG. Therefore, we also compared the effect of EDTA, heparin, and no anticoagulant (by using Teflon-coated vessels in which clotting was not initiated) on LPS binding and neutralization.

Our data indicate that $CAP18_{106-138}$–IgG binds and neutralizes LPS in whole blood that is not treated with anticoagulants. Binding and neutralization were significantly increased in blood that was anticoagulated with EDTA and significantly decreased in blood that was anticoagulated with heparin. Our in vitro studies with $CAP18_{106-138}$–IgG indicate that it will be worthwhile to evaluate the protective efficacy of this and other peptide-IgG conjugates as a means of binding and clearing LPS from the bloodstream (7, 10). Our results also suggest that considerable caution should be used in interpreting assays that measure the binding and neutralization of LPS in whole blood in the presence of calcium-binding anticoagulants or heparin.

MATERIALS AND METHODS

LPS. *Escherichia coli* O18K+ and O25 were the kind gifts of Alan Cross (University of Maryland Cancer Center, Baltimore). Radiolabeled LPS was extracted from bacteria that had been biosynthetically radiolabeled by growth in the presence of [^{3}H]acetate (Du Pont, New England Nuclear, Boston, Mass.) as previously described (43). The LPS was extracted as described by Rudbach (30), by using the hot-phenol method (46). Following extraction with phenol, the LPS preparation was treated with DNase, RNase, and protease as described by Romeo et al. (29) to remove contaminating proteins and nucleic acids. With the identical methodology, more than 99% of the radiolabeled LPS in previous preparations of LPS remained in the water phase after a 1:1 ether-water extraction at pH 5, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the LPS, followed by autoradiography, resulted in regularly spaced bands in a pattern characteristic of LPS. The specific activities of the LPS from *E. coli* O18K+ and O25 were 1.1×10^7 and 6.3×10^6 cpm/mg, respectively. Unlabeled LPS was prepared in the identical manner by using bacteria grown without tritiated acetate.

Anticoagulants and buffers. Heparin sodium was obtained from Elkins-Sinn (Cherry Hill, N.J.). Bovine serum albumin (BSA), EDTA, RPMI 1640 medium, Hanks balanced salt solution without Ca^{2+} and Mg^{2+} (HBSS), L-glutamine, fetal calf serum, dimethyl sulfoxide (DMSO), and phosphate-buffered saline (PBS) were obtained from Sigma Chemical Co. (St. Louis, Mo.).

Preparation of $CAP18_{106-138}$ and $CAP18_{106-138}$–IgG. $CAP18_{106-138}$ was synthesized essentially as previously described (11, 17). Peptides were synthesized by FMOC (9-fluorenylmethoxycarbonyl) solid-state peptide chemistry with automated peptide synthesizers (Exel [MilliGen/Millipore, Bedford, Mass.] and PS3 [Rainin Instrument, Woburn, Mass.]). Coupling was done by using a fivefold molar excess of FMOC-amino acid over the amount of resin with either benzotriazole-1-yl-oxy-TRIS-(dimethylamino)-phosphoniumhexafluorophosphate or benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphoniumhexafluorophosphate as the activator of carboxyl residues (16). Reagents were purchased from NovaBiochem (La Jolla, Calif.) and Bachem (Torrance, Calif.). FMOC-Trp(Boc) (FMOC–tryptophan–*t*-butoxycarbon; NovaBiochem) was used for the coupling of tryptophan in peptides containing arginine. Peptides were cleaved from the resin by reagent R (90% trifluoroacetic acid, 5% thioanisole, 3% dithioethane, 2% anisole) for 2 to 4 h at room temperature. Crude peptides were precipitated from reagent R with cold ethyl ether, washed twice more with the same ether, air dried, and dissolved in distilled water. Peptides were purified to homogeneity by reverse-phase high-pressure liquid chromatography on 300A wide-bore C_{18} preparative columns. The amount of peptide was quantified by dry-mass or quantitative amino acid analysis. A cysteine was added to the C-terminal end of the peptide for subsequent coupling.

$CAP18_{106-138}$ was coupled to human IgG (Gamimmune N; Cutter/Miles, Elkhart, Ind.) by using the heterobifunctional agent SPDP (Pierce Chemicals) as previously described (11), essentially in accordance with the manufacturer's directions as originally reported by Carlsson et al. (5). Pyrogen-free precautions were observed throughout. Briefly, IgG (50 mg/ml) was diluted to 5 mg/ml with saline and dialyzed against PBS-EDTA (20 mM sodium phosphate, 150 mM NaCl, 1 mM EDTA, pH 7.2) to remove the reducing sugar maltose present in this commercial preparation of IgG. A molar excess of SPDP (20 mM in DMSO, freshly made) was added to the PBS-EDTA-dialyzed IgG in five equal aliquots with gentle vortexing, after which the sample was allowed to incubate (room temperature, 30 min) with slow, end-over-end rotation to allow formation of an amide bond between the SPDP and the IgG. The solution was next desalted by extensive dialysis against PBS-EDTA, after which the A_{280} and A_{343} of 50 μl of SPDP-coupled IgG in 350 μl of carbonate buffer were measured. The degree of SPDP coupling was determined by adding 20 μl of 50 mM dithiothreitol. After 10 min, the A_{343} was measured again. The concentration of pyridine 2-thione (2-Py-S) released upon reduction was calculated in accordance with the formula [2-Py-S] = (change in $A_{343} \times 8$) ÷ 8,080 × 1.05. The measured IgG concentration was corrected for A_{280} attributable to the 2-Py-S groups that had been added to IgG by using the formula Corrected [IgG = [IgG] − ([2-Py-S] × 5,100). From this information, the molar ratio of 2-Py-S to IgG was determined as follows: [2-Py-S]/[IgG] = ([2-Py-S] ÷ corrected [IgG]) × 150,000. Different quantities of $CAP18_{106-138}$ were added to vary the ratio of moles of peptide to moles of SPDP-coupled IgG. The degree of thiol-disulfide exchange was estimated by measuring the A_{343} of the reaction mixture after overnight incubation with peptide at room temperature to calculate the [2-Py-S] displaced by peptide (5). The number of uncoupled 2-Py-S groups remaining on the peptide-IgG conjugate was measured by observing the change in A_{343} after adding dithiothreitol as described above. It was found to be necessary to add a two- to threefold molar excess of peptide to Py-2-S groups to drive the exchange reaction to completion. We estimated that the conjugate reported here contained 2.3 mol of peptides per mol of IgG. Slight aggregation of the final $CAP18_{106-138}$–IgG product was noted after extensive dialysis against PBS. Therefore, samples were filtered (0.2-μm-pore-size Whatman filter) before storage at −70°C.

TNF production by whole blood in Teflon tubes. After venipuncture, human blood was collected in syringes containing either heparin (25 U/ml or as indicated elsewhere in this report), EDTA (25 mM or as indicated elsewhere in this report), or no anticoagulant. The blood was immediately diluted 1:4 by addition of 4 parts HBSS. Diluted blood (980 μl) was placed into Teflon-coated inserts (Teflon inserts for 24-well plates; Savillex Corporation, Minnetonka, Minn.) in 24-well tissue culture plates (Becton Dickinson Labs, Lincoln Park, N.J.). There was no detectable coagulation of the blood for at least 4 h. A solution of 10 μl of $CAP18_{106-138}$ or $CAP18_{106-138}$–IgG diluted in HBSS was added to each insert to make the final concentrations indicated elsewhere in this report. Ten minutes later, 10-μl volumes of dilutions of LPS were added and mixed by gentle agitation of the plates to make a final volume of 1.0 ml. Tissue culture plates were then incubated at 37°C in 5% CO_2. After incubation for 4 h, supernatants were centrifuged at 100 × *g* for 10 min and the samples were stored at −80°C until TNF was measured.

Biologically active TNF was measured by using the WEHI 164.13 bioassay (9) as modified by Abukhabar et al. (1). This assay utilizes a reverted clone of the WEHI 164.13 cell line, WEHI 164.13K, which possessed enhanced TNF sensitivity (8 pg/ml) when cultured in the presence of actinomycin D.

WEHI 164.13K cells were used to seed complete medium at 2×10^4 cells/100 μl of medium in each well of a 96-well flat-bottom microtiter plate and incubated for 18 h at 37°C in 5% CO_2 at 95% humidity. Serial 1:2 dilutions of samples were made in assay medium (RPMI 1640 with 3% defined fetal calf serum, 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer, 2 mM L-glutamine, 100 μg of streptomycin per ml, 100 U of penicillin per ml, 1 μg of actinomycin D per ml). Samples (100 μl) were added to each well and incubated for 20 h. On the following day, 25 μl of a 2-mg/ml concentration of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide in PBS was added per well of cells and incubated for an additional 4 h. After removal of the medium, 100 μl of DMSO was added to each well to dissolve the formazan crystals. The A_{570} of each well was determined in a microplate reader. Absorbance was plotted against the reciprocal of the sample dilution. Units of TNF were obtained by comparing the dilution of sample giving 50% cytotoxicity with that of a recombinant TNF standard that was included in each assay. Conversion of units to picograms of TNF was based on a factor of 10 pg of recombinant TNF = 1 U.

Amounts of antigenic TNF-α in some of the human plasma samples were measured by enzyme-linked immunosorbent assay (ELISA) with a kit (Quantikine; R & D Systems, Minneapolis, Minn.) as described in the manufacturer's directions. For these experiments, an LPS concentration of 100 ng/ml was utilized.

Preparation of magnetic anti-human IgG beads. Magnetic beads (BioMag Amine Terminated 8-4100) were obtained from PerSeptive Diagnostics (Cambridge, Mass.). Magnetic anti-human IgG beads were prepared in accordance with the manufacturer's instructions. Briefly, rabbit anti-human IgG (Jackson

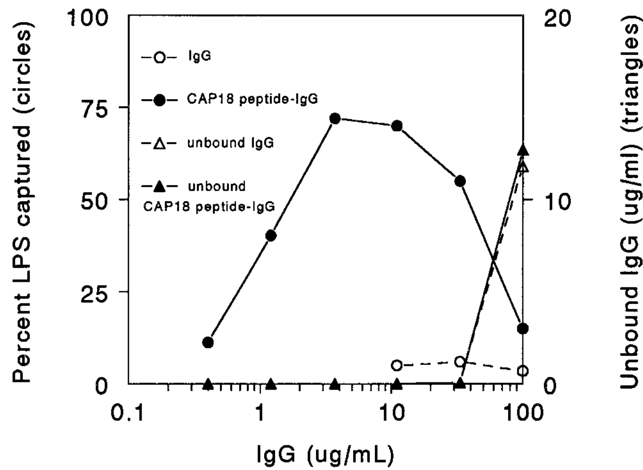


FIG. 1. Effect of IgG concentration in magnetic bead LPS capture assay on capture of IgG and [^{3}H]LPS. Dilutions of CAP18$_{106-138}$–IgG (CAP18 peptide-IgG; 0.4 to 100 μg/ml) and sham-coupled human IgG (IgG; 0.37 to 100 μg/ml) were incubated in rabbit whole blood anticoagulated with 5 mM EDTA in Teflon screw-top tubes with tritiated LPS (2 μg/ml) at 37°C for 30 min. Anti-human IgG magnetic beads (60 μl per tube) were added, and the mixture was incubated overnight at 4°C, after which the magnet was applied and the percent [^{3}H]LPS captured was calculated as described in Materials and Methods. Unbound IgG (that not captured by the magnetic beads) was measured in the supernatant by ELISA after application of the magnet and is indicated on the right *y* axis.

Immunoresearch Laboratories, West Grove, Pa.) was dialyzed overnight at 4°C with coupling buffer (10 mM pyridine in pyrogen-free water, pH 6). BioMag beads were washed three times with coupling buffer by placing the beads in a strong magnetic field (magnet obtained from PerSeptive Diagnostics) and carefully aspirating the remaining solution, after which the magnet was removed and more buffer was added. After washing, 5% glutaraldehyde was added to the beads and the mixture was shaken vigorously. The glutaraldehyde was then removed, the beads were again washed three times with coupling buffer, and rabbit anti-human IgG was combined with the beads at 5 mg/ml in coupling buffer and mixed gently at room temperature by slow end-over-end rotation for 16 to 20 h. Beads were separated with the magnet, and glycine quenching solution (1 M glycine in pyrogen-free water, pH 6) was added to the beads, which were then rotated end over end at room temperature for 30 min. The beads were then washed three times with washing buffer (10 mM Tris, 0.1% NaN_3, 0.1% [wt/vol] BSA, 150 mM NaCl, 1.0 mM EDTA in pyrogen-free water, pH 7.4) prior to storage at 4°C in wash buffer. The beads were again washed three times with PBS or saline prior to use.

Fluid-phase LPS capture assay in rabbit whole blood. To measure the ability of CAP18$_{106-138}$–IgG to bind LPS in whole blood, we developed a radioimmunoassay using magnetic beads covalently coupled to rabbit anti-human IgG (described above) to capture human IgG (and bound, tritiated LPS) in blood. Fresh blood was drawn from rabbits into syringes containing heparin (25 U/ml or as indicated elsewhere in this report), EDTA (25 mM or as indicated elsewhere in this report), or no anticoagulant. The blood was immediately diluted 1:4, and 190 μl was placed into a 3-ml Teflon vial with a rounded inside bottom (Savillex Corporation). Five microliters of CAP18$_{106-138}$–IgG was then added to the vials and gently mixed, after which 5 μl of [^{3}H]LPS (*E. coli* O25) was added. The final concentrations of peptide-IgG and IgG control were both 5 μg/ml, and the final concentration of [^{3}H]LPS was 2 μg/ml. The tops of the Teflon vials were screwed on tightly, and each vial was gently rotated at 37°C for 30 min, after which the vials were placed on ice. Six hundred microliters of a solution containing 1.0% BSA in HBSS and anti-human IgG magnetic beads (sufficient to bind all of the added human IgG) was added, and the mixture was incubated at 4°C overnight to capture the peptide-IgG conjugate. On the following day, after vortexing of each Teflon vial, the samples were transferred into 1.5-ml microcentrifuge tubes and placed in a strong magnetic field (magnet obtained from Perseptive Diagnostics) to separate beads containing captured CAP18$_{106-138}$–IgG and bound [^{3}H]LPS from unbound [^{3}H]LPS in the 20% blood. While still applied to the magnet, the portion of whole blood not containing beads was carefully aspirated and set aside. The beads were then washed four times by removal of the magnet, addition of HBSS, vortexing, and application of the magnet. The presence of tritium in the whole blood and beads was then determined by liquid scintillation counting. The red color of the 20% whole blood and the black color of the beads resulted in unacceptable quenching of the signal in each solution. Accordingly, we added 80 μl of bleach (5.25% sodium hypochlorite; Chlorox Co., Oakland, Calif.) to 320 μl of whole blood for approximately 10 min to clarify the blood before counting. After washing of the magnetic beads, 290 μl of a 0.1% sodium dodecyl sulfate solution was added to 30 μl of beads and the mixture was incubated for 30 min at 37°C to cleave the [^{3}H]LPS from the beads. The beads were then applied to the magnet, and the remaining solution containing eluted [^{3}H]LPS was counted. Remaining quenching was less than 10 and 1% for the blood and beads, respectively. The amount of quenching in each experiment was determined by the internal counting method by using known amounts of [^{3}H]LPS to spike solutions containing whole blood or beads, and counts were adjusted accordingly. Percent capture was calculated as follows: cpm of beads/(cpm of beads + cpm of whole blood). Percent recovery was calculated as follows: (cpm of beads + cpm of whole blood)/total cpm added. In all experiments, there was greater than 75% recovery.

In a series of initial experiments, we determined the optimal capture conditions in whole blood for 2 μg of [^{3}H]LPS per ml. Figure 1 represents the relationship between IgG concentration and percent LPS capture. When the bead volume was fixed at 60 μl of beads per tube, the percentage of tritiated LPS captured increased directly with increasing IgG, reaching a plateau at an IgG concentration of 3.7 to 11 μg/ml. At greater IgG concentrations, the percent LPS capture decreased and free IgG was detected by an IgG capture ELISA specific for human IgG analogous to that previously described (32), with rabbit anti-human IgG as the coating antibody and peroxidase-coupled anti-human IgG for the detecting antibody. Thus, the maximum bead capacity was 0.06 to 0.18 μg of IgG/μl of magnetic beads and an IgG concentration of 3.7 to 11 μg/ml was optimal for capture of 2 μg of [^{3}H]LPS per ml with 60 μl of magnetic beads per tube. We therefore utilized 5 μg of IgG per ml in this assay.

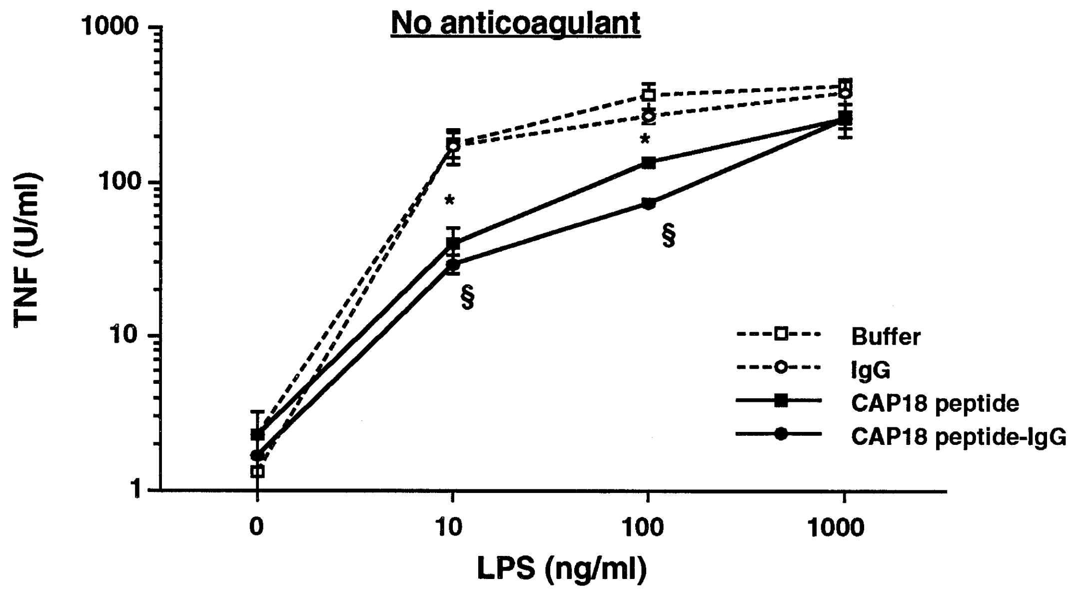
Statistics. Statistical analyses were performed by analyses of variance (ANOVA) followed by Scheffe F test with Stat View II (Abacus Concepts, Inc., Berkeley, Calif.).

RESULTS

Effect of anticoagulation with EDTA or heparin on the ability of CAP18$_{106-138}$–IgG to suppress LPS-induced TNF production. We chose to study CAP18$_{106-138}$–IgG at a concentration of 50 μg/ml in this first group of experiments, reasoning that this concentration should be easily achievable after intravenous infusion. This concentration of CAP18$_{106-138}$–IgG contains 2.7 μg of CAP18$_{106-138}$ peptide per ml. LPS-induced TNF production was dose dependent, and both CAP18$_{106-138}$ and CAP18$_{106-138}$–IgG inhibited LPS-induced TNF production in blood without any anticoagulant (Fig. 2, top) and in blood anticoagulated with 5 mM EDTA (Fig. 2, middle). The neutralizing effects of CAP18$_{106-138}$ and CAP18$_{106-138}$–IgG were augmented by EDTA. In contrast, heparin at a concentration of 5 U/ml completely blocked the neutralization of LPS-induced TNF production by CAP18$_{106-138}$ and CAP18$_{106-138}$–IgG (Fig. 2, bottom). Thus, EDTA enhanced and heparin inhibited the ability of CAP18$_{106-138}$ and CAP18$_{106-138}$–IgG to suppress LPS-induced TNF production.

These experiments were performed with blood from a single human donor and blood stimulated with LPS from *E. coli* O25. It was therefore of interest to determine if our results were applicable to other bacterial strains, other species, and other donors. We obtained similar results with LPS from *E. coli* O18 in human blood and also in blood obtained from rabbits (data not shown). To study a range of human donors, we repeated the same experiments with a single concentration of LPS by using blood from six different healthy donors. For these exper-

FIG. 2. Effect of LPS-induced TNF production in 20% whole blood in the absence of anticoagulants (top), in blood anticoagulated with 5 mM EDTA (middle), and in blood anticoagulated with 5 U of heparin per ml (bottom). Teflon tubes contained dilutions of LPS and buffer, 50 μg of human IgG per ml (IgG), 2.7 μg of CAP18$_{106-138}$ per ml (CAP18 peptide), or 50 μg of CAP18$_{106-138}$–IgG per ml (CAP18 peptide-IgG). Tubes were incubated for 4 h at 37°C, after which TNF was measured as described in Materials and Methods. Each result is the mean ± standard error of the mean of at least three independent experiments. Symbols: *, $P < 0.05$; ‡, $P < 0.01$ versus buffer; §, $P < 0.05$; ‖, $P < 0.01$ versus IgG by ANOVA.


No anticoagulant
TNF (U/ml)
1000
100
10
1
0
10
100
1000
LPS (ng/ml)
*
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Buffer
IgG
CAP18 peptide
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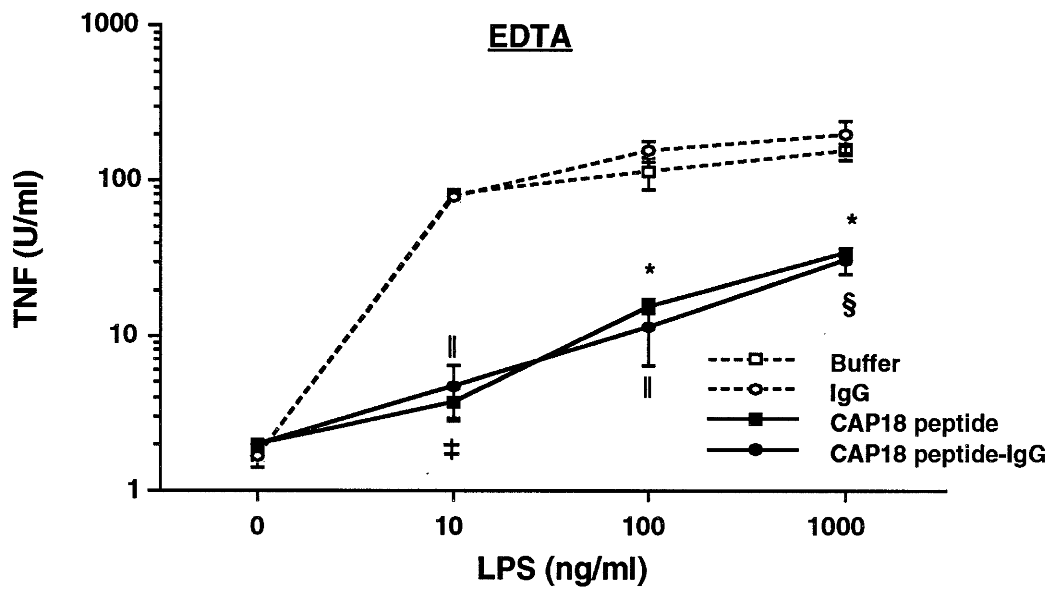
EDTA
TNF (U/ml)
1000
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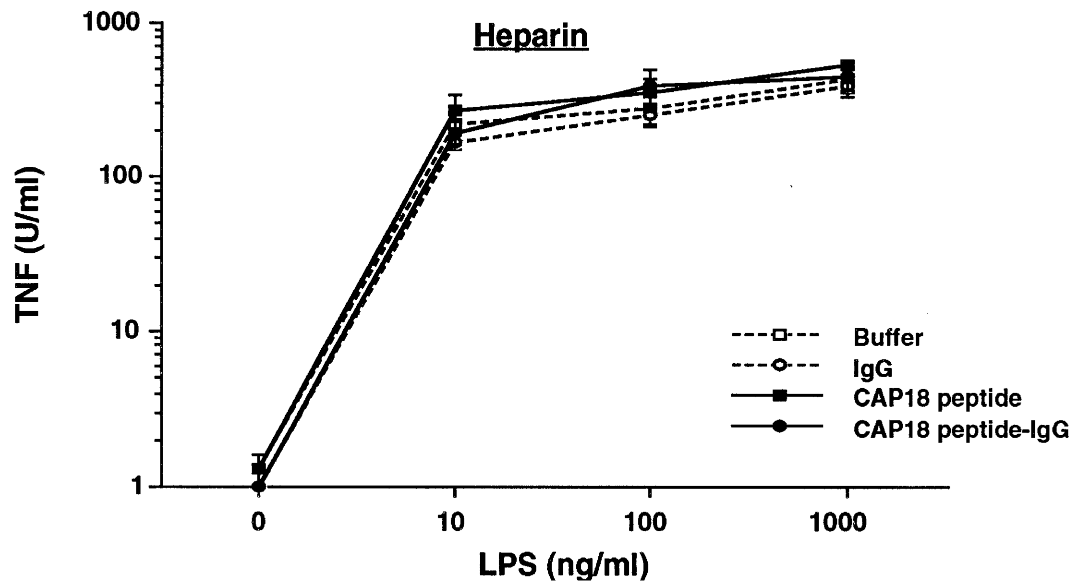
Heparin
TNF (U/ml)
1000
100
10
1
0
10
100
1000
LPS (ng/ml)
Buffer
IgG
CAP18 peptide
CAP18 peptide-IgG

TABLE 1. Effects of anticoagulants on LPS-induced TNF

Treatment	Mean amt of TNF (pg/ml) ± SEM		
	No anticoagulant	EDTA (5 mM)	Heparin (5 U/ml)
Buffer	1,049 ± 157	263 ± 35[a]	1,024 ± 103
IgG	1,069 ± 79	262 ± 34[a]	1,121 ± 88
CAP18 peptide	533 ± 86[b]	123 ± 14[a,b]	1,069 ± 86[a]
CAP18 peptide-IgG	713 ± 160[c]	187 ± 22[c,d]	1,192 ± 84[d]

[a] $P < 0.01$ versus no anticoagulant by unpaired *t* test.
[b] $P < 0.01$ versus buffer by ANOVA.
[c] $P < 0.05$ versus IgG by ANOVA.
[d] $P < 0.05$ versus no anticoagulant by unpaired *t* test.

iments, we utilized an immunological assay (ELISA) to measure TNF in the plasma. We obtained similar results with these donors (Table 1), although the effect of EDTA itself was even stronger in this series of experiments.

Effect of concentrations of EDTA and heparin on the ability of $CAP18_{106-138}$ or $CAP18_{106-138}$–IgG to suppress LPS-induced TNF production. Because a range of anticoagulant concentrations are utilized in clinical and laboratory studies, we evaluated the dose effects of EDTA and heparin on LPS-induced TNF production in the presence or absence of $CAP18_{106-138}$ or $CAP18_{106-138}$–IgG. As noted above, EDTA alone partly inhibited LPS-induced TNF. EDTA enhanced (Fig. 3) and heparin inhibited (Fig. 4) the activity of CAP18 and $CAP18_{106-138}$–IgG in a dose-dependent manner. Even low concentrations of EDTA (0.5 mM) and extremely low concentrations of heparin (0.05 U/ml) altered the neutralizing effects of $CAP18_{106-138}$ and $CAP18_{106-138}$–IgG in the system.

Binding of LPS by $CAP18_{106-138}$–IgG in 20% whole blood. $CAP18_{106-138}$ (21) and $CAP18_{106-138}$–IgG (11a) bind LPS in aqueous buffer systems. However, these assays do not reflect the fluid-phase conditions found in circulating blood. To measure binding of $CAP18_{106-138}$–IgG in blood, we developed a fluid-phase radioimmunoassay using magnetic beads covalently coupled to rabbit anti-human IgG to quantify binding of tritiated LPS. We found that 5 μg of $CAP18_{106-138}$–IgG per ml captured greater than 50% of 2 μg of [^{3}H]LPS per ml in 20% whole rabbit blood in the Teflon tubes in the absence of an anticoagulant (Fig. 5). A low concentration of heparin (0.5 U/ml) decreased the percentage of LPS bound. In contrast, a low concentration of EDTA (0.5 mM) increased the amount of LPS bound (Fig. 5). Both anticoagulants altered LPS binding in a dose-dependent manner. Similar results were obtained with tritiated LPS from *E. coli* O18 (data not shown).

To evaluate whether heparin could displace LPS from preformed complexes of LPS–$CAP18_{106-138}$–IgG, we compared the capture of LPS in blood to which the different anticoagulants had been added alone or in combination at different times (Fig. 6). We found that heparin blocked the capture of [^{3}H]LPS by $CAP18_{106-138}$–IgG in the presence of 5 mM EDTA when added at the same time as the LPS. However, if $CAP18_{106-138}$–IgG complexes were allowed to form by incubating the LPS and the $CAP18_{106-138}$–IgG for 30 min in EDTA, after which heparin was added, there was no significant

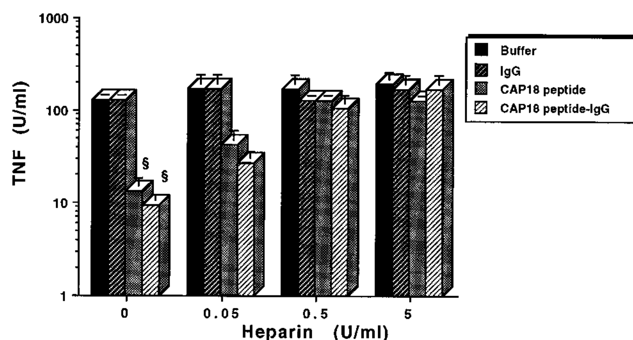

FIG. 4. Dose effect of heparin on LPS-induced TNF production in whole blood. Teflon tubes contained 20% whole blood with the indicated concentrations of heparin, 100 ng of *E. coli* O25 LPS per ml and buffer, 50 μg of human IgG per ml (IgG), 2.7 μg of $CAP18_{106-138}$ per ml (CAP18 peptide), or 50 μg of $CAP18_{106-138}$–IgG per ml (CAP18 peptide-IgG). Note that a lower dose of LPS was used here than in the experiments of Fig. 3. Tubes were incubated for 4 h at 37°C, after which TNF was measured as described in Materials and Methods. Each result is the mean ± the standard error of the mean of at least three independent experiments. Symbols: §, $P < 0.05$ versus buffer and IgG by ANOVA.

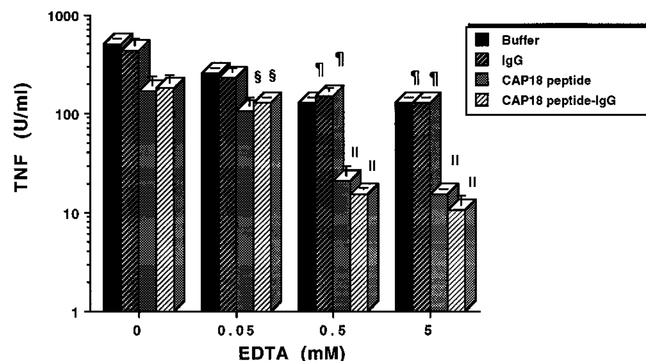

FIG. 3. Dose effect of EDTA on LPS-induced TNF production in whole blood. Teflon tubes contained 20% whole blood with the indicated concentrations of EDTA, 1.0 μg of *E. coli* O25 LPS per ml and buffer, 50 μg of human IgG per ml (IgG), 2.7 μg of $CAP18_{106-138}$ per ml (CAP18 peptide), or 50 μg of $CAP18_{106-138}$–IgG per ml (CAP18 peptide-IgG). Tubes were incubated for 4 h at 37°C, after which TNF was measured as described in Materials and Methods. Each result is the mean ± the standard error of the mean of at least three independent experiments. Symbols: ¶, $P < 0.05$ versus no EDTA by unpaired *t* test; §, $P < 0.05$ versus buffer and IgG; ‖, $P < 0.01$ versus buffer and IgG by ANOVA.

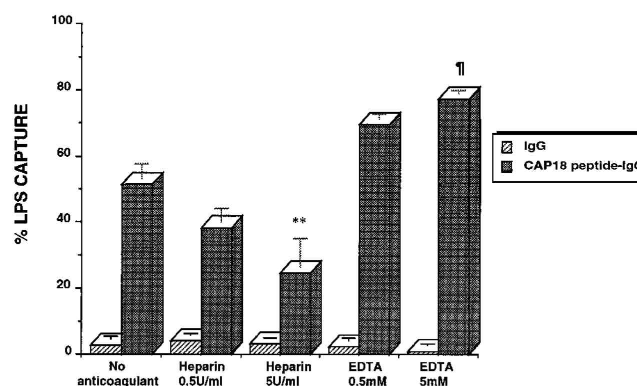

FIG. 5. Effect of anticoagulants on the binding IgG and $CAP18_{106-138}$–IgG to LPS in whole blood. Teflon tubes containing 5 μg of IgG or $CAP18_{106-138}$–IgG per ml (CAP18 peptide-IgG) in 20% whole rabbit blood were incubated with 2 μg of tritiated *E. coli* O25 LPS per ml in the absence of an anticoagulant or in the presence of heparin or EDTA. After 30 min of incubation, 60 μl of magnetic beads covalently coupled with anti-human IgG was added and the solution was incubated overnight at 4°C. The beads containing the captured IgG (and bound, tritiated LPS) were then separated, and the percent bound LPS was calculated as described in Materials and Methods. Each result is the mean ± the standard error of the mean of at least three independent experiments. Symbols: ¶, $P < 0.05$; **, $P < 0.01$ versus no anticoagulant by unpaired *t* test.

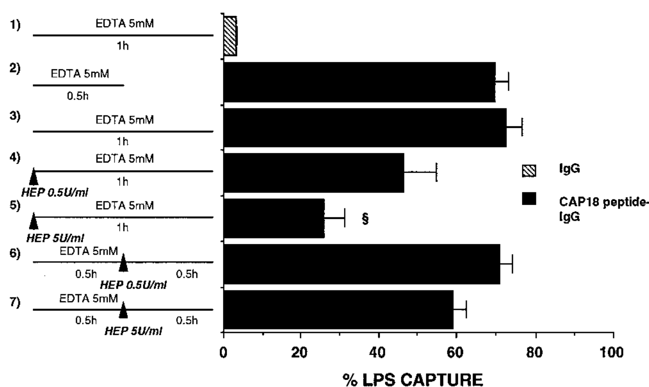

FIG. 6. Effect of heparin (*HEP*) on complexes of LPS–$CAP18_{106-138}$–IgG (CAP18 peptide-IgG) in the presence of EDTA. Teflon tubes containing 5 μg of IgG or $CAP18_{106-138}$–IgG per ml were incubated in 20% rabbit whole blood with 2 μg of tritiated LPS per ml for 1 h under the following conditions: 1, human IgG with 5 mM EDTA for 1 h; 2, CAP18 peptide-IgG with 5 mM EDTA for 0.5 h; 3, CAP18 peptide-IgG with 5 mM EDTA for 1 h; 4, CAP18 peptide-IgG with 5 mM EDTA and 0.5 U of heparin per ml for 1 h; 5, CAP18 peptide-IgG with 5 mM EDTA and 5.0 U of heparin per ml for 1 h; 6, CAP18 peptide-IgG with 5 mM EDTA for 0.5 h and heparin at 0.5 U/ml added for an additional 0.5 h; 7, CAP18 peptide-IgG with 5 mM EDTA for 0.5 h and heparin at 5.0 U/ml added for an additional 0.5 h. After 30 min of incubation, 60 μl of magnetic beads covalently coupled with anti-human IgG was added and the solution was incubated overnight at 4°C. The beads containing the captured IgG (and bound, tritiated LPS) were then separated, and the percent bound LPS was calculated as described in Materials and Methods. Each result is the mean ± the standard error of the mean of at least three independent experiments. Symbols: §, $P < 0.05$ versus the values of conditions 2, 3, 6, and 7 by ANOVA.

effect on the $[^3H]$LPS captured. These data suggest that a very low dose of heparin inhibits the ability of $CAP18_{106-138}$–IgG to bind LPS but is insufficient to disrupt preformed complexes of LPS–$CAP18_{106-138}$–IgG.

DISCUSSION

$CAP18_{106-138}$ is a synthetic peptide mimicking amino acids in positions 106 to 138 of CAP18 that binds, neutralizes, and kills a wide variety of gram-negative bacteria and protects in a mouse model of endotoxin shock (19, 21, 26, 37, 41). Accordingly, this peptide is a candidate for an agent that might be useful in the treatment of gram-negative sepsis. To increase the effective half-life of the peptide, to decrease the chance of renal toxicity, and to add an Fc fragment in the hope of increasing clearance of LPS and bacteria, we covalently coupled $CAP18_{106-138}$ to human IgG to create $CAP18_{106-138}$–IgG. In studies to be reported elsewhere, we found that this compound retains the biological activities of $CAP18_{106-138}$ in aqueous buffers (11). Although these findings represent a positive first step, factors such as the amphipathic nature of LPS and the interaction of LPS with blood elements, including proteins, lipoproteins, and cells, suggested that such an agent might function differently in blood. Accordingly, the present study was undertaken to evaluate whether $CAP18_{106-138}$–IgG would retain the ability to bind and neutralize LPS in the bloodstream. Because the use of different anticoagulants was a potential complicating factor (4, 24, 28, 34), we developed new assays that measured binding and neutralization in the presence and absence of the anticoagulants EDTA and heparin.

Our data indicate that $CAP18_{106-138}$–IgG neutralizes LPS in 20% whole blood in the absence of anticoagulants. Since the neutralization of the peptide-IgG conjugate was roughly equivalent to estimated molar equivalents of free $CAP18_{106-138}$ peptide (relative to peptide in the conjugate) and since uncoupled normal IgG did not neutralize, all of the neutralization by the conjugate can be attributed to the peptide portion. Our data therefore suggest that activity of the peptide, at least with respect to the neutralization of LPS-induced TNF, is not dramatically altered by conjugation to IgG. We observed similar findings in both biological and immunoreactive assays of TNF in our studies.

Our results also indicate that $CAP18_{106-138}$–IgG captures $[^3H]$LPS in 20% whole blood in a similar molar relationship as it does in buffer (11). However, because no assay is available to measure the binding of free peptide to LPS in blood, we cannot assess if the conjugation interferes with binding. Nevertheless, our results indicate that $CAP18_{106-138}$–IgG can bind LPS in the presence of a complicated mixture of proteins, lipoproteins, and cells and, accordingly, that it might be able to enhance clearance of LPS in addition to neutralizing its biological activity.

The use of Teflon tubes allowed us to compare the activities of the peptide and peptide-IgG conjugate in blood in the presence and absence of anticoagulants. EDTA treatment itself partially suppressed LPS-induced TNF production in 20% whole blood in comparison with no anticoagulant (Table 1 and Fig. 3) (28). Some or all of this suppression may be due to facilitation of the interaction of LPS with lipoproteins. LPS is amphipathic and forms large aggregates in aqueous suspensions. It is disaggregated in plasma prior to binding to high-density lipoproteins (23, 40). EDTA dramatically increases the rate of LPS-lipoprotein binding, presumably by increasing the disaggregation of LPS (44). LPS that is bound to lipoprotein does not bind to macrophages and is 100- to 1,000-fold less active than free LPS in inducing TNF (6).

The inhibition of LPS-induced TNF production by $CAP18_{106-138}$ and $CAP18_{106-138}$–IgG was markedly enhanced in the presence of EDTA (Fig. 3). EDTA also increased the binding of LPS by $CAP18_{106-138}$–IgG in whole blood compared to blood without anticoagulants (Fig. 5), suggesting that the increased neutralization was due to increased binding. Presumably, the EDTA facilitates the binding of LPS to $CAP18_{106-138}$ by disaggregating the LPS in a similar manner as it does for LPS-lipoprotein binding.

Heparin did not inhibit LPS-induced TNF induction in 20% whole blood. However, heparin inhibited the neutralization of LPS by $CAP18_{106-138}$ and $CAP18_{106-138}$–IgG (Fig. 4) and the binding of $[^3H]$LPS by $CAP18_{106-138}$–IgG (Fig. 5 and 6). The most direct explanation for this finding is that heparin competes for the same binding site as LPS on $CAP18_{106-138}$. Heparin is a sulfated polysaccharide with a net negative charge. CAP18 binds heparin and was, indeed, initially purified by heparin affinity chromatography (14). It was recently reported that the binding of heparin to short, synthetic peptides mimicking the sequence of BPI correlates with the ability of the same peptides to inhibit LPS-induced coagulation of *Limulus* amoebocyte lysate in aqueous buffers (22). $CAP18_{106-138}$ therefore shares properties with several other mammalian proteins that bind to LPS and heparin with a binding domain characterized by a cluster of basic and hydrophobic amino acids (15, 22). A very low dose of heparin (0.5 U/ml) suppressed the LPS binding by $CAP18_{106-138}$–IgG but did not influence the percent capture by $CAP18_{106-138}$–IgG if it had already bound to LPS. Although we did not attempt to calculate binding affinities, these findings suggest that the complexes of LPS and $CAP18_{106-138}$–IgG are relatively stable once formed.

Our findings indicate that it is possible, and may be preferable, to evaluate agents that bind and neutralize LPS in the absence of anticoagulants in Teflon tubes. We found that the tubes and 24-well inserts did not cause macrophage activation

by adhesion in the time frame studied (13), could be rendered pyrogen free by washing, and could be utilized up to five times before minor abrasions were noted that resulted in partial coagulation of the blood. Many of the previously published studies evaluating the stimulation of cytokines in whole blood with LPS or gram-negative bacteria have utilized EDTA (24), citrate (45), or heparin (3, 27, 47) as an anticoagulant. Our findings raise the possibility that the ability of some LPS-binding agents to neutralize in this assay may be artifactually enhanced by the presence of anticoagulants that bind divalent cations (EDTA and citrate) and inhibited by anticoagulants that might compete with the LPS-binding domains of the agent studied (heparin).

Our data indicate that $CAP18_{106-138}$–IgG binds and neutralizes smooth LPS in whole blood without anticoagulants. Both EDTA and heparin alter the interactions of LPS with $CAP18_{106-138}$–IgG in whole blood. Assays of LPS binding and neutralization performed in the absence of anticoagulants may be preferable than the systems currently utilized, which may lead to erroneous conclusions.

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