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Saturable CD14-Dependent Binding of Fluorescein-Labeled Lipopolysaccharide to Human Monocytes

ANNET TROELSTRA,* PETER ANTAL-SZALMAS,† LIA A. M. DE GRAAF-MILTENBURG, ANNEMARIE J. L. WEERSINK, JAN VERHOEF, KOK P. M. VAN KESSEL, AND JOS A. G. VAN STRIJP

Eijkman-Winkler Institute for Medical Microbiology, Utrecht University, Utrecht, The Netherlands

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We used rough lipopolysaccharide (ReLPS) to construct a fluorescein-labeled LPS (FITC-LPS) with a very high labeling efficiency that bound to isolated human monocytes in a CD14-dependent fashion and that in this respect behaved indistinctively from native LPS. The CD14-dependent binding could be inhibited either by a 1,000-fold excess of unlabeled LPS or by polymyxin B, bactericidal/permeability-increasing protein, cationic protein 18, or soluble CD14. Although this FITC-LPS preparation no longer possessed the ability to prime neutrophils for the production of reactive oxygen species or to stimulate human monocytes to produce tumor necrosis factor, activation of the *Limulus* amoebocyte lysate cascade was comparable to activation by native LPS. Binding to monocytes was enhanced by human pooled serum (HPS) or LPS-binding protein (LBP) for LPS concentrations up to 100 ng/ml and was completely CD14 dependent. For LPS concentrations exceeding 100 ng/ml, binding was still partially CD14 dependent, but not HPS or LBP dependent. CD14-dependent association of LPS with monocytes was shown to be totally saturable. In conclusion, we found an HPS- or LBP-dependent binding of FITC-LPS to monocytes that was CD14 dependent at up to 100 ng of LPS per ml, and saturation of binding was shown.

Lipopolysaccharide (LPS, or endotoxin) is the major component of the outer membrane of gram-negative bacteria and is able to activate a variety of biochemical pathways (2). A very important phenomenon is the activation of monocytes and other phagocytes, one of the major events that under certain circumstances can lead to the complex and life-threatening clinical syndrome of septic shock. The search for a specific membrane receptor for LPS on these cells has led to the discovery of the involvement of CD14 (41, 42). This glycosylphosphatidylinositol-anchored receptor of 55 kDa is found on the membranes of monocytes and macrophages and in a 10-fold-lower amount on polymorphonuclear granulocytes (12). CD14 is present in the serum of healthy individuals as a soluble receptor (sCD14) at a concentration of 2 to 6 μg/ml (23) and in three- to four-times-higher concentrations in patients with acute infections (1, 22). CD14-negative cells, such as endothelial or epithelial cells, that are nonresponsive to LPS can be activated by LPS in the presence of sCD14 via a mechanism that is not yet completely understood (9, 13).

The interaction of LPS with CD14 is enhanced by LPS binding protein (LBP), an acute-phase protein that is synthesized by hepatocytes and that is found in normal human serum in concentrations of 1 to 10 μg/ml. LBP concentration is elevated during infection (35, 41). LBP is known to transfer LPS to CD14, giving rise to amplified responses of cells to LPS (28, 33), and recently has been shown to mediate LPS neutralization as well (14, 29, 43).

Binding studies have been of great value in unraveling the interactions of LPS with the cell surface, and a variety of methods and cell types have been used for this. Although LPS binding has been described as being inhibited by specific monoclonal antibodies (MAbs) directed at CD14, saturable LPS binding was not consistently observed. LPS concentrations giving rise to saturable binding differed greatly for promonocytic cell line THP-1 cells (from 100 to 120 [20] to 500 ng [19] of [^{3}H]LPS per ml), for Chinese hamster ovary cells expressing human CD14 (CHO hCD14 cells) (from 80 to 120 [10] to 500 ng [19] of [^{3}H]LPS per ml), and for human monocytes (from 700 to 900 [7] to 200 to 2000 ng [11] of [^{3}H]LPS per ml. Unsaturable binding was described for human peripheral blood monocytes incubated with concentrations of up to 10 μg of ^{125}I-LPS per ml (38). Using fluorescein isothiocyanate-labeled LPS (FITC-LPS) and flow cytometry offers the advantage of studying LPS binding to different cell types at the same time on a per-cell basis. Unfortunately, relatively high LPS concentrations have to be used in order to detect LPS binding to monocytes, most probably due to the inefficient FITC labeling of LPS. Several authors described unsaturable binding of FITC-LPS to monocytes, i.e., at 1 to 100 μg of FITC-LPS per ml (39) and at up to 50 μg of FITC-LPS per ml (6); one study showed saturation at 1 μg/ml (15).

In the present study we labeled rough LPS (ReLPS) with FITC by a new technique (8, 30, 32), leading to a highly efficient labeling with a LPS-to-FITC ratio close to 1:1. This preparation enabled us to study the interaction between LPS and monocytes at a wide range of LPS concentrations, including the concentration range that has been described as being present in the blood of patients with gram-negative sepsis (37).

MATERIALS AND METHODS

Materials. ReLPS from *Salmonella minnesota* Re595 and polymyxin B were purchased from Sigma Chemical Co. (St. Louis, Mo.). We are grateful for the following gifts: recombinant human LBP and sCD14 from H. Lichenstein (AMGEN, Thousand Oaks, Calif.), bactericidal/permeability-increasing protein (BPI) from P. Elsbach (New York University, New York, N.Y.), and rabbit cationic protein 18 (CAP 18) from J. Larrick (Palo Alto Institute of Molecular Medicine, Mountain View, Calif.).

* Corresponding author. Mailing address: Eijkman-Winkler Institute for Medical Microbiology, AZU Room G04.515, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands. Phone: (31) 30-2506525. Fax: (31) 30-2541770. E-mail: a.troelstra@lab.azu.nl.

† Present address: 3rd Department of Medicine, University Medical School of Debrecen, 4004 Debrecen, Hungary.

TABLE 1. Properties of different LPS preparations

FITC-LPS preparation[a]	KDO content[b] (mM)	FITC content[c] (mM)	Free FITC content[d] (%)	FITC/LPS ratio	Comparison with native LPS		
					LAL activity (%)	TNF induction (%)	CL[e] response (%)
New	294 ± 29	341 ± 32	<0.1	1:0.9	133 ± 25	<0.1	<0.1
Old[f]	294 ± 29	7 ± 2	<1	1:41.7	115 ± 21	95 ± 13	97 ± 11

[a] Old and new designations are described in the text. The FITC-LPS content of both preparations was 1 mg/ml.
[b] The KDO contents were measured, and then samples were adjusted to a level of 1 mg of LPS per ml (294 mM). The KDO determination has a standard deviation of 10% at these low concentrations of LPS; this variation is calculated throughout the assay.
[c] As determined by A_{492} measurements.
[d] As determined by gel filtration (see text).
[e] CL, chemiluminescence.
[f] Preparation as described by Skelly et al. (31).

Serum and MAbs. Blood was drawn from healthy human volunteers after informed consent, and normal human serum (NHS) was obtained after pooling the sera of 10 donors and stored until use at −70°C.

Hybridoma cells secreting murine MAb 60bca (immunoglobulin G [IgG_1]; directed against CD14), 3c10 (IgG2b; anti-CD14), 26ic (anti-CD14; IgG2b κ), 44a (IgG_1; directed against CD11b), and W6/32 (IgG2a; anti-HLA-A, -B, and -C) were obtained from the American Type Culture Collection, Rockville, Md. MAbs 357-101-4 and 2-179-E11 (both anti-human tumor necrosis factor alpha [TNF-α]) were obtained from the European Collection of Animal Cell Cultures (Porton Down, United Kingdom). Culture supernatants were collected, and MAbs were purified over a protein G column (Pharmacia, Uppsala, Sweden). Other MAbs used, which were commercially available, were MEM18 (anti-CD14; IgG1; Sanbio, Uden, The Netherlands) and LeuM3 and LeuM3-PE (IgG2b; anti-CD14; Becton Dickinson, Mountain View, Calif.). Anti-TNF MAb 2-179-E11 was biotinylated with NHS-LC biotin (Pierce, Rockford, Ill.) according to the manufacturer's specification.

Cells. Mononuclear cells were isolated from peripheral blood obtained from healthy human volunteers after informed consent. Blood was collected into heparin tubes (Greiner, Frickenhausen, Germany) and diluted 1:1 with phosphate-buffered saline (PBS) (pH 7.4), and the mixture was loaded on a Ficoll (Pharmacia) and Histopaque (density, 1.119 g/ml; Sigma) gradient. After centrifugation for 20 min at 250 × *g* at room temperature, the mononuclear fraction and the interface of neutrophils were collected and washed with RPMI medium (Gibco BRL, Gaithersburg, Md.) (pH 7.4) containing 0.05% human serum albumin (HSA; CLB, Amsterdam, The Netherlands). The remaining erythrocytes were lysed for 45 s with distilled water, after which concentrated PBS was added to reestablish the isotonic condition. Cells were washed and resuspended in RPMI medium at 10^7 cells/ml and tested for viability and purity. The viability of the cells, judged by trypan blue or propidium iodide exclusion, exceeded 95%. The purity of the cell fraction was checked by microscopy of cytospin slides and was over 98% for the neutrophil fraction. The mononuclear fraction consisted of 15 to 25% monocytes.

The MonoMac-6 cell line was kindly provided by H. W. L. Ziegler-Heitbrock (Institute of Immunology, University of Munich, Munich, Germany). MonoMac-6 cells were cultured in 5% CO_2 at 37°C in Iscove modified Dulbecco medium (Gibco BRL) containing 10% fetal calf serum.

Preparation of labeled LPS. ReLPS (4 mg) was made monomeric by treatment with 2 ml of 0.5% triethylamine (Rathburn Chemicals Ltd., Walkerburn, United Kingdom). After sonication for 15 min on ice, 200 μl of 100 mM EDTA (Merck, Darmstadt, Germany) was added. After adjustment to pH 5 with 10 μl of 1 N HCl, 800 μl of 0.25 M borate buffer (pH 10.5) containing 20 mg of FITC (isomer I; Sigma Chemical Co.) was added. The mixture was sonicated again for 1 min and after the addition of 1 ml of 1.6% sodium deoxycholate (Merck) was incubated for 18 h at 37°C while rotating. Aggregates were pelleted at 10,000 × *g*, and the supernatant was concentrated in a dialysis bag with polyethylene glycol 6000 (BDH Biochemical, Poole, United Kingdom). After dialysis against PBS the preparation (2 ml) was run over a 3-cm-diameter desalting column (containing Excellulose GF-5; Pierce) to separate the FITC-LPS from free FITC molecules. The fractions containing FITC-LPS were pooled, concentrated, and dialyzed against PBS. Desalting and concentration of FITC-LPS were repeated once more. The concentration of FITC in the final preparation was determined spectrophotometrically at 492 nm, with an extinction coefficient for FITC (E_{492}) of 8.5×10^4 M^{-1} cm^{-1}. To determine the amount of LPS, the level of 2-keto-3-deoxyoctulosonic acid (KDO) was measured by the thiobarbiturate assay (3, 4), with a standard range of unlabeled ReLPS. The biological activity of the FITC-LPS preparation was tested in a *Limulus* assay (Endotoxin Coatest; Chromogenix AB, Mölndal, Sweden) according to the manufacturer's instructions, calibrated with unlabeled ReLPS. Biological activity was further assayed as TNF induction in the MonoMac-6 cell line and the ability to prime neutrophils for an enhanced oxidative response, as described below.

TNF induction. The ability of FITC-LPS to induce TNF was tested with the MonoMac-6 cell line. MonoMac-6 cells (5×10^5) were mixed with 50 μl of serial dilutions of FITC-LPS in the presence of 1% NHS in RPMI medium supplemented with 0.1% HSA. After incubation at 37°C for 2.5 h, the supernatant fluid was collected and analyzed in a TNF-α-specific enzyme-linked immunosorbent assay (26). In short, a microtiter plate was coated with anti-TNF MAb 357-101-4 and TNF was detected with biotinylated monoclonal 2-179-E11 and peroxidase-labeled streptavidin (Southern Biotechnology Associates, Birmingham, Ala.). The concentration of TNF was determined by comparison with a serially diluted standard of recombinant human TNF-α (Sigma Chemicals).

Neutrophil priming. The priming of neutrophils for an enhanced chemiluminescence response was performed as described before (34). In short, neutrophils were diluted in Hanks balanced salt solution (HBSS) with 2% HSA and primed for 30 min at 37°C with 1 ng of LPS per ml in the presence of human pooled serum (HPS) or LBP. Next, the sample was transferred to a test tube and placed in the luminometer (Autolumat LB 953; Berthold GmbH & Co., Wildbad, Germany). By using automated injection, cells were stimulated with 10^{-6} M formyl-methionyleucyl-phenylalanine in the presence of 180 μM luminol. The chemiluminescence response was measured automatically during a 10-min time interval, and data were analyzed with the AXIS software package (Berthold GmbH & Co.). Absolute counts were obtained by calculating the area under a 10-min segment of the chemiluminescence curve.

Flow cytometric binding assay. In a total volume of 50 μl, 3×10^5 mononuclear cells were incubated with increasing concentrations of FITC-LPS (0.1 to 2,500 ng/ml) in the presence of NHS, LBP, or HBSS containing 0.1% bovine serum albumin (Organon Teknika, Turnhout, Belgium). Samples were incubated for 30 min at 37°C, gently shaking. For blocking experiments cells were first incubated for 15 min on ice with 10 μg of MAb 60bca per ml directed against CD14. For the binding inhibition studies, 2.5 ng of FITC-LPS per ml was preincubated for 15 min at 37°C with 4% HPS and either increasing amounts of native LPS, 3 mM EDTA, 10 μg of polymyxin B per ml, 1 μg of rabbit CAP 18 per ml, 1 μg of human BPI per ml, or 10 μg of sCD14 per ml. To quench extracellular fluorescence, trypan blue treatment of the cells was performed as described earlier (39). Subsequently, cells were allowed to bind and flow cytometry was performed. The mean fluorescence of 10,000 monocytes was measured in a flow cytometer (FACScan; Becton Dickinson). Monocytes were distinguished from lymphocytes on the basis of their forward and sideward scatter properties and occasionally on the basis of double labeling with phycoerythrin-labeled MAb leuM3 directed against CD14.

RESULTS

Evaluation of the FITC-LPS preparation. As we were interested in the LPS-monocyte interaction at very low concentrations of LPS, we needed a preparation with better FITC labeling than the "old preparation" having a labeling efficiency of 1 molecule of FITC per 40 molecules of LPS, as described before (39). Therefore, LPS molecules were converted to the monomeric state and were kept in the monomeric state during the labeling steps by using a combination of described procedures. The resulting "new FITC-LPS preparation" was analyzed for its KDO content as a function-independent estimation of the LPS concentration, and the optical density at 492 nm was determined as an assessment of FITC content. Gel filtration on Superose 12 (FPLC; Pharmacia) coupled to a flowthrough fluorometer (Perkin-Elmer Ltd., Beaconsfield, United Kingdom) was used to estimate the amount of free FITC. To evaluate the biological activity of this preparation, experiments testing the ability to induce TNF in a monocytic cell line, to

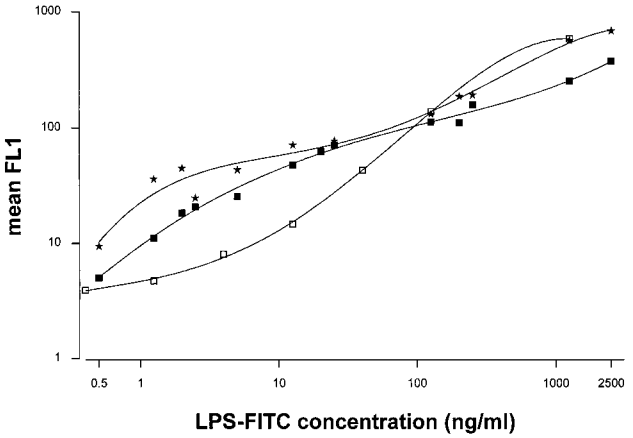

FIG. 1. Binding of FITC-LPS is LBP dependent. Monocytes were incubated with different concentrations of FITC-LPS in the presence of 1% HPS (solid squares), 100 ng of LBP per ml (stars), or buffer (open squares) for 30 min at 37°C and were analyzed by flow cytometry. Each data point represents the mean of three independent experiments using monocytes from different donors. Because of the extreme differences in fluorescence, the *y* axis is displayed with a logarithmic scale.

prime neutrophils, and to activate the *Limulus* cascade were performed. Table 1 summarizes the results from these determinations. For comparison, the old and new preparations were adjusted to the same LPS concentration on the basis of their KDO contents. KDO-versus-FITC data were used to calculate the labeling efficiency of the new LPS preparation. The amount of FITC per LPS molecule was estimated to be 1.16 for the new preparation. As determined by gel filtration, the fraction of free FITC was <0.1%. The ability to stimulate the Limulus amoebocyte lysate (LAL) cascade was completely intact. However, the ability to stimulate human cells in a CD14-dependent fashion, as evaluated by the induction of TNF and the priming of neutrophils, was severely impaired for this new FITC-LPS preparation.

Binding to monocytes. We studied the interaction between FITC-LPS and monocytes in a broad range of FITC-LPS concentrations. In Fig. 1, the enhancing effect of HPS or LBP on the LPS-monocyte interaction is illustrated. In the presence of low concentrations of LBP (100 ng/ml) or HPS (1%), 10- to 30-fold-less LPS resulted in the same fluorescence signal observed in the absence of LBP or HPS. The association was dependent on the presence of either LBP or HPS for LPS concentrations up to 100 ng/ml. At LPS concentrations of 100 ng/ml or higher, the signal appeared to be totally independent of LBP. Figure 2A depicts dose-dependent LPS binding to monocytes in both the absence and presence of 10 μg of anti-CD14 MAb 60bca per ml. Control antibodies did not block the interaction between cells and LPS (data not shown). Up to a concentration of 100 ng/ml, LPS binding was completely CD14 dependent. Above this concentration, only part of the interaction was inhibited by anti-CD14 MAb 60bca (also with higher concentrations of blocking MAbs) and interaction occurred also in the absence of LBP or HPS. Although the total binding was not saturable, the CD14-mediated interaction (calculated as the difference between total binding and CD14-independent binding in Fig. 2A) was saturable at LPS concentrations over 100 ng/ml (Fig. 2B). This was independent of the concentration of HPS: both at 1 and 4%, the same saturation curves were found. In Fig. 3, representative fluorescence histograms are shown, illustrating the CD14 dependency of LPS binding to monocytes. In contrast to earlier reports (15) this FITC-LPS preparation always bound to the monocyte population as a whole; we never observed binding to subpopulations.

Trypan blue quenching was performed in order to discriminate between surface binding and internalization of FITC-LPS. After quenching, the fluorescence signal was reduced to only 5 to 20%, so 80 to 95% was indeed due to surface-bound FITC-LPS. This finding was confirmed by experiments with monocytes preincubated with buffer containing 5 mM 2-deoxyglucose, 10 mM sodium azide, and 2 mM sodium fluoride in order to inhibit internalization. This treatment did not reduce the association of LPS to the cells by more than 5%.

Serum dependence. Figure 4 represents the binding of 2.5 ng of FITC-LPS per ml to monocytes at 37°C in the presence of an HPS concentration varying from 0.1 to 75%. Optimal association was found at between 4 and 10% HPS. At higher HPS concentrations (between 30 and 75%) the signal was roughly half of that at 4%. When the binding was performed in the

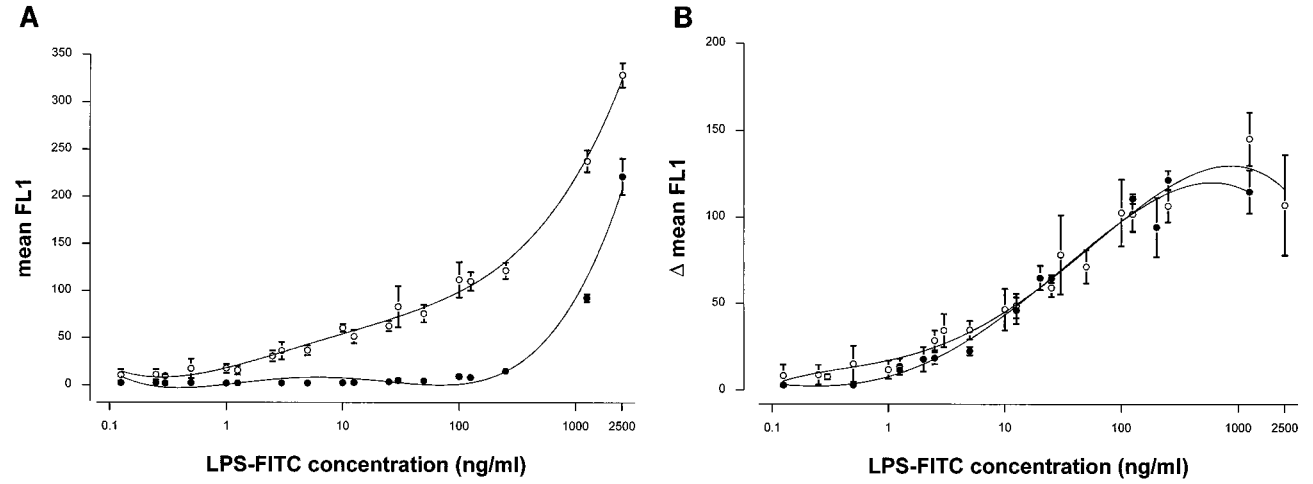

FIG. 2. Saturated CD14-dependent binding of FITC-LPS to monocytes. (A) Monocytes were incubated with different concentrations of FITC-LPS and 4% HPS for 30 min at 37°C in the presence (solid circles) or absence (open circles) of the blocking anti-CD14 MAb 60bca and were analyzed by flow cytometry. (B) The difference in binding with and without anti-CD14 was determined in the presence of 1% HPS (solid circles) and 4% HPS (open circles). Each data point represents the mean of three to seven independent experiments with different donors ± the standard error of the mean.

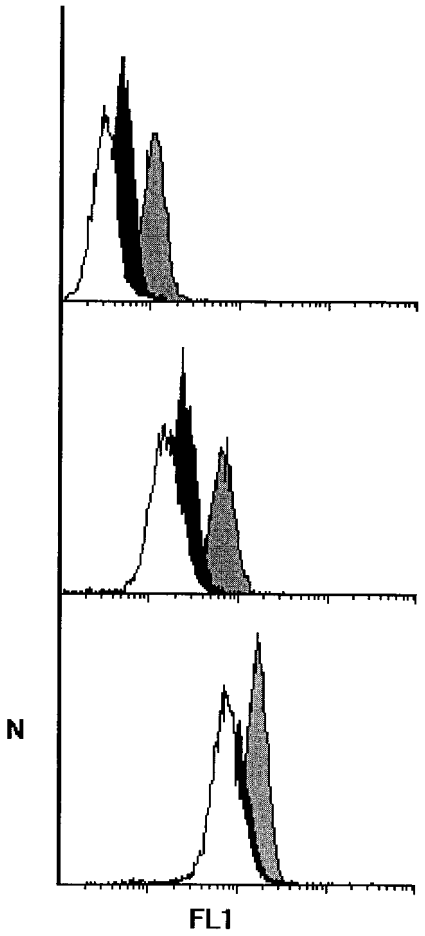

FIG. 3. CD14-dependent binding of FITC-LPS to mononuclear cells. Shown are fluorescence histograms of a representative experiment of FITC-LPS binding to monocytes incubated with (from top to bottom) 10 ng, 100 ng, or 1 μg of FITC-LPS per ml. Background LPS binding in the absence of HPS is depicted in the white histograms. Gray histograms show binding in the presence of 1% HPS, and black histograms show binding in the presence of both HPS and blocking anti-CD14 MAb 60bca.

presence of a CD14-blocking MAb, no association of LPS with monocytes was observed at all HPS concentrations tested.

Modulation of binding. Using 2.5 ng of FITC-LPS per ml, we studied the specificity of the monocyte-LPS interaction (Fig. 5). Anti-CD14 MAbs 60bca, 3c10, and MEM18 are known to interfere with CD14-dependent activation processes, and preincubation of the mononuclear cell with each one of these MAbs did result in complete inhibition of LPS binding (data shown for 60bca only). MAbs LeuM3 and 26ic, two monoclonal anti-CD14 antibodies that are known not to interfere with CD14-dependent activation, indeed failed to block binding. Neither control MAb W6/32 (directed against HLA) nor MAbs against complement or Fc receptors inhibited FITC-LPS binding (data not shown). Binding to monocytes in the presence of HPS or LBP was not affected by the addition of 3 mM EDTA, confirming that CR3 was not involved in this process. sCD14 only partially inhibited the binding to monocytes, which is in accordance with the effect we observed with neutrophil priming (34). sCD14 competes with the membrane-bound receptor for LPS (14, 29) and also, at low LBP concentrations, mediates the association of LPS to the membrane resulting in a response. Therefore, total inhibition was not expected. The addition of unlabeled LPS almost completely prevented the binding of FITC-LPS to monocytes, although a 1,000-fold excess of unlabeled over labeled LPS was needed. Polymyxin B, CAP 18, and BPI have all been described as interacting with LPS and blocking its toxicity. In our binding assay they all prevented binding at concentrations that were described earlier as preventing activation as well (16–18, 24, 25, 31, 40).

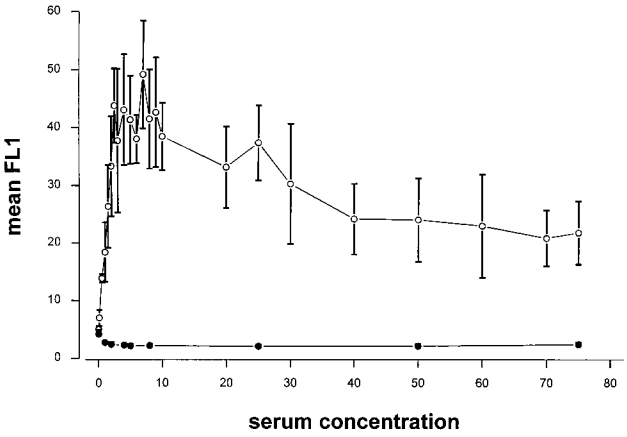

FIG. 4. HPS enhances FITC-LPS binding. Different concentrations of HPS were incubated with 2.5 ng of FITC-LPS per ml and monocytes for 30 min at 37°C in the presence (solid circles) or absence (open circles) of anti-CD14 MAbs. Binding was measured by flow cytometry. Data represent the means of three or more independent observations ± the standard errors of the means.

DISCUSSION

In the present paper experiments with a new, very efficiently labeled FITC-LPS preparation are described. A flow cytometric assay was used to study the association of FITC-labeled

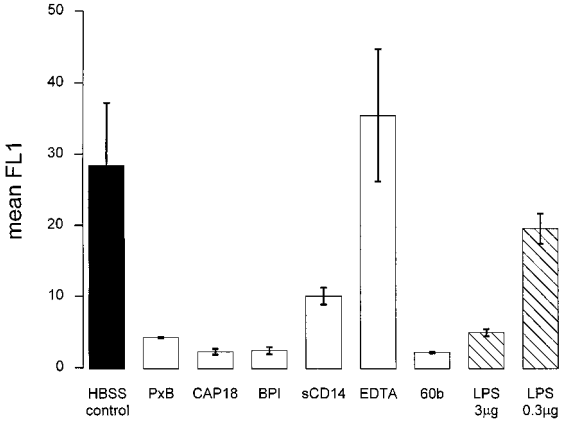

FIG. 5. Inhibition of FITC-LPS binding. Monocytes were incubated with 2.5 ng of FITC-LPS for 30 min at 37°C in HBSS with 4% HPS as a control sample. FL1 values reflect the amounts of FITC-LPS bound to the monocytes as measured by flow cytometry. In parallel samples, 2.5 ng of LPS per ml was preincubated with 4% HPS and either 10 μg of polymyxin B (PxB) per ml, 1 μg of rabbit CAP 18 (CAP18) per ml, 1 μg of human BPI (BPI) per ml, different concentrations of nonlabeled LPS, or 10 μg of sCD14 per ml before the LPS was added to the cells. The binding experiment was also performed in the presence of 3 mM EDTA to investigate the role of bivalent anions. In another sample, cells were preincubated with 10 μg of anti-CD14 MAb 60bca (60b) per ml to study the role of this receptor in the binding. Bars represent the means ± the standard errors of the means of three independent experiments using monocytes from different donors.

ReLPS with isolated human peripheral blood monocytes. Low concentrations of FITC-LPS associated with monocytes only in the presence of HPS or LBP. By increasing the concentration of LPS, the binding became independent of both LBP and HPS as well as of CD14. However, the lowest concentration of LPS at which the CD14-independent binding first occurred (>200 ng/ml) was higher than the concentration of LPS at which LBP-independent association was first observed (>50 to 100 ng/ml). For example, at a concentration of 200 ng/ml, LPS association was still largely CD14 dependent but it could occur in the absence of LBP or HPS. For LPS concentrations exceeding 1 μg/ml the interaction was almost totally CD14 independent. This explains the current confusion in the literature in which LBP-independent binding or activation is more often observed than CD14-dependent binding, a phenomenon that may be due to the relatively high concentrations of LPS (i.e., 500 to 1,000 ng/ml) often used.

We observed that the association of LPS with monocytes is a saturable phenomenon to the extent that CD14 is involved. Binding to monocytes is saturable for FITC-LPS concentrations of 100 to 200 ng/ml, which is in good agreement with former studies (10, 20). The CD14-independent binding was not saturable at all: the more LPS was added, the more association occurred, as described previously (6, 39). Since LPS in relatively high concentrations forms aggregates, this observation strongly suggests that LPS in a low concentration interacts with CD14 as a monomer and that the CD14-independent interaction is an association of LPS aggregates with the monocyte via receptors or structures other than CD14.

The binding of this new FITC-LPS preparation to monocytes could be inhibited with polymyxin B, CAP 18, BPI, and partially with sCD14, as described previously (14, 29, 34). Preincubation of cells with native LPS inhibited FITC-LPS association with monocytes in a dose-dependent fashion, suggesting that the mechanism for the association of FITC-LPS with monocytes is the same as that for the association with native LPS.

Preferably, we would have performed the binding studies at 4°C to eliminate internalization of LPS. However, hardly any binding was observed at 4°C. We hypothesize that at 37°C most of the LPS is present in vesicular structures from which it can be transferred to the cell by LBP. The LPS structure is probably so rigid at 4°C that LPS monomers are not mobile enough to be transferred by LBP to CD14 on monocytes. According to trypan blue quenching experiments the internalized fraction of FITC-LPS in our assays was negligible, a finding confirmed by monocyte preincubation studies with metabolic inhibitors.

Since serum consists of a large variety of proteins, many of which interact with LPS, we expected to find a role on the cell surface for receptors other than CD14, especially at higher HPS concentrations. Natural anti-LPS antibodies might interact with LPS, directing uptake via Fc receptors. Also, the activation of both the classical and alternative complement pathways by LPS has been described (5). Although evidence for a deposition of C3 fragments on the endotoxin moiety itself has never been presented, one might expect that there is some deposition of C3b and C3bi. This would favor the interaction with CR1 (CD35) and CR3 (CD11b/CD18), respectively.

However, under the conditions used in our experiments, none of these interactions seemed to occur, not even at higher HPS concentrations, as MAbs against complement or Fc receptors did not interfere with LPS binding (data not shown). Although the binding of LPS at higher HPS concentrations was less than at 4% HPS, anti-CD14 MAbs completely blocked this interaction. We suppose that other serum factors such as high-density lipoproteins interfere with FITC-LPS binding (43), causing the lower level of FITC-LPS binding seen for higher serum concentrations. Concerning the interaction of LPS in concentrations comparable to the endotoxin levels of patients with gram-negative sepsis with human monocytes in the presence of HPS or LBP, CD14 is the most important receptor involved.

We performed our experiments with LPS from *S. minnesota* Re595, a rough-mutant LPS. Pollack et al. (27) studied the interaction of smooth- versus rough-mutant LPS with human monocytes and showed the differential impact of these LPS phenotypes. We decided not to concentrate on this subject, but in our system also, it is likely that different results could have been found for the smooth-LPS phenotype.

With respect to the biological activity of our FITC-LPS preparation, the ability to stimulate the LAL cascade was totally intact. However, the capacity to stimulate human cells in a CD14-dependent fashion, as evaluated in the TNF induction assay and in the neutrophil priming assay, was severely impaired. Interestingly, these results clearly demonstrate that binding to CD14 and activation via CD14 are independent processes. As to the mechanism of impaired TNF induction and neutrophil priming we can only speculate. How FITC binds to the LPS molecule is not known exactly. It is possible that the FITC molecule blocks or sterically hinders a region of the LPS molecule that is involved in cell activation but that does not affect stimulation of the LAL cascade and CD14-dependent cell binding. We compared the biological activity of our new FITC-LPS preparation with that of native LPS and with that of the old FITC-LPS preparation, which was considered much less efficient. One can speculate that the biological activity of the old FITC-LPS preparation is due to the 41 nonlabeled FITC molecules that are mixed with the one labeled molecule (which may be biologically impaired) present in the 1:42-labeled old FITC-LPS preparation. The loss of biological activity could be a logical result of such an efficient labeling procedure.

Investigations of the bioactive regions of LPS with regard to structure-activity relationships have been described by several authors (20, 21, 36). It was shown that synthetic tetraacyl lipid A precursor Ia (also called compound 406, LA-14-PP, or lipid IVa) does not induce production of TNF, interleukin 1, or interleukin 6 in human monocytes and antagonizes LPS induction of the production of these monokines. Whether lipid IVa has activity in the LAL assay has not, to our knowledge, been determined. The inhibition of LPS effects by lipid IVa or by deacylated LPS did not result in the inhibition of CD14-dependent binding and uptake of native LPS (21). These papers confirm that binding to CD14 and activation are independent processes.

We constructed an LPS preparation that was very efficiently labeled with FITC; the amount of FITC per LPS molecule was estimated to be very close to 1:1, corresponding to an efficiency which is at least 40 times higher than that for preparations made according to an often-used method (31, 39). This enabled us to study the association of LPS with cells in concentrations of LPS that are comparable to the LPS concentration that can be found in patients with a gram-negative sepsis (37). Our technique also had the advantage of being a nonradioactive assay.

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