Effect of Anticoagulants on Binding and Neutralization of Lipopolysaccharide by the Peptide Immunoglobulin Conjugate CAP18\textsubscript{106-138}–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\textsubscript{106-138}–IgG to bind and neutralize LPS in whole blood in the presence and absence of anticoagulants was studied. Both CAP18\textsubscript{106-138} and CAP18\textsubscript{106-138}–IgG significantly suppressed LPS-induced tumor necrosis factor (TNF) production in whole blood in the absence of anticoagulants. EDTA potentiated the ability of CAP18\textsubscript{106-138} and CAP18\textsubscript{106-138}–IgG to decrease LPS-induced TNF production in a dose-dependent manner. In contrast, heparin inhibited the ability of CAP18\textsubscript{106-138} and CAP18\textsubscript{106-138}–IgG to suppress LPS-induced TNF production. EDTA also enhanced the ability of CAP18\textsubscript{106-138}–IgG to capture in a fluid-phase binding assay that utilizes magnetic anti-IgG beads to capture CAP18\textsubscript{106-138}–IgG (and bound \(^{[3}H\)LPS) in whole blood. In contrast, heparin inhibited the binding dose dependently. We conclude that CAP18\textsubscript{106-138}–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.
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–IgG 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, 45, 47). Here we describe the ability of CAP18/106-138–IgG to neutralize LPS-induced tumor necrosis factor (TNF) and bind to [3H]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. In 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 [14C]glutamate (Du Pont, New England Nuclear, Boston, Mass.) as previously described (43). The LPS was extracted as described by Buffa et al. (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 preparations, 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 × 10^6 and 6.3 × 10^5 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 Ca2+ and Mg2+ (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 (Exell/MillGen/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-pyrrolidino-phosphoniumhexafluorophosphate or benzotriazole-1-yl-oxy-tris-pyrroldino-phosphoniumhexafluorophosphate as the activator of carbonyl residues (16). Reagents were purchased from NovaBiochem (La Jolla, Calif.) and Bachem (Torrance, Calif.). FMOC-Trp(Boc) (FMOC–tryptophan–boc-carbobenzoxy; 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% thiouacetic acid, 3% dithioethanol, 2% anisole) for 2 to 4 h at room temperature. Crude peptides were precipitated from reagent R with 9 volumes of EtOH, washed twice more with the same ether, air-dried, and dissolved in distilled water. Peptides were purified to homogeneity 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–IgG 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).
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 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 [3H]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.

RESULTS

Effect of anticoagulation with EDTA or heparin on the ability of CAP18106-138-IgG to suppress LPS-induced TNF production. We chose to study CAP18106-138-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 CAP18106-138-IgG contains 2.7 μg of CAP18106-138 peptide per ml. LPS-induced TNF production was dose dependent, and both CAP18106-138 and CAP18106-138-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 CAP18106-138 and CAP18106-138-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 CAP18106-138 and CAP18106-138-IgG (Fig. 2, bottom). Thus, EDTA enhanced and heparin inhibited the ability of CAP18106-138 and CAP18106-138-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. 1. Effect of IgG concentration in magnetic bead LPS capture assay on capture of IgG and [3H]LPS. Dilutions of CAP18106-138-IgG (CAP18 peptide-IgG; 0.4 to 100 μg/ml) and sham-coupled human IgG (IgG; 0.37 to 11 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 [3H]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.

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 CAP18106-138 per ml (CAP18 peptide), or 50 μg of CAP18106-138-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.
TABLE 1. Effects of anticoagulants on LPS-induced TNF production.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean aml of TNF (pg/ml) ± SEM</th>
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<tr>
<td>No anticoagulant</td>
<td>EDTA (5 mM)</td>
</tr>
<tr>
<td>Buffer</td>
<td>1,049 ± 157</td>
</tr>
<tr>
<td>IgG</td>
<td>1,069 ± 79</td>
</tr>
<tr>
<td>CAP18 peptide</td>
<td>533 ± 86c</td>
</tr>
<tr>
<td>CAP18 peptide-IgG</td>
<td>713 ± 160f</td>
</tr>
</tbody>
</table>

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.

Effect of concentrations of EDTA and heparin on the ability of CAP18106-138 or CAP18106-138–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 CAP18106-138 or CAP18106-138–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 CAP18106-138–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 CAP18106-138 and CAP18106-138–IgG in the system.

Binding of LPS by CAP18106-138–IgG in 20% whole blood. CAP18106-138 (21) and CAP18106-138–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 CAP18106-138–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 CAP18106-138–IgG per ml captured greater than 50% of 2 μg of [3H]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–CAP18106-138–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 [3H]LPS by CAP18106-138–IgG in the presence of 5 mM EDTA when added at the same time as the LPS. However, if CAP18106-138–IgG complexes were allowed to form by incubating the LPS and the CAP18106-138–IgG for 30 min in EDTA, after which heparin was added, there was no significant
IgG or CAP18106-138–IgG per ml were incubated in 20% rabbit whole blood with m(CAP18 peptide-IgG) in the presence of EDTA. Teflon tubes containing 5 VOL. 65, 1997 BINDING AND NEUTRALIZATION OF CAP18-IgG IN WHOLE BLOOD 2165
tubes and 24-well inserts did not cause macrophage activation.

effect on the [3H]LPS captured. These data suggest that a very low dose of heparin inhibits the ability of CAP18106-138–IgG to bind LPS but is insufficient to disrupt preformed complexes of LPS–CAP18106-138–IgG.

DISCUSSION

CAP18106-138 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 in-
creasing clearance of LPS and bacteria, we covalently coupled CAP18106-138–IgG to human IgG to create CAP18106-138–IgG. In studies to be reported elsewhere, we found that this compound retains the biological activities of CAP18106-138 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 function differently in blood. Accordingly, the present study was undertaken to evaluate whether CAP18106-138–IgG would retain the ability to bind and neutralize LPS in the blood-
stream. Because the use of different anticoagulants was a po-
tential 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 CAP18106-138–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 CAP18106-138 Pepti-
dide (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 dra-
matically 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 CAP18106-138–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 CAP18106-138–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 ac-
tivity.

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 it-
self 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 fa-
cilitation 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 CAP18106-138 and CAP18106-138–IgG was markedly enhanced in the presence of EDTA (Fig. 3). EDTA also increased the binding of LPS by CAP18106-138–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 CAP18106-138 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 CAP18106-138 and CAP18106-138–IgG (Fig. 4) and the binding of [3H]LPS by CAP18106-138–IgG (Fig. 5 and 6). The most direct explanation for this finding is that heparin competes for the same binding site as LPS on CAP18106-138. 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). CAP18106-138 therefore shares properties with several other mammalian proteins that bind to LPS and heparin with a binding domain char-
acterized 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 CAP18106-138–IgG but did not influence the percent capture by CAP18106-138–IgG if it had already bound to LPS. Although we did not attempt to calculate binding affin-
ities, these findings suggest that the complexes of LPS and CAP18106-138–IgG are relatively stable once formed.

Our findings indicate that it is possible, and may be prefer-
able, 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 artifically 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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REFERENCES


