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Binding of Lipopolysaccharide (LPS) to an 80-Kilodalton Membrane Protein of Human Cells Is Mediated by Soluble CD14 and LPS-Binding Protein

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Activation of cells by bacterial lipopolysaccharide (LPS) plays a key role in the pathogenesis of gram-negative septic shock. The 55-kDa glycoprotein CD14 is known to bind LPS and initiate cell activation. However, there must be additional LPS receptors because CD14 is linked by a glycosylphosphatidyl inositol anchor to the cell membrane and therefore unable to perform transmembrane signalling. Searching for potential LPS receptors, we investigated the binding of LPS to membrane proteins of the human monocytic cell line Mono-Mac-6. Membrane proteins were electrophoretically separated under reducing conditions, transferred to nitrocellulose, and exposed to LPS, which was visualized with anti-LPS antibody. Smooth- and rough-type LPS, as well as free lipid A, bound to a variety of proteins in the absence of serum. However, in the presence of serum, additional or preferential binding to a protein of approximately 80-kDa was observed. Experiments with differently acylated lipid A structures showed that the synthetic tetraacyl compound 406 was still able to bind, whereas no binding was detected with the bisacyl compound 606. The 80-kDa membrane protein was also detected on human peripheral blood monocytes and endothelial cells. The serum factors mediating the binding of lipid A to the 80-kDa membrane protein were identified as soluble CD14 and LPS-binding protein. From these results, we conclude that this 80-kDa protein is a candidate for the hypothetical molecule for LPS and/or LPS-CD14 recognition and signal transduction.

Lipopolysaccharide (LPS; endotoxin), an essential outer membrane glycolipid of gram-negative bacteria, is a potent inducer of inflammation and is involved in the pathogenesis of septic shock (36, 37). Lipid A represents the biologically active principle and is structurally conserved among different types of LPS (12, 37). LPS-activated mononuclear phagocytes release mediators, such as interleukin-1, interleukin-6, and tumor necrosis factor alpha (31, 32). This activation requires recognition of LPS at the cell surface and transmembrane signalling. So far, several cellular proteins have been reported to bind LPS or lipid A (15, 22–24, 29, 33, 43, 46, 47). However, only membrane-bound CD14 (mCD14) has been shown to be involved in the initiation of a secretory response after recognition of LPS (6, 7, 49). This LPS binding is enhanced by LPS-binding protein (LBP) (14, 39) and probably other serum proteins such as septin (48). Since CD14 is connected with the cell membrane only by a glycosylphosphatidyl inositol anchor, which is not able to transduce a signal to the cell interior, an additional signal transduction molecule is assumed to exist (28, 42, 44).

On the other hand, cell types which do not express CD14 on their surfaces, e.g., endothelial cells, can also be stimulated by LPS (1, 10, 16, 34, 35). In such cases, soluble CD14 (sCD14) binds LPS (14); these complexes are thought to interact with a cell membrane component that is capable of mediating signal transduction (10, 34, 35). In the case of monocytes as well as endothelial cells, LPS in connection with CD14 (soluble or membrane-bound form) seems to be recognized by a further molecule. However, no recognizing molecule has not been identified or characterized so far.

We have examined membrane proteins of various cell types for LPS and lipid A binding to select candidates for these hypothetical signal transducing molecules. It is shown that both monocytes and endothelial cells express an 80-kDa protein which is capable of binding lipid A in the presence of sCD14 and LBP.

MATERIALS AND METHODS

Cells. The monocytic cell line Mono-Mac-6 (50) was kindly provided by H. W. L. Ziegler-Heitbrock (Institute of Immunology, University of Munich, Munich, Germany). Cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml).

Human vascular endothelial cells were isolated from umbilical cord veins by treatment with collagenase (20, 30) and cultured in medium 199 with 5% fetal calf serum, endothelial cell growth factor (50 μg/ml), heparin (25 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), and 2 mM L-glutamine.

All cultures were maintained in a humidified incubator at 37°C with 5% CO_2. Human mononuclear cells from healthy donors were isolated by density gradient centrifugation (3) with Ficoll-Paque. After three washes with Hanks' balanced salt solution, cells were resuspended in Hanks' balanced salt solution with 0.1% bovine serum albumin (BSA) at 2×10^6 cells per ml and separated by counterflow centrifugation in a JE-6B-elutriator (Beckman Instruments, Munich, Germany). The purity of the monocyte fraction was >95%, as determined by fluorescence-activated cell sorter analysis after staining with anti-CD14 monoclonal antibody (MAb) My4 (Coulter Electronics, Krefeld, Germany).

Reagents and antibodies. All fine chemicals were from Sigma (Deisenhofen, Germany), Serva (Heidelberg, Germany), or Merck (Darmstadt, Germany). The anti-CD14 MAb MEM-18 (2) was a kind gift from V. Horejsi (Institute of Molecular Genetics, Prague, Czech Republic). MAb A6 recognizes the phosphorylated carbohydrate backbone of free lipid A independently of the acylation pattern (26). Anti-LPS MAb WN1 222-5 is cross-reactive with smooth- and rough-form LPSs of *Escherichia coli* and *Salmonella enterica* (8) and was kindly provided by F. E. Di Padova (Sandoz Pharma Ltd., Basel, Switzerland). Anti-BSA MAb BSA-33 and alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G were purchased from Sigma.

Normal human sera (NHS) were obtained from healthy volunteers. sCD14-

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depleted serum was prepared by passing NHS diluted with an equal volume of phosphate-buffered saline (PBS) through a Sepharose 4B column to which anti-CD14 MAb MEM-18 had been coupled. The eluate contained <1 ng of sCD14 per ml, as determined by capture enzyme-linked immunosorbent assay (IBL, Hamburg, Germany).

sCD14 was purified from NHS by affinity chromatography, as previously described (2). Human LBP was purified from acute-phase human serum by preparative isoelectric focusing and anion-exchange chromatography (38).

LPS, lipid A, and lipid A partial structures. Smooth LPSs of *Salmonella abortus equi*, *Salmonella friedenau*, and *E. coli* serotypes O4, O6, O12, O15, and O16 were prepared by the phenol-water procedure (45), purified by repeated ultracentrifugation, and converted into sodium salt form by electrodialysis (11). Rough LPSs of *S. minnesota* chemotypes Ra (strain R60) and Rb_2 (strain R345) as well as *E. coli* K-12, R1, R2, R3, and R4 were obtained by the phenol-chloroform-petroleum ether method (13).

Lipid A was obtained from *E. coli* Re mutant F515 by treating phenol-chloroform-petroleum ether-extracted LPS with acetate buffer (4).

Synthetic bisphosphorylated hexaacyl lipid A (*E. coli* type), also known as LA-15-PP or compound 506, was synthesized as described previously (18). Synthetic tetraacyl lipid A precursor Ia (LA-14-PP or compound 406), a partial structure of LA-15-PP lacking nonhydroxylated fatty acids, was synthesized as previously described (19). Compound LA-19-PP (compound 606) represents a synthetic bisacylated partial structure corresponding to de-O-acylated lipid A (17).

LPS, lipid A, and synthetic compounds were solubilized in water and stored in aliquots at 1 mg/ml and 4°C.

Preparation of plasma membranes. Plasma membranes of Mono-Mac-6 cells, human monocytes, and human endothelial cells were isolated by a method described elsewhere (21, 25), with modifications. Briefly, cells were washed three times with lysis buffer (20 mM Tris-HCl, 1 mM $MgCl_2$, 250 mM sucrose, 1 mM phenylmethylsulfonyl fluoride, 100 μM leupeptin [pH 7.4]) and lysed by nitrogen cavitation (450 lb/in^2, 30 min) in a bath of melting ice. The homogenate was centrifuged at 1,000 × *g*, applied to a sucrose cushion (35% g/v), and centrifuged for 1 h at 24,000 × *g* and 4°C. Membranes collected at the sucrose-buffer interface were pelleted by a final centrifugation step (100,000 × *g*, 30 min, 4°C). After determinations of protein concentrations (5), aliquots were stored at −70°C.

In addition, plasma membranes of Mono-Mac-6 cells were occasionally prepared by hypo-osmotic lysis, differential centrifugation, and centrifugation on a sucrose cushion (41), but no differences in experimental results were observed.

Detection of LPS-/lipid A-binding proteins. For identification of LBS- or lipid A-binding proteins, the method of Kirikae et al. (22) was used with some modifications. Membrane proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (27) on a 9% separating gel. After electrophoresis, proteins were blotted onto nitrocellulose membranes with a semidry apparatus for 1 h at 2 mA/cm^2. Blotting buffer contained 25 mM Tris and 192 mM glycine (pH 8.3), with 20% methanol and 0.02% SDS. Nitrocellulose membranes were blocked with PBS containing 5% nonfat milk (PBS/M) for 2 h at 25°C and then immersed with various concentrations of LPS, lipid A, or lipid A partial structures in PBS/M–0.1% octylglucoside, with 10% NHS in some cases. After incubation for 90 min at 25°C and four washes in PBS containing 0.05% Tween 20, bound LPS or lipid A were detected by MAbs WN1 222-5 or A6, respectively. Visualization was performed by incubation with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G. For substrate reactions, 5-bromo-4-chloroindoxyl phosphate–nitroblue tetrazolium was used.

RESULTS AND DISCUSSION

Western blot (immunoblot) analysis of lipid A binding to membrane proteins of the Mono-Mac-6 cell line. Since lipid A constitutes the biologically active principle of LPS (12, 37), it was reasoned that a putative LPS receptor binds to lipid A. Therefore, our first experiments aimed at the detection of lipid A binding to membrane proteins of the human monocytic cell line Mono-Mac-6 which had been electrophoretically separated and blotted onto nitrocellulose membranes. Lipid A-binding proteins were detected by incubation with free lipid A and subsequent anti-lipid A antibody (Fig. 1). To ensure comparable results, all nitrocellulose strips in a given experiment were obtained from a single run, i.e., from the same membrane sample. In the absence of serum, lipid A bound to several proteins with molecular masses of about 35 to 50 kDa. Only when NHS was added, preferential binding to a protein of about 80 kDa was observed. The binding of lipid A was blocked by preincubation with LPS at 100-fold-higher concentration.

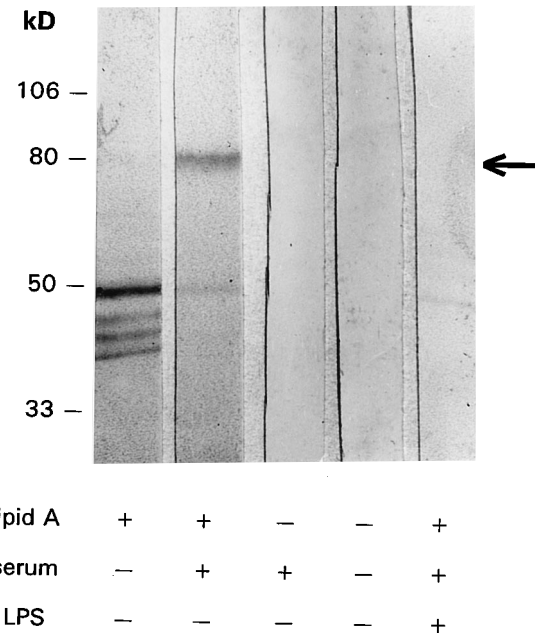

FIG. 1. Western blot analysis of lipid A binding to membrane proteins of the Mono-Mac-6 cell line. Isolated cell membranes were electrophoresed on 9% polyacrylamide gels and blotted onto nitrocellulose membranes. Nitrocellulose strips were incubated with lipid A (0.5 μg/ml) and/or 10% NHS as indicated. Preincubation with LPS (*S. friedenau*; 50 μg/ml) was performed for 10 min in the presence of 10% NHS, and then lipid A (final concentration, 0.5 μg/ml) was added. All incubations were done in PBS/M–0.1% octylglucoside. Lipid A-binding proteins were visualized with anti-lipid A MAb A6. The position of the 80-kDa protein is indicated by an arrow on the right.

Without lipid A, no binding was observed in either the presence or absence of serum. This excludes nonspecific interaction between serum or detecting antibodies and blotted proteins. An LPS-binding protein of comparable size has been described (15); however, it has recently been shown to be cell surface-associated albumin (9). To exclude the possibility that the 80-kDa protein is albumin, we electrophoresed and blotted Mono-Mac-6 cell membranes and BSA. One blot was stained for protein. On a parallel blot, we tested for lipid A-binding proteins as described above, and on a third blot, we tested for reactivity with anti-BSA antibody (Fig. 2). No binding of lipid A to BSA was detected in the presence of serum. In contrast, in the lane with Mono-Mac-6 membranes, a band of 80-kDa occurred (Fig. 2B). As expected, anti-BSA antibody recognized BSA, whereas no reaction was observed with Mono-Mac-6 cell membrane proteins (Fig. 2C). In addition, Fig. 2 shows that albumin can be clearly differentiated from the 80-kDa lipid A-binding protein by its electrophoretic mobility. Taken together, these data clearly exclude the possibility that the detected 80-kDa protein is cell-bound albumin.

The binding of lipid A to membrane proteins (35 to 50 kDa) observed in the absence of serum largely disappeared in the presence of serum. Obviously, this is low-affinity binding and can thus be blocked with serum. Although it cannot be excluded that this low-affinity binding is also specific, we assume that it represents nonspecific hydrophobic interaction with lipid A.

Binding to the 80-kDa protein occurred exclusively in the presence of serum and was still detectable at lipid A concentrations as low as 30 ng/ml (data not shown), making an unspecific reaction unlikely.

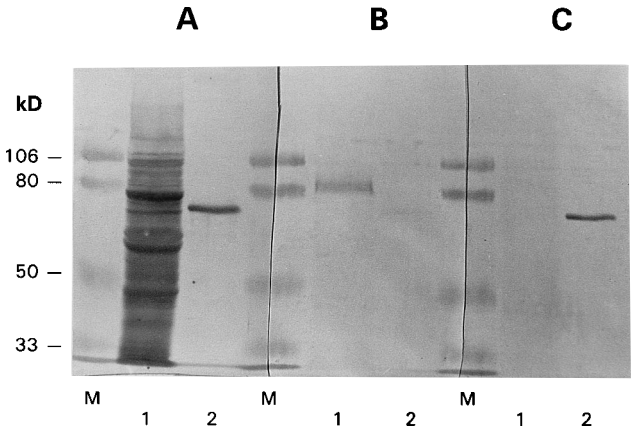

FIG. 2. Comparison between Mono-Mac-6 cell membranes and BSA. Mono-Mac-6 cell membranes (lanes 1; 20 μg of protein) and BSA (lanes 2; 0.5 μg) were electrophoretically separated and transferred to nitrocellulose. The blot was stained with amido black for protein (A), anti-lipid A MAb A6 after incubation with lipid A in the presence of 10% NHS (B), and antibody against BSA (C). Lanes M, marker proteins. Molecular masses (in kilodaltons) are indicated on the left.

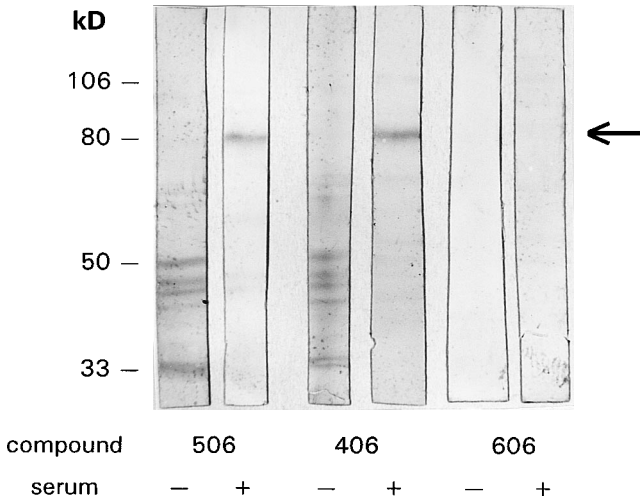

FIG. 4. Western blot analysis of binding of lipid A substructures. Nitrocellulose strips with electrophoretically separated membrane proteins of the Mono-Mac-6 cell line were incubated with compound 506, 406, or 606 (1 μg/ml), as indicated, in the presence (+) or absence (−) of 10% NHS. Binding to membrane proteins was visualized with MAb A6 (recognizing all three compounds). The position of the 80-kDa protein is indicated by an arrow on the right.

Analysis of LPS binding to membrane proteins of the Mono-Mac-6 cell line. To examine whether the observed binding to the 80-kDa protein was restricted to free lipid A, a variety of smooth- and rough-type LPSs were tested. Figure 3 shows a typical example of the observed binding pattern. Without serum, LPS bound to several membrane proteins (90, 65, 60, and 35 to 50 kDa). Only in the presence of serum, additional binding to an 80-kDa protein occurred. In controls without LPS, unspecific reactions of serum and detecting antibodies were excluded. We tested the binding of wild-type (S-form) LPSs of *S. friedenau*, *S. abortus equi*, and *E. coli* serotypes O4, O6, O12, O15, and O18, as well as the binding of rough mutant

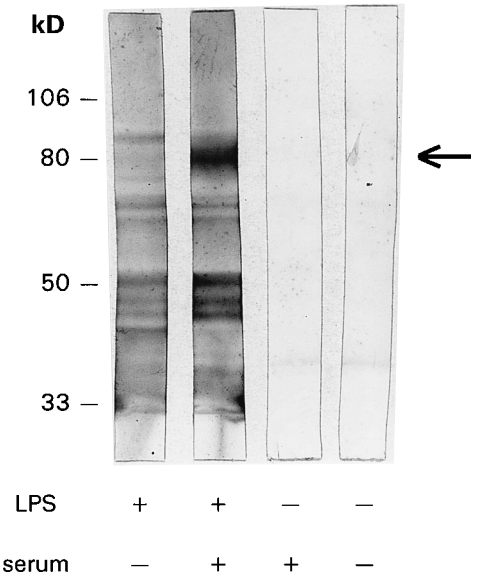

FIG. 3. Western blot analysis of LPS binding to membrane proteins of the Mono-Mac-6 cell line. Nitrocellulose strips with electrophoretically separated membrane proteins were incubated with *S. minnesota* R60 LPS (1 μg/ml) and/or 10% NHS as indicated. All incubations were done in PBS/M–0.1% octylglucoside. LPS-binding proteins were visualized with anti-LPS MAb WN1 222-5. The position of the 80-kDa protein is indicated by an arrow on the right.

(R-form) LPSs of *S. minnesota* R60 and R345 and *E. coli* R1, R2, R3, R4, and K-12. All LPSs bound to the 80-kDa protein only in the presence of serum. Because of its high sugar content, S-form LPS is much less hydrophobic than lipid A; nevertheless, binding to the membrane protein was still detectable in the presence of serum, representing a further piece of evidence for specificity.

Binding of synthetic lipid A partial structures. For further characterization of binding, several synthetic lipid A partial structures were tested in comparison with synthetic *E. coli*-type hexaacyl lipid A (compound 506), which showed the same properties and activities as its natural counterpart in the blot assay (Fig. 4). The tetraacyl lipid A precursor Ia (compound 406) also bound only in the presence of serum, whereas compound 606 (a synthetic bisacylated partial structure) did not bind to the 80-kDa protein. Therefore, binding of lipid A partial structures to the 80-kDa protein is dependent on the acylation pattern.

sCD14 and LBP mediate binding of lipid A to the 80-kDa protein. To identify the serum factor(s) that mediates binding of lipid A or LPS to the 80-kDa protein, purified serum proteins were tested in this system. Several serum proteins are known to influence binding of LPS to cells (10, 39, 48). Thus, we decided to examine whether sCD14 and LBP are involved in mediating binding. In Fig. 5, lanes 1 and 2 show lipid A binding to membrane proteins of the Mono-Mac-6 cell line in the absence (lane 1) and presence (lane 2) of serum; once again, lipid A bound to the 80-kDa protein only in the presence of serum (lane 2). Neither sCD14 nor LBP alone substituted for serum (Fig. 5, lanes 3 and 4). However, sCD14 and LBP together were sufficient to enable binding (Fig. 5, lane 5). In accordance with this observation, neither sCD14-depleted serum (lane 6) nor serum preincubated with the anti-CD14 MAb MEM-18 (which blocks LPS binding to CD14) (lane 7) provided binding. Taken together, these data show that binding of lipid A to the 80-kDa membrane protein requires the presence of both LBP and sCD14.

Several investigators have hypothesized that CD14 is part of a receptor complex with other proteins that act as signal trans-

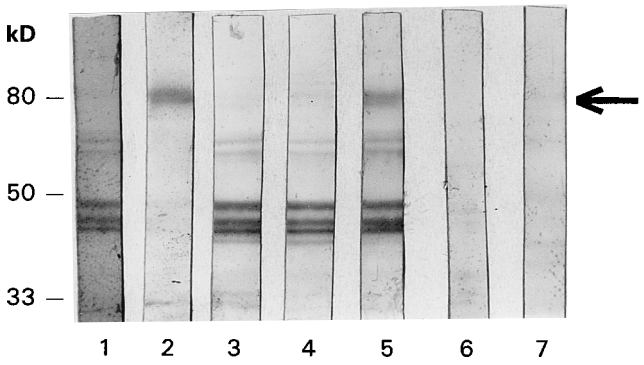

FIG. 5. Influence of purified sCD14 and LBP on lipid A binding to Mono-Mac-6 cell membrane proteins. Nitrocellulose strips with electrophoretically separated membrane proteins were incubated with lipid A (0.5 μg/ml) alone (lane 1), lipid A (0.5 μg/ml) and 10% NHS (lane 2), lipid A and purified sCD14 (1 μg/ml) (lane 3), lipid A and purified LBP (1 μg/ml) (lane 4), sCD14 and LBP (both at 1 μg/ml) (lane 5), 10% sCD14-depleted serum (lane 6), and 10% serum preincubated with anti-CD14 MAb MEM-18 (200 μg/ml) (lane 7). Lipid A-binding proteins were visualized with anti-lipid A MAb A6. The position of the 80-kDa protein is indicated by an arrow on the right.

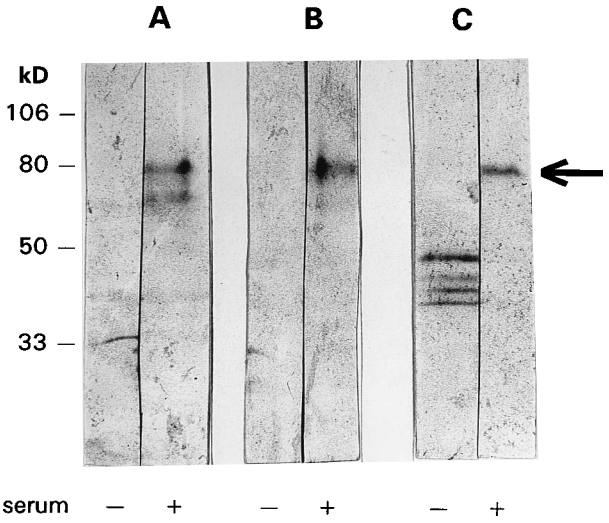

FIG. 6. Detection of the 80-kDa protein in human monocytes (A), human umbilical cord vein endothelial cells (B), and Mono-Mac-6 cells (C). Isolated cell membranes of each indicated cell type were electrophoresed on 9% polyacrylamide gels and blotted to nitrocellulose membranes. Nitrocellulose strips were incubated with lipid A (0.5 μg/ml) in the presence (+) or absence (−) of 10% NHS. Lipid A binding was detected with anti-lipid A MAb A6. The position of the 80-kDa protein is indicated by an arrow on the right.

ducers (28, 44). In our system, mCD14 is fixed on nitrocellulose and cannot be involved in lipid A or LPS binding to the 80-kDa protein. However, it is possible that in intact cells, mCD14 fulfills the function which sCD14 has in our system, i.e., the transfer of lipid A to the 80-kDa protein. In this context, the results of Stefanová and Horejsí, who found coprecipitation of an 80-kDa protein with different glycosylphosphatidyl inositol-anchored proteins, are of interest; however, CD14 was not examined (40).

Detection of the 80-kDa protein on different cell types. To study the presence of the 80-kDa protein on other cell types, purified human monocytes and human umbilical cord endothelial cells were examined. The results of experiments involving the Mono-Mac-6 cell line, human peripheral blood monocytes, and human umbilical cord vein endothelial cells are shown in Fig. 6C, A, and B, respectively. It is evident that the 80-kDa protein is detectable on all of the cell types tested. However, unlike the Mono-Mac-6 cell line, monocytes and endothelial cells possess an additional lipid A-binding protein of about 65 kDa. In this context, it is important to recall that endothelial cells do not express mCD14 but can be stimulated by sCD14-LPS complexes (1, 14, 16). The formation of sCD14-LPS complexes is enhanced by LBP (14, 16). A receptor on the endothelial cell surface which recognizes these complexes and provides further signalling is assumed to exist (10, 34, 35). In our opinion, the detected 80-kDa protein is a candidate for this receptor molecule.

Until now, neither the putative second receptor on monocytic cells nor the assumed receptor on endothelial cells has been detected or biochemically characterized. We have shown that the 80-kDa membrane protein described here binds LPS and free lipid A only in the presence of serum. LBP and sCD14 combined, in the absence of additional serum factors, were sufficient to mediate binding. We detected the 80-kDa protein on endothelial cells, a cell type for which sCD14-mediated LPS binding has already been shown in vitro (10, 34). Therefore, we regard this protein as a candidate for the putative receptor molecule that is responsible for transmembrane signalling during LPS activation of cells. However, assessment of the physiological significance of this protein is not yet possible, because its binding properties were examined after denaturing SDS-PAGE and blotting. Purification and further characterization of the 80-kDa protein and development of immunological reagents able to recognize it will identify its role in LPS-induced cellular activation.

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