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J Immunol 2003; 171:2594-2601; doi: 10.4049/jimmunol.171.5.2594
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C1 Inhibitor Prevents Endotoxin Shock Via a Direct Interaction with Lipopolysaccharide

Dongxu Liu,* Shenghe Cai,* Xiaogang Gu,* Jennifer Scafidi,* Xiao Wu,† and Alvin E. Davis III3*  

C1 inhibitor (C1INH) is beneficial in animal models of endotoxemia and sepsis. However, the mechanisms(s) of C1INH protection remain(s) ill-defined. In this study, we demonstrated that both active C1INH and reactive center-cleaved, inactive C1INH protected mice from lethal Gram-negative endotoxemia. Both forms of C1INH blocked the LPS-binding-protein-dependent binding of Salmonella typhimurium LPS to the murine macrophage cell line, RAW 264.7, and suppressed LPS-induced TNF-α mRNA expression. Inhibition of LPS binding to RAW 264.7 cells was reversed with anti-C1INH Ab and was more efficient when C1INH was incubated first with LPS rather than with the cells. C1INH also suppressed LPS-induced up-regulation of TNF-α mRNA in whole human blood. The interaction of C1INH with LPS was directly demonstrated both by ELISA and by nondenaturing PAGE, but deletion of the amino-terminal 97-aa residues abrogated this binding. Therefore, C1INH, in addition to its function as a serine protease inhibitor, has a novel anti-inflammatory function mediated via its heavily glycosylated amino-terminal non-serpin domain. The Journal of Immunology, 2003, 171: 2594–2601.

Lipopolysaccharide is a major constituent of the outer membrane of Gram-negative bacteria and is a key molecule in the pathogenesis of Gram-negative endotoxemia, sepsis, and septic shock (1). Gram-negative endotoxemia is accompanied by contact system activation, complement activation, production of cytokines, and other evidence of unregulated inflammatory responses (2, 3). LPS activates mononuclear phagocytes to produce and release inflammatory mediators, of which TNF-α appears to be very important for the development of endotox shock (4). LPS interacts with the LPS-binding protein (LBP)⁴ and transfers LPS to CD14 (5–8). The formation of LPS-CD14 complexes initiates intracellular signaling by binding to Toll-like receptors expressed on mononuclear phagocytes and other cells (9). When pure LPS or bacterial outer membrane fragments are injected into the bloodstream, a large fraction of the LPS is cleared by the liver within 10 min (10, 11), whereas most of the remaining LPS binds rapidly to plasma proteins, such as lipoproteins, which inhibit its biologic activity (12–15).

C1 inhibitor (C1INH) is the only inhibitor of the classical complement pathway proteases, C1r and C1s (16), and is the major inhibitor of factor XII and prekallikrein of the contact system (17, 18). The complement system has been implicated in both the pathogenesis of, and protection from, endotoxin shock (19). The contact system also appears to play a role in the mediation of septic shock (20). Levels of proteolytically inactivated C1INH are increased in fatal septic shock, which suggests an increased turnover and a relative secondary deficiency of biologically active C1INH (21). C1INH can be inactivated by limited proteolytic cleavage by elastase released from activated neutrophils (19, 22). The inactivation of C1INH may occur locally in inflamed tissue and thereby contribute to increased local complement activation (22). The direct biologic effects, if any, of inactivated C1INH remain unknown. Therapy with C1INH has been shown to improve outcome in several animal models of sepsis (19, 23–27). In addition, preliminary data suggest that C1INH may have beneficial effects in septic shock in humans (19).

In this study, we demonstrated that native, active C1INH and reactive center-cleaved, inactive C1INH (iC1INH) protected mice from lethal Gram-negative endotoxemia. This protection was associated with inhibition of LPS-triggered macrophage expression of TNF-α mRNA. Furthermore, C1INH interacts directly with LPS, and this binding appears to be a function of the amino-terminal mucin domain of the protein. These data provide evidence that C1INH, in addition to its function as a serine protease inhibitor, serves as an anti-inflammatory effector via this new mechanism.

Materials and Methods

Mouse endotoxemia model

C57BL/6J mice (male and female, 6–8 wk, 18–22 g; Charles River Breeding Laboratories, Wilmington, MA) were injected i.p. with a lethal dose of Salmonella typhimurium LPS (20 mg/kg; Sigma-Aldrich, St. Louis, MO) following treatment i.p. or i.v. with C1INH (200 μg/mouse; Advanced Research Technologies, San Diego, CA) or iC1INH (200 μg/mouse). In other experiments, mice were injected i.p. with a mixture of LPS (20 mg/kg) and C1INH (200 μg/mouse) or iC1INH (200 μg/mouse). Control mice were injected with LPS (i.p.) or C1INH (i.v.) alone. Mice were monitored for 5 days. Each of the treated groups was compared with the control group that received LPS alone using the log rank test (GraphPad Prism version 3.0; GraphPad Software, San Diego, CA). All experiments were performed in compliance with relevant laws and institutional guidelines and were approved by the Center for Blood Research Animal Care and Use Committee.

Flow cytometry

The murine macrophage cell line RAW 264.7 (American Type Culture Collection, Manassas, VA) was incubated with FITC-conjugated S. typhimurium LPS (175 ng/ml, Sigma-Aldrich) in the presence of active C1INH (10–150 μg/ml), C1INH-C1s complexes, iC1INH (1–150 μg/ml), or a recombinant full-length C1INH (40 μg/ml), and a truncated C1INH (50

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Received for publication January 10, 2003. Accepted for publication June 20, 2003.

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0022-1767/03/$02.00

The Journal of Immunology

2594–2601.
μg/ml) with deletion of the amino-terminal 97 aa (28) in DMEM containing 10% FBS (15 min, 37°C; Life Technologies, Grand Island, NY). C1INH-C1s complexes were prepared by incubation of C1INH (150 μg/ml) with C1s (150 and 300 μg/ml, 60 min, 37°C; Advanced Research Technologies) and iC1INH was generated by incubation of active C1INH with trypsin attached to cross-linked agarose (15 min, 37°C; Sigma-Aldrich) (29, 30). In other experiments, the macrophages were incubated with FITC-conjugated LPS and C1INH that was pretreated with rabbit anti-human C1INH Ab (1 h, 37°C; DAKO, Glostrup, Denmark).

Fluorescence microscopy

Macrophages were plated on glass coverslips (Fisher Scientific, Pittsburgh, PA), treated with FITC-conjugated LPS (175 ng/ml) in the presence of DMEM containing 10% FBS without or with C1INH (50, 100, and 150 μg/ml, 15 min, 37°C) (31, 32), washed with PBS, and fixed with 4% formaldehyde. Fluorescence localization was evaluated by Axiophot fluorescence microscopy (Zeiss, Oberkochen, Germany) with a green fluorescence filter set.

RT-PCR

Total RNA was isolated from the RAW 264.7 macrophages induced with LPS (175 ng/ml) in the presence or absence of C1INH and iC1INH and was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (New England Biolabs, Beverly, MA) with oligo(dT)20 primers (1 h, 37°C; Invitrogen, Carlsbad, CA). PCR primers were designed to generate mouse TNF-α and β-actin fragments with lengths of 200 bp (TNF-α, sense: 5′-ATGAGCACAGAAAGCATGATCC-3′ and antisense: 5′-AGG GCCATTTGGGAACTCCTCTC-3′; β-actin, sense: 5′-TGGATGACGAT AT CCACGGTGC-3′ and antisense: 5′-AGGGTCAGGATACCTCTT-3′). PCR products were analyzed on 1.2% (w/v) agarose gels containing 0.5 μg/ml ethidium bromide and visualized under UV light. Band density was analyzed and quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). In addition, human peripheral venous blood from a normal volunteer was collected in EDTA (1 mg/ml whole blood). A aliquot of the whole blood were treated with LPS at a final concentration of 175 ng/ml in the absence and the presence of added C1INH (5–150 μg/ml) for 15 min at 37°C. Total RNA was isolated from the blood leukocytes and was reverse transcribed using Moloney murine leukemia virus reverse transcriptase with oligo(dT)20 primers. PCR primers were designed for human TNF-α (sense: 5′-ATGAGCACCTGAAACATGATCCGGGACGTG-3′ and antisense: 5′-AGGGTCCTTCGGAATCTTCTCTTG-3′) and human β-actin (sense: 5′-ATGAGCATGATACCGCCGCCTGCTGC-3′ and antisense: 5′-AGGGTGAGGTGCCTCTCTGCTG-3′).

ELISA

Plates (Costar; Corning, Corning, NY) were coated with S. typhimurium LPS (100 μl at a variety of concentrations) at 4°C overnight. Control plates were incubated with BSA (100 μg/ml; New England Biolabs), IgG (20 μg/ml trypsin (Sigma-Aldrich), or PBS in the absence of C1INH. C1INH (100 μl of 150 μg/ml) was incubated with LPS-coated plates for 1 h at room temperature in the presence or absence of FBS (10–100 μl) or human LBP peptides (5–40 ng/ml; Cell Sciences, Norwood, MA). In addition, various concentrations of iC1INH and C1INH (100 μl) were incubated with LPS (100 μl of 87.5 ng/ml-coated plates for 1 h at room temperature. Rabbit anti-human C1INH Ab (1/1000) was incubated for 1 h at room temperature, after which plates were incubated with Immunopure goat anti-rabbit IgG (H + L) conjugated with HRP (1/100,000; Pierce, Rockford, IL). In other experiments, recombinant truncated C1INH (100 μl of 50 μg/ml) and full-length C1INH (100 μl of 40 μg/ml) were incubated with LPS (100 μl of 175 ng/ml)-coated plates for 1 h at room temperature. After washing with PBS, 3,3′-diaminobenzidine (Sigma-Aldrich) substrate was added and the color reactions were developed for 5 min at room temperature and terminated with 3 N HCl. Absorbance was measured at 490 nm using the Revelation Microsoft in an MRX microplate reader (DYNEX Technologies, Chantilly, VA).

FIGURE 1. The effect of native, active C1INH and reactive center loop-cleaved, inactive C1INH on survival of mice in Gram-negative endotoxemia. Mice (C57BL/6J) were injected with either LPS i.p. following treatment with C1INH i.p., or with mixtures of LPS and C1INH i.p. (○). Control mice were injected with LPS i.p. alone (●) or with C1INH i.v. (n = 4) alone (○). The indicated p values are for each treatment group in comparison to the group treated with LPS only.
To determine whether the C1INH used in these studies might be contaminated with high-density lipoprotein, purified C1INH was tested with anti-apolipoprotein A1 Ab using a solid-phase capture sandwich ELISA as compared with standard human plasma (1/10^4 dilution). Apolipoprotein A1 could not be detected in the C1INH preparation (data not shown).

**C1INH blocks LPS binding to macrophages**

FITC-labeled LPS (175 ng/ml), in the presence of 10% FBS as a source of LBP, binds to the murine macrophage cell line RAW 264.7, but does not bind in the absence of FBS (Fig. 2A). C1INH, at concentrations of 37.5–150 µg/ml, which are within the physiological concentration range in human plasma (21), completely blocked this binding (Fig. 2A). The fluorescence intensity also was decreased when macrophages were pretreated with C1INH (150 µg/ml) for 15 min at 37°C, after which the C1INH was removed and LPS added. However, the effect was only observed at a lower concentration of LPS (40 ng/ml) and was not apparent at 175 ng/ml LPS (Fig. 2B). This suggested that C1INH interacts primarily with endotoxin rather than with a cellular receptor. Anti-C1INH Ab (175 µg/ml) completely abrogated the effect of C1INH (75 µg/ml) on LPS binding to macrophages, whereas BSA (100 µg/ml) did not interfere (Fig. 2C). To visualize the binding of LPS to macrophages, RAW 264.7 cells were cultured on microscope slides in the presence of FITC-conjugated LPS (175 ng/ml) that had been incubated with or without C1INH. The fluorescent signals were decreased at a concentration of 50 µg/ml C1INH and were eliminated at 100–150 µg/ml C1INH (Fig. 2D). LPS-induced TNF-α mRNA was detected in RAW 264.7 cells using RT-PCR. LPS-mediated up-regulation of TNF-α mRNA was completely suppressed following treatment with 150 µg/ml C1INH (Fig. 3A). Dose-response analysis showed that LPS (175}

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**FIGURE 2.** The effect of C1INH on the binding of FITC-conjugated LPS to the murine macrophage cell line RAW 264.7. RAW 264.7 macrophages were incubated in DMEM containing 10% FBS at 37°C (FITC-LPS binding, thick line; control, shaded field for A–C). A, The binding of LPS (175 ng/ml) to RAW 264.7 cells in the presence of C1INH (10, 37.5, 75, and 175 µg/ml). B, The binding of LPS (40 and 175 ng/ml) to RAW 264.7 cells after incubation of the cells with C1INH (150 µg/ml) for 60 min at 37°C. C, Anti-C1INH Ab (175 µg/ml) reversed the inhibitory effect of C1INH (75 µg/ml) on the binding of LPS (175 ng/ml) to macrophages, but BSA (100 µg/ml) did not interfere with C1INH-mediated inhibition (150 µg/ml) of LPS (175 ng/ml) binding to macrophages. D, The effect of C1INH (50, 100, and 150 µg/ml) on the binding of LPS (175 ng/ml) to macrophages analyzed by fluorescence microscopy.
ng/ml)-induced expression of TNF-α mRNA was completely inhibited with 37.5 μg/ml of intact active C1INH and with 10 μg/ml iC1INH (Fig. 3). Similarly, C1INH at concentrations of 37.5–150 μg/ml suppressed LPS-induced TNF-α mRNA expression by cells in whole human blood (Fig. 3 D).

Interaction of C1INH with LPS

We immobilized LPS in microtiter wells at a variety of concentrations and measured the amount of C1INH binding in the presence or absence of BSA (100 μg/ml) or IgG (20 μg/ml). The binding of C1INH (150 μg/ml) was maximal to 175 ng/ml LPS and neither BSA nor IgG interfered with this binding (Fig. 4A). Dose-response analysis of C1INH and iC1INH binding to LPS (87.5 ng/ml) showed similar binding curves, although maximal binding may occur with a somewhat lower concentration of iC1INH (37.5 μg/ml) than of intact C1INH (75 μg/ml; Fig. 4B). FBS (10–100 μl) and the human LBP peptide (5–40 ng/ml) reduced C1INH binding by ~80 and 75%, respectively (Fig. 4C and D). Binding of the negatively charged LPS to proteins results in a characteristic anodal shift in the mobility of the protein on native PAGE (31, 32). C1INH-LPS mixtures, when analyzed by native PAGE/Western blot with anti-C1INH Ab (Fig. 4E) and by native PAGE following incubation of C1INH with 3H-LPS (Fig. 4F), also demonstrated an anodal shift, which increased with increasing amounts of LPS. LPS does not alter the electrophoretic mobility of a variety of other proteins, including OVA, α2-macroglobulin, and catalase (32). C1INH incubated with LPS for 30 min at 37°C before the addition of C1s had no effect on either the rate or extent of complex formation with C1s in comparison to C1INH incubated with C1s in the absence of endotoxin (Fig. 4G). Therefore, although Gram-negative endotoxin LPS binds directly to C1INH, it neither enhances nor suppresses the ability of C1INH to complex with target protease.

The C1INH amino-terminal domain is responsible for the interaction with LPS

To investigate whether an intact reactive center loop is required for inhibition of LPS binding to macrophages, we prepared C1INH-C1s complexes and iC1INH (1, 5, 10, 37.5, 75, and 150 μg/ml) by

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**FIGURE 3.** C1INH-mediated inhibition of LPS-induced TNF-α mRNA expression in the murine macrophage cell line RAW 264.7. Total RNA from macrophages was isolated after treatment with LPS (175 ng/ml). RT-PCR was performed using mouse TNF-α cDNA and β-actin cDNA primers. A, RAW 264.7 cells were induced with LPS for 0, 5, 15, 30, 60, and 120 min at 37°C in the presence of C1INH (150 μg/ml). B, RAW 264.7 cells were induced with LPS for 30 min at 37°C in the presence of C1INH (150, 75, 37.5, 10, 5, 1, and 0 μg/ml). C, RAW 264.7 cells were induced with LPS for 30 min at 37°C in the presence of iC1INH (150, 75, 37.5, 10, 5, 1, and 0 μg/ml). D, Total RNA from whole human blood cells was isolated after treatment with LPS (175 ng/ml) in the presence of C1INH (150, 75, 37.5, 10, 5 and 0 μg/ml). RT-PCR was performed using human TNF-α and β-actin oligonucleotide primers.

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cleaving C1INH at P1-P1' with trypsin. Both C1INH-C1s complexes (Fig. 5A) and iC1INH (Fig. 5B) retained the ability to block FITC-LPS (175 ng/ml) binding to RAW 264.7 cells. Therefore, an intact reactive center loop is not required for this inhibition.

Binding of a truncated C1INH molecule consisting of amino acid residues 98–478 (28) to LPS, as assessed by ELISA, was reduced by 85% compared with full-length C1INH (Fig. 6A). The truncated C1INH also did not block the binding of LPS to RAW 264.7 cells (Fig. 6B), but full-length C1INH in the presence of the truncated protein was able to block binding (data not shown). Mixtures of recombinant full-length C1INH and truncated C1INH with LPS were analyzed using native PAGE followed by Western blot. The recombinant full-length C1INH-LPS mixture showed an anodal shift (Fig. 6C) very similar to that of native C1INH (Fig. 4E). The electrophoretic mobility of the recombinant truncated C1INH, on the other hand, was unchanged when mixed with LPS (Fig. 6D). Therefore, the amino-terminal domain appears to be required for the inhibition of LPS-mediated macrophage activation that is a result of the interaction of C1INH with Gram-negative endotoxin LPS.

Discussion

C1INH is an acute phase protein with a mean plasma level of ~250 mg/L. It may increase up to 2.5-fold during inflammation (33). Antigenic levels of C1INH tend to be normal in patients with...
LPS transports LPS to macrophage CD14 to form LPS-CD14 complexes that initiate intracellular signaling reactions by binding to Toll-like receptors. This results in the release of a variety of inflammatory products, including TNF-α. LPS can also interact with a variety of structurally diverse proteins in plasma, including proteases, complement components, lipoproteins, MD-2, L-selectin, P-selectin, and CD14 (31–36). The binding of LPS to RAW 264.7 cells is dependent on the presence of LBP in serum; in the absence of serum, little binding is observed (37). The binding of LPS by other proteins may affect the binding activity of LPS to LBP or the transfer of LPS to macrophages. The data presented here suggest the possibility that, in addition to protection via inhibition of complement and contact system activation, C1INH also may protect from endotoxin shock via a direct interaction with endotoxin. We demonstrated that plasma-derived and recombinant full-length C1INH (Fig. 6, A and C) bind with relatively high affinity to purified bacterial endotoxin LPS from S. typhimurium (Fig. 4, A, B, E, and F) and that this binding inhibits the binding of LPS to RAW 264.7 cells (Figs. 2, A and D, and 6B), probably by preventing the interaction of endotoxin with LBP. This inhibition prevents macrophage activation as shown by suppression of TNF-α mRNA synthesis by the RAW 264.7 cells (Fig. 3, A–C) and by leukocytes in whole human blood (Fig. 3D).

Somewhat surprisingly, the ratio of C1INH to LPS that is protective in vivo is lower than that required to inhibit LPS binding to RAW 264.7 cells or to bind to LPS in the gel shift experiments. Treatment with C1INH at a C1INH:LPS molar ratio of 1:3 reduced mortality from 100 to 45–50% (Fig. 1). LPS is present in aqueous solution in micellar form with an effective molecular mass >1 million. Upon interaction with proteins to which it binds, it dissociates to individual subunits with $M_r \sim 4000$ (32). In the in vitro experiments, the molar ratio of C1INH ($M_r \sim 100,000$) to LPS was $\sim 2:1$ (Fig. 4F) and $\sim 4:1$ (Fig. 4E) in the gel shift experiments, which probably provide the best estimate of stoichiometry. A ratio of 9:1 C1INH:LPS was required to inhibit binding of LPS to RAW 264.7 cells (Fig. 2A) or to inhibit TNF-α expression by these cells (Fig. 5B). The reason for the difference between the in vivo and in vitro experiments is not obvious. However, in vivo, multiple physiologic mechanisms contribute to protection from endotoxin including, among others, the binding of endotoxin to lipoproteins (12–15) and complement-mediated clearance (26). In addition, the animal also has circulating C1INH that would contribute. Therefore, because of these other mechanisms, the quantity of C1INH required for an effect in vivo might be less than would be predicted based on the in vitro experiments in which only the direct binding by C1INH would be operative.

Another important question is whether cleaved iC1INH, in fact, provides a greater degree of protection than does the intact active protein. A single dose of i.v. iC1INH increased survival from 50 to 60%, a difference that was not statistically significant (Fig. 1). However, mixing the iC1INH with LPS before i.p. administration resulted in an improvement in survival from 65 to 100%, a difference that was statistically significant ($p = 0.0074$). These findings are consistent with the in vitro studies that demonstrated that a lower concentration of iC1INH was required to inhibit TNF-α production by RAW 264.7 cells (Figs. 3, B and C), to bind to LPS in ELISA (Fig. 4B), and to inhibit LPS binding to RAW 264.7 cells (Figs. 2A and 5B).

Because LPS can interact with a number of different proteins in plasma, it is possible that a contaminant in C1INH preparations could be responsible for the results observed here. This seems extremely unlikely for the following reasons. First, the inability to detect apolipoprotein A1 suggested that the C1INH preparations are not contaminated with high-density lipoproteins, which also...
can bind LPS and prevent macrophage activation (12–15). Second, the inhibition of binding of LPS to RAW 264.7 cells by the C1INH preparation was reversed with an Ab to C1INH. Third, several of the effects (inhibition of binding of LPS to RAW 264.7 cells, binding of C1INH to LPS in an ELISA, complex formation demonstration by gel shift analysis) were duplicated with a recombinant full-length C1INH protein. It is unlikely that the recombinant C1INH would contain the same contaminating proteins as the plasma-derived C1INH. It therefore seems most likely that the findings described here are the result of a property of C1INH and not of a contaminant.

The primary recognition element for serpin-protease association is a 15-aa residue-exposed segment known as the reactive center loop. The specificity of serpins is determined by the amino acid sequence within the reactive center loop, particularly the amino acids from approximately P5 through P5′ (38). As might be expected, this region therefore reveals little homology among different serpins. Reactive center loop cleavage by either target or non-target proteases results in a structural rearrangement with complete insertion of the loop into the five-stranded β sheet A. In the case of nontarget proteases, the active protease is released, while target proteases remain covalently bonded to the serpin via the P1 residue (39, 40). In either case, the ability to inhibit additional protease is lost. We demonstrated that C1INH-C1s complexes or C1INH inactivated by cleavage of the reactive center loop with trypsin retained the ability to block the binding of LPS to macrophages. C1INH has an amino-terminal heavily glycosylated mucin-like domain (aa 1–120) that contains seven repeats of the tetrapeptide sequence Glx-Pro-Thr-Thr or variants thereof (28, 41). This domain does not influence complex formation with target proteases (28). The functional significance of the amino-terminal extension of C1INH is unknown, although several possibilities have been suggested (28). Deletion of the amino-terminal 97 aa residues abrogated the ability of C1INH to bind to LPS. Therefore, one potential role for this domain is to participate in host protection from Gram-negative sepsis via this direct binding activity.

FIGURE 6. The effect of recombinant truncated C1INH on LPS binding. A, Recombinant truncated C1INH (50 µg/ml) had greatly reduced binding to LPS (175 ng/ml) as assessed by ELISA. B, FITC-conjugated LPS (175 ng/ml) binding to RAW 264.7 macrophages was not inhibited with recombinant truncated C1INH (50 µg/ml; LPS binding, thick line; control, shaded field). C, LPS (10 and 20 µg) clearly altered the electrophoretic mobility of recombinant full-length C1INH (40 µg/ml) as measured by native PAGE/Western blot (lanes 1 and 2). D, LPS (10 and 20 µg) did not change the electrophoretic mobility of recombinant truncated C1INH (50 µg/ml) as detected by native PAGE/Western blot (lanes 1 and 2).
The above-described results indicate that protease inhibitory activity is not required for the binding of LPS to C1INH and that the binding site(s) is(are) almost certainly contained within the amino-terminal domain. Therefore, it is likely that C1INH contributes to protection from Gram-negative endotoxin shock via three mechanisms: inhibition of excessive complement activation which would limit the amount of C5a generated (42); inhibition of contact system activation which would limit the amount of activated plasma kallikrein, factor XIIa, and bradykinin generated (19, 20); and by direct inhibition of endotoxin binding to macrophages which thereby suppresses macrophage activation.

Acknowledgments

We thank Dr. Chester Alper and Dr. Eileen Remold-O’Donnell for critical review of this manuscript.

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