Localization of the Lipopolysaccharide-binding Protein in Phospholipid Membranes by Atomic Force Microscopy*

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Lipopolysaccharides (LPS; endotoxin) activate immunocompetent cells of the host via a transmembrane signaling process. In this study, we investigated the function of the LPS-binding protein (LBP) in this process. The cytoplasmic membrane of the cells was mimicked by lipid liposomes adsorbed on mica, and the lateral organization of LBP in these membranes and its interaction with LPS aggregates were characterized by atomic force microscopy. Using cantilever tips functionalized with anti-LBP antibodies, single LBP molecules were localized in the membrane at low concentrations. At higher concentrations, LBP formed clusters of several molecules and caused cross-linking of lipid bilayers. The addition of LPS to LBP-containing liposomes led to the formation of LPS domains in the membranes, which could be inhibited by anti-LBP antibodies. Thus, LBP mediates the fusion of lipid membranes and LPS aggregates.

Membrane-embedded proteins or serum proteins interacting with membranes very often play a particular role in cell functioning (e.g. in signal transduction induced by various pathogenic factors). In these cases, signaling occurs across the cytoplasmic membrane via one or more transmembrane or membrane-bound proteins. The participating components are usually known; however, their lateral organization and the mechanism of their interaction are unknown.

The cell wall of Gram-negative bacteria consists of the cytoplasmic and an additional outer membrane. This outer membrane is an extremely asymmetric bilayer with respect to the lipid composition. The inner leaflet is composed of a phospholipid mixture, and the outer leaflet is composed of glycolipids, in most cases lipopolysaccharide (LPS). When released from the bacterial surface into the blood circulation of the host, LPS plays an important role in the pathogenesis and manifestation of Gram-negative inflammation, in general, and of septic shock, in particular. It is therefore also named endotoxin. The important biological aim of this study was to get an understanding of an early step in the activation mechanism of immune cells (mononuclear cells, MNCs) by LPS. A comparison of the capacity of LPS monomers and aggregates to induce cytokines such as tumor necrosis factor-α showed that the aggregates play a biologically important role in the initial step of cell activation (1). One important step in the signal transduction process is the interaction between LPS and the acute phase serum protein LPS-binding protein (LBP). LBP is synthesized by hepatocytes (2) and different epithelial cells (3, 4) and has been described in the literature as a shuttle protein for monomers of LPS or lipid A, the endotoxic principle of LPS, toward the surface of MNCs (5–9). Our group provided evidence for binding of LBP to and intercalation into lipid membranes composed of negatively charged phosphatidylserine (PS) or zwitterionic phosphatidylcholine (PC) and also in LPS aggregates by using fluorescence resonance energy transfer and surface plasmon resonance experiments (10). Furthermore, we also provided evidence for a directed transmembrane configuration of LBP within symmetric phospholipid bilayers, mimicking the cytoplasmic membrane, by electrical measurements on reconstituted planar membranes (11). Because of our results, it is likely that LBP is not only present as an acute phase protein in serum but also assumes a transmembrane configuration in the cytoplasmic membrane of immune cells and in this configuration acts as fusion protein for LPS aggregates.

Depending on its concentration, LBP plays a dual role in vivo as well as in vitro; it can both potentiate and decrease cell response to bacterial LPS. Whereas at low concentrations, LBP causes an increase of the cell activating capacity of LPS, at high LBP concentrations, it causes a decrease of LPS-induced cell responses (10, 12). The inhibitory mechanism is discussed controversially. In part, it can be explained by the ability of LBP to neutralize the bioactivity of LPS by transferring it to plasma lipoproteins (13, 14). However, further inhibitory mechanisms are likely to occur, because LBP can inhibit LPS-induced cell activation also under serum-free conditions (10). Possible mechanisms might include the formation of large complexes of LPS and LBP (LPS + LBP) with an increased binding energy of the LPS in these complexes (15). Other data support the hypothesis that LBP can reduce cell response to LPS by inhibiting LPS transfer to mCD14 and finally to the Toll-like receptor 4/MD-2 signaling receptor (7).

In this work, we investigated the orientation and the lateral organization of LBP after intercalation into reconstituted lipid membranes and the LBP-mediated intercalation of endotoxin into these membranes by atomic force microscopy (AFM). The host cell membrane was mimicked by lipid liposomes prepared of PC or PS. This membrane reconstitution system reduces the complex biological membrane and, thus, allows us to investigate the interaction mechanisms on a molecular level. After reincubation of the liposomes with LBP and in the presence and absence of lipid A, they were adsorbed on mica. The AFM (16) allows us to acquire the surface topography of biological samples under native conditions in buffer with high resolution (17, 18).

In addition to the ability of the AFM technique to image surfaces, we used this technique for force spectroscopy experiments to investigate the thickness of membranes, showing that LBP induced a cross-linking of several membranes. Functionalized cantilevers with antibodies bound to their tip by 3-(trimethoxysilyl)propyl methacrylate (MEMO) were used for the localization of specific proteins on the surface (19–21). Binding experiments between single LBP molecules, embedded in...
the lipid matrix, and the modified tip were performed. These experiments clearly identified LBP as a membrane-embedded protein forming domains of several molecules at higher concentrations.

Our data provide clear evidence that LBP mediates the fusion between lipid membranes and LPS aggregates that might be an important initial step in the activation of MNCs (22).

MATERIALS AND METHODS

Atomic Force Microscopy—Imaging was performed with an MFP-3D atomic force microscope from Asylum Research (Santa Barbara, CA) in an aqueous environment. The AFM was placed on an inverted optical fluorescence microscope (Olympus IX70, Tokyo, Japan), and the setup was located on an air-buffered graphite table. Imaging in AC mode was performed with MSCP-AUNM silicon nitride cantilevers with a spring constant \( k = 0.1 \text{ N/m} \) and a resonant frequency \( f_{\text{res}} \approx 38 \text{ kHz} \) in air (Veeco Instruments GmbH, Mannheim, Germany). Imaging in liquid was performed in AC (tapping) mode using frequencies of about 8 kHz. The frequency was chosen to result in an amplitude 5% lower than the amplitude at the resonance frequency. The set point was always adjusted to guarantee minimum forces applied to the sample. Further image processing (flattening and plane fitting) was done with the MFP-3D software under IGOR Pro (Lake Oswego, OR). The sample was placed on mica glued to a small Petri dish top (Sarstedt, Nümbrecht, Germany). Force spectroscopy was performed in an aqueous environment with an AC240TS cantilever (\( k = 2 \text{ N/m} \), \( f_{\text{res, air}} = 70 \text{ kHz} \); Olympus Optical Co., Ltd. Tokyo, Japan). AFM images of single LBP molecules adsorbed on mica were obtained in DC (contact) mode using a MultiMode III setup (Veeco/Digital Instruments, Santa Barbara, CA) and the MSCT-AUNM cantilever.

Lipids and Proteins—PS and PC from egg yolk lecithin (sodium salt) were purchased from Avanti Polar Lipids (Alabaster, AL) and Sigma, respectively, and were used without further purification.

For the formation of lipid A aggregates, lipid A from \( S. \) enterica serovar Minnesota (\( S. \) minnesota) strain R595 (R595 LPS) (chemical structures according to Ref. 23) was used. LPS was extracted by the phenol/chloroform/petalroleum ether method (24), purified, lyophilized, and transformed into the triethylamine salt form. Lipid A was isolated from LPS by acetate buffer (pH 4.4) treatment (0.1M, 100 °C lyophilized, and transformed into the triethylamine salt form. Lipid A (chemical structures according to Ref. 23) was used. LPS was extracted for 45 min, followed by extensive rinsing with methanol and purified water. Finally, the cantilevers were incubated in a solution containing 0.2% ammonium peroxodisulfate by weight, 0.2% \( \text{N},\text{N}',\text{N}''\text{,N}'''\text{-tetramethyl}-\text{ethylenediamine} \) by volume and 5 \( \mu \text{g/ml} \) antibody for 45 min.

Preparation of Liposome-derived Bi-/Multilayers—2 ml of phospholipid liposomes (100 \( \mu \text{mol} \)) were placed on the freshly cleaved mica glued into the lid of a Petri dish. The liposomes were then stored overnight on a shaker at 4 °C. For further use, the preparation was washed intensively with 5 ml Hepes, at pH 7, room temperature; [PS] = 100 mM, [LBP] = 2 mM, cantilever: MSCT-AUNM E (\( k = 100 \text{ mN/m} \)).

RESULTS

Single LBP Molecules on Mica—LBP was physisorbed from 200 \( \text{pm} \) solutions in deionized water on mica and imaged in air. LBP molecules adsorbed mainly as individual molecules, showing a stretched conformation with a length of about 10 nm, a width of about 4 nm, and a height of 2–3 nm (Fig. 1A). This shape and the dimensions are in good agreement with the calculated structure of LBP (27).

LBP Molecules Intercalated in Lipid Matrices—Imaging bilayers solely composed of PS or PC exhibited flat surfaces with maximal height differences of <0.4 nm. The thickness of the bilayers was 5 ± 0.5 nm. This value was determined by scratching off the bilayer from the mica surface using low force to avoid destruction of mica layers (Fig. 1B). The
subsequent addition of 2 nM LBP to already formed PS bilayers led to small elevations of 2–3 nm, with a length of ~22 nm and a width of ~15 nm (Fig. 1C). Preincubation of PS liposomes (100 μM) with 2 nM LBP ([LBP]/[PS] = 1:5×10^6, M/M) prior to the adsorption process onto the mica surface led to formation of circular domains with a diameter of ~200 nm and a mean height of ~2 nm (maximum of 10 nm) in the lipid bilayers (Fig. 1D). Analogous experiments using PC liposomes yielded comparable results with a slightly decreased number of domains (data not shown). The formation of these nanodomains was concentration-dependent. At an LBP concentration of 20 pM ([LBP]/[PS] = 1:5×10^6, M/M), no domains or individual molecules could be detected in/on the membranes in height images. At concentrations up to 2 μM LBP, the number of domains and with that the roughness of the images increased; the size of the domains, however, remained unchanged (data not shown). Experiments with different molar ratios of [LBP]/[PS] were performed in the range of 1:5×10^5 to 5×10^7 M/M. Several images of the same position did not show any time-dependent changes of the topography; thus, it is unlikely that adsorption of molecules occurred during scanning.

**LBP-induced Formation of Multilayers**—Besides scratching off the bilayer from the mica, force spectroscopy allows the determination of the thickness of the lipid layers adsorbed onto the mica surface. In Fig. 2, a representative force spectroscopy experiment is shown for (PS + LBP) layers prepared from 100 μM PS liposomes preincubated with 2 nM LBP for 15 min at 37 °C. Approaching the surface, the force between the cantilever tip and the sample started to increase at a distance of about 40 nm from the mica surface. Thus, the preincubulation of PS liposomes with LBP led to the formation of multilayers of at least six lipid bilayers. After reaching a plateau, a further increase of the force was observed at distances of <6 nm from the mica surface. This effect probably resulted from the bilayer adsorbed directly onto the solid support. Also, the preincubation of 100 μM lipid A aggregates with 2 nM LBP led to the formation of multilayers. However, the surface was very rough, and the thickness of the multilayers assumed values up to 3 μm (data not shown).

**Detection of LBP in the Lipid Matrix by Force Spectroscopy**—The observation that no LBP molecules could be detected in PS preparations from liposomes preincubated with LBP at a low concentration ([LBP] = 20 pM, [LBP]/[PS] = 1:5×10^6, M/M) (Fig. 3A) may have different reasons: (i) the LBP concentration is too low to observe individual molecules, (ii) the lateral diffusion of LBP molecules in the bilayer is too high to localize them with the AFM, or (iii) the molecules are embedded in the lipid matrix, and the height difference between LBP molecules and the PS membrane is below the resolution of the AFM. To clarify this important question, a chemically modified cantilever was used, and the same sample was imaged again. Scanning with the modified MSCT-AUNM cantilever led to “force images” showing areas with stronger interaction between the cantilever tip than observed with pure PS bilayers (Fig. 3B). The areas in the height image, where the antibody and/or the linker molecule bound to LBP, appear higher, because the amplitude is reduced by the specific interaction. This effect leads to a retraction of the cantilever by the feedback loop of the AFM, resulting in higher height values. Thus, these images may be called force or recognition images (19). Force spectroscopy with the functionalized cantilever was performed on pure PS membranes or on LBP-containing PS membranes outside the regions in which LBP was detected. The respective force images show only binding interactions in a range of 0–20 nm (Fig. 4A, black curve). In comparison, the force curves performed in the regions showing stronger interactions in the force images (Fig. 4A, gray curve) or anywhere on (PS + LBP) layers completely covered with LBP molecules (Fig. 4B, two representative curves) exhibited the interaction with the PS bilayer and, moreover, the rupture of bonds at distances of about 20–60 nm. The latter were caused by interactions between the antibodies and/or the linker and the LBP molecules in the membrane. The average binding force was (127 ± 20) pN at a pulling velocity of 0.77 μm/s (Fig. 4C). The distribution had a width of (52 ± 25) pN.

**LBP-mediated Incorporation of Lipid A**—It is thought that LBP mediates the intercalation of LPS or lipid into cell membranes as an early step in the cell activation by these virulence factors. To investigate this important biological question, PS liposomes were first preincubated with 2 nM LBP, and subsequently lipid A aggregates (50 or 100 μM) were added, and the resulting (PS + LBP) + lipid A preparations were then adsorbed on mica. Height images showed two different kinds of structures (Fig. 5, A and B). On the one hand, hemispheres were observed with diameters up to 20 μm and heights up to 16 nm (Fig. 5A), and on the other hand, larger flat structures with heights of ~1.5 nm (Fig. 5B). Both kinds of structures were also observed after the subsequent addition of lipid A to already formed (PS + LBP) multilayers. Interestingly, the formation of the flat structures could also be created by scanning on the higher hemispheres, demonstrating that in the stack of multilayers the last bilayer is less influenced by the solid support and behaves more like a fluid system.

Formation of the observed structures was inhibited by incubation of (PS + LBP) with 100 ng/ml α-LBP antibodies for 15 min at 37 °C prior to the addition of lipid A (data not shown). Force-distance curves on the (PS + LBP) + lipid A) samples (Fig. 5C) showed the formation of multilayers, which were thicker by 10–15 nm than the (PS + LBP) multilayers (Fig. 2).

**DISCUSSION**

It is an essential capability of all cells to respond in a certain manner to external stimuli. This response involves various signal transduction pathways comprising different proteins, some of which are located at or in the cytoplasmic membrane and are responsible for transmembrane signaling. In past years, different methods have been developed to localize these signaling proteins and to characterize their functions. Thus, different light microscopic fluorescence techniques allow the localization of proteins at a lateral resolution in the range of 30 nm and more (28). For the identification of a colocalization of proteins with distances of less than 20 nm, fluorescence resonance energy transfer spectroscopy has proven to be a useful tool (29). However, these techniques do not provide topographical information of protein clusters or so-called rafts. With electron microscopy, images of fixed samples in vacuum can be obtained with high resolution. The sizes of lipid rafts, which are being discussed as hosting signaling proteins, have been determined with optical tweezers (30). High resolution under physiological conditions...
can be obtained with atomic force microscopy. Furthermore, a functionalization of the cantilever tip with particular chemical groups or proteins (e.g. antibodies) can be used to specifically localize proteins (19, 31).

With the aim of characterizing the role of LBP in the LPS-induced activation of MNCs, we performed imaging and force spectroscopy, applying AFM to solid supported reconstituted lipid bilayers resembling the membrane of MNCs. The interactions between LPS, LBP, sCD14, mCD14, and host cells have been investigated intensively in the last years in vitro and also in vivo experiments. In this study, we correlated already published biological data with data obtained with reconstituted membrane systems. Due to the reduction of complexity of the biological system, reconstitution systems allow the determination of the underlying mechanisms on a molecular level. Our results and the proposed model allow us to understand the observations from biological experiments described earlier.

In earlier investigations into the influence of LBP on the LPS-mediated activation, a dual role of LBP has been proposed; at low concentrations, LBP enhances the activation of MNCs, whereas at high concentrations, it causes a decrease (12). We could show that the enhanced activation is mediated by LBP molecules intercalated in the cytoplasmic membrane of the MNCs and that the neutralization of LPS is mediated by soluble LBP in the serum (10).

In the following, we will discuss three interactions: (i) between soluble LBP and phospholipids as a prerequisite for the activation of MNCs, (ii) between soluble LBP and lipid A as the interaction leading to a neutralization of endotoxin, and (iii) between LBP intercalated in phospholipid membranes and lipid A as the first step in the activation of MNCs.

Interaction between Soluble LBP and Phospholipid Membranes—In earlier studies, it has been shown that LBP interacts with phospholipids and that it intercalates into phospholipid bilayer membranes (6) in a transmembraneous orientation (11). However, nothing was known so far about the lateral organization and orientation of LBP in membranes.

In general, two preparation methods can be used to determine the interaction between proteins and lipid matrices: (i) first the lipid liposomes are adsorbed on mica, and the protein is subsequently added to the preformed planar lipid bilayer. Both procedures do not necessarily lead to identical results, and, in fact, this was the case for the interaction between LBP and PS liposomes. When the bilayers were adsorbed first, the LBP molecules bound probably as individual molecules to the bilayer surface and did not form larger protein aggregates (Fig. 1C), although the size of the individual dots was larger than that of molecules imaged directly on mica (Fig. 1A). This observation might result from a dimerization of LBP. However, it is more likely that it results from artifacts (e.g. small movements of individual LBP molecules in the bilayer). Fig. 1, A and C, also demonstrate that LBP does not form protein aggregates in solution. The lateral diffusion of lipids and proteins in a solid supported membrane is reduced (32, 33), and, thus, the best preparation method mimicking the natural system is the one in which the components are preincubated at 37 °C prior to adsorption on mica. Under these conditions, the phospholipids used in this study were in the fluid state.

In preparations according to the latter method and containing low amounts of LBP ([LBP]/[PS] = 1:5 \times 10^6 M/M), no LBP molecules could be detected in the height images (Fig. 3A). This does not necessarily imply that no LBP was in the membrane, and, in fact, using a functionalized cantilever, LBP molecules were specifically detected in the same bilayer preparation (Fig. 6C). Because of the reduced resolution of the modified cantilever (34), the results give no definite answer of whether single LBP molecules or small aggregates were detected. Furthermore, there is no information on how many linker molecules and αLBP antibodies were attached to the cantilever or were simultaneously in contact with LBP. Assuming an effective area of about 0.6 nm²/phospholipid molecule, the number of lipid molecules in the outermost layer of the membrane shown in Fig. 3B was about 1.7 \times 10^6. With the given molecular ratio of [LBP]/[PS] of 1:5 \times 10^6, M/M, ~0.3 LBP molecules should be detectable in a 1-μm² scan. In fact, about 20 specific interaction spots were observed, implying that the probability to detect LBP molecules in the outermost leaflet of the adsorbed multilayer is about 50 times higher. The observed interaction spots probably result from individual LBP molecules which are embedded in the lipid membrane (Fig. 6B). This could result from a...
higher accessibility of the outermost lipid layer for soluble LBP molecules in the buffer. The orientation of LBP in the membrane could not yet be clearly defined. Nevertheless, the results obtained from planar lipid bilayer experiments support the assumption of a transmembraneous orientation of LBP. In planar bilayer experiments, a polyclonal LBP antiserum, which is known to bind to the C-terminal as well as to the N-terminal parts of LBP, interacts with LBP on both sides of the planar membrane, although LBP was added only to one side of the membrane (11).

An increase in LBP concentration led to the formation of small domains in the bilayer with diameters of about 200 nm (Figs. 1D and 6a). As shown in force spectroscopy experiments, LBP was also present outside of these domains. A further increase of the LBP concentration did not change the domain structure but led to an increase of the overall roughness of the bilayer. An intercalation of and domain formation by LBP in zwitterionic PC membranes has also been observed. Thus, the negative charge of PS is not essential; however, it increases the amount of intercalated LBP, and this observation is in good agreement with published data (10). In this context, it is noteworthy that the cytoplasmic membrane contains not only zwitterionic but also negatively charged lipids, mainly PS and cardiolipin (35, 36); however, the distribution to the two leaflets is not exactly known.

These findings are in good agreement with data obtained in Western blotting and cytometry experiments showing LBP associated with the cell membrane of macrophages and HEK293 cells (37). Furthermore, the bactericidal/permeability-increasing protein BPI, which reveals a 45% amino acid identity with LBP, has been shown to be associated with human peripheral blood monocytes (38).

Neither the orientation of the LBP molecules in the lipid membranes at low nor at high concentrations can be determined from our experiments, and, thus, the schematic diagrams in Figs. 1, 2, and 6 show a number of possible orientations of LBP molecules in the membrane.

Force spectroscopy experiments also showed that LBP leads to a cross-linking of several phospholipid bilayers (Figs. 2 and 6d). This effect is a first hint for the function of LBP as a fusion protein. In good agreement with the indirect measurements performed on planar membranes (11), these experiments demonstrate that LBP molecules must have at least two binding sites for phospholipids.

Interaction between Soluble LBP and Lipid A Aggregates—An intercalation of LBP into lipid A aggregates and moreover a cross-linking of several layers of lipid A could be observed. The latter effect leads to a dramatic decrease of the accessible amount of lipid A by an increase of
LBP Localization in Membranes

FIGURE 6. Model for the interaction mechanism between PS, LBP, and lipid A. a, at high concentrations, LBP intercalates into PS or PC membranes and forms aggregates of several molecules protruding from the membrane surface. At low concentrations, LBP cannot be observed in height images (b); however, single LBP molecules can be detected by a cantilever functionalized by MEMO-linker molecules and αLBP antibodies (c). The orientation of LBP in the membrane is not clear yet (indicated by light red LBP molecules). d, LBP induces the formation of multilamellar structures (e) and functions as a fusion protein for phospholipid membranes and lipid A aggregates. f, the LBP-induced fusion can be inhibited by αLBP antibodies.

volume to surface ratio. This effect and an increase in the binding energy of the lipid A molecules in the aggregates provoked by the insertion of LBP molecules is probably an important effect in the neutralizing activity of LBP at higher serum concentrations observed in in vivo (12) and in vitro experiments (10). Further mechanisms of LPS neutralization by LBP, including the transfer of LPS to plasma lipoproteins, are discussed in the literature (14).

Interaction between LBP-containing PS Liposomes and Lipid A Aggregates—To understand the function of LBP in the LPS-induced signal transduction, the phospholipid liposomes preincubated with LBP were used to mimic the membrane of MNCs. After the addition of lipid A, the endotoxic principle, the preparation was adsorbed on mica. The height images show lipid surfaces with a particular domain structure (Figs. 5 and 6e). These domains did not appear when αLBP antibodies were added prior to the lipid A addition (Fig. 6f). Thus, incubation of lipid A aggregates with PS liposomes preincubated with LBP led to fusion between PS liposomes and lipid A aggregates. An important question is whether the fusion was induced by LBP intercalated into the PS membrane or by soluble LBP that was still present in the buffer. Earlier experiments using flow chambers to remove all unbound LBP molecules from the buffer demonstrated that only membrane-intercalated LBP molecules caused the binding of LPS aggregates (10). The domains exhibited different structures, hemispheres and flat structures (Fig. 5, A and B), which might be due to different stages of the fusion process. It may be proposed that LBP acts with its positively charged NH$_2$-terminal domain as a fusion protein between anionic lipid membranes and aggregates. Our observation is in contrast to some interpretations in the literature (5), where LBP has been described as a shuttle protein, removing single LPS molecules from LPS aggregates, transporting them to the cell surface of MNCs, returning to an LPS aggregate, and so on. It has been shown that cells expressing a membrane-anchored glycosylphosphatidylinositol-LBP were unresponsive to LPS under serum-free conditions (40). This fact may be explained by the incapability of glycosylphosphatidylinositol-anchored proteins to mediate the fusion of aggregates and membranes. Post et al. (41) have recently published data showing an LBP/CD14-dependent transfer of bacterial proteins from bacterial membrane blebs to host membranes. These findings can easily be explained by the proposed LBP-mediated fusion of the blebs and the host cell membrane.

Force Spectroscopy between Functionalized Cantilevers and Membrane-embedded LBP Molecules—A short range interaction (up to 0–20 nm) was measured between the modified tip and the PS surface, and, in addition, a long range interaction (up to 60 nm) with a binding force of $127 \pm 20$ pN was detected between the tip and LBP. Control experiments showed that the linker molecule MEMO also binds with a long range interaction to LBP but not to PS (data not shown). Probably not all MEMO molecules were saturated with antibodies, and, thus, the long range interactions may result from binding between LBP and either αLBP antibodies or the linker molecules. For the interpretation of our data, it is only important to know that the long range binding is specific for LBP and not for PS molecules. The technique used in these experiments to label the cantilever is, thus, so far limited to the detection of LBP molecules in a lipid matrix not containing further proteins. A prerequisite for the specific localization of proteins in cell membranes at a higher resolution would be a reduction of the number of linker molecules and with that of antibodies to the cantilever tip.

Three different bonds might break: (i) the covalent bonds between cantilever and αLBP antibodies/MEMO, (ii) the bonds between LBP and the membrane, and (iii) those between LBP and αLBP antibodies/MEMO. A force of $127$ pN is too low to break the covalent bond between αLBP antibodies/MEMO and the cantilever tip ($>1$ nN) (42). To further elucidate the source of this force, force spectroscopy experiments were repeated in a reproducible manner on positions showing either an unspecific or a specific interaction. From these experiments, it appears unlikely that LBP molecules were pulled out of the surface, but rather that the bonds between LBP and the αLBP antibodies or the linker molecules broke. The pulling distances up to the point where the bonds broke were surprisingly long. Probably, they were even too long to be explained by simple stretching of LBP molecules and αLBP antibodies. The distances can, however, be explained by the formation of long linker chains due to intermolecular cross-linking or by assuming that the linker molecules were stretched and that parts of the membrane patches, in which the LBP molecules were embedded, were stretched until breaking of the bond. However, it also must be considered that more than one LBP molecule and/or more than one αLBP antibody/MEMO is involved in the interaction.

In summary, on the basis of our experiments with model membranes, we propose that LBP intercalates into the cytoplasmic membrane of MNCs in a membrane-embedded or even transmembrane orientation and mediates the fusion between LPS aggregates and the host cell membrane. The importance of this membrane step is supported by the observations (i) that each change in the chemical composition of LPS or lipid A, such as a reduction of the number of charges and/or the number and distribution of fatty acids, causes dramatic decreases of their bioactivity down to a complete loss, (ii) that for antagonistic action, a cylindrical rather than a conical conformation of the lipid A moiety of the endotoxin molecules is a prerequisite (39), and (iii) that the active unit of endotoxin is the aggregate rather than the monomer (1). By the interaction of endotoxin with further surface proteins (e.g., the Toll-like receptor 4 and the MaxiK channel, transmembrane signaling is triggered).

Our present investigations demonstrate that a protein can be detected in a lipid membrane utilizing chemically modified AFM cantilevers. It may be expected that this method will become an important tool for the investigation of protein clusters and the formation of rafts.
REFERENCES

1. Mueller, M., Lindner, B., Kussumoto, S., Fukase, K., Schromm, A. B., and Seydel, U. (2004) J. Biol. Chem. 279, 26307–26313
2. Ramadori, G., Meyer zum Buschenfelde, K. H., Tobias, P. S., Mathison, J. C., and Ulevitch, R. J. (1996) Pathobiology 58, 89–94
3. Vreugdenhil, A. C., Dentener, M. A., Snoek, A. M., Greve, J. W., and Buurman, W. A. (1999) J. Immunol. 163, 2792–2798
4. Braas, D. M., Savov, J. D., Whitehead, G. S., Maxwell, A. B., and Schwartz, D. A. (2004) J. Allergy Clin. Immunol. 114, 586–592
5. Schumann, R. R., Leong, S. R., Flad, H.-D., Carroll, S. F., and Schindler, H. (1999) FEBS Lett. 456, 225–229
6. Schromm, A. B., Brandenburg, K., Rietschel, E. T., Flad, H.-D., Carroll, S. F., and Seydel, U. (1996) FEBS Lett. 399, 267–271
7. Thompson, P. A., Tobias, P. S., Viriyakosol, S., Kirkland, T. N., and Kitchens, R. L. (2003) J. Biol. Chem. 278, 28367–28371
8. Dunzendorfer, S., Lee, H. K., Soldau, K., and Tobias, P. S. (2004) FASEB J. 18, 1117–1119
9. Kato, A., Ogasawara, T., Homma, T., Saito, H., and Matsumoto, K. (2004) J. Biol. Chem. 279, 27832–27836
10. Gutsmann, T., Muller, M., Carroll, S. F., MacKenzie, R. C., Wiese, A., and Seydel, U. (2001) Infect. Immun. 69, 6942–6950
11. Gutsmann, T., Haberer, N., Seydel, U., and Wiese, A. (2001) Biol. Chem. 382, 425–434
12. Lamping, N., Dettmer, R., Schroeder, N. W. J., Pfeil, D., Hallatschek, W., Burger, R., and Schumann, R. R. (1998) J. Clin. Invest. 101, 2065–2071
13. Vesey, C. J., Kitchens, R. L., Wolfbauer, G., Albers, J. J., and Munford, R. S. (2000) Infect. Immun. 68, 2410–2417
14. Wurfel, M. M., Kunitske, S. T., Lichenstein, H., Kane, J. P., and Wright, S. D. (1994) J. Exp. Med. 180, 1035–1035
15. Gegner, J. A., Ulevitch, R. J., and Tobias, P. S. (1995) J. Biol. Chem. 270, 5320–5325
16. Binning, G., Quate, C. F., and Gerber, C. (1986) Phys. Rev. Lett. 56, 930–933
17. Schabert, A. H., Henn, C., and Engel, A. (1995) Science 268, 92–94
18. Muller, D. J., and Engel, A. (1999) J. Mol. Biol. 285, 1347–1351
19. Stohr, C., Wang, H., Bash, R., Ashcroft, B., Nelson, J., Gruber, H., Lohr, D., Lindsay, S. M., and Hinterdorfer, P. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 12503–12507
20. Hinterdorfer, P., Baumgartner, W., Gruber, H. J., Schilcher, K., and Schindler, H. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 3477–3481
21. Kienberger, F., Mueller, H., Pastushenko, V., and Hinterdorfer, P. (2004) EMBO Rep. 5, 579–583
22. Dunzendorfer, S., Lee, H. K., Soldau, K., and Tobias, P. S. (2004) J. Immunol. 173, 1166–1170
23. Wiese, A., Münstermann, M., Gutsmann, T., Lindner, B., Kawahara, K., Zähringer, U., and Seydel, U. (1998) J. Membr. Biol. 162, 127–138
24. Galanos, C., Liderizt, O., and Westphal, O. (1968) Eur. J. Biochem. 9, 245–249
25. Theofan, G., Horwitz, A. H., Williams, R. E., Liu, P.-S., Chan, L, Birr, C., Carroll, S. F., Mézáros, K., Parent, J. B., Kasler, H., Abeele, S., Trown, P. W., and Gazzano-Santoro, H. (1994) J. Immunol. 152, 3623–3629
26. Cai, X.-E., and Yang, J. (2002) Biophys. J. 82, 357–365
27. Beamer, L. J., Carroll, S. F., and Eisenberg, D. (1998) Protein Sci. 7, 906–914
28. Schmidt, T., Schütz, G. J., Baumgartner, W., Gruber, H. J., and Schindler, H. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 2926–2929
29. Jares-Erijman, E. A., and Jovin, T. M. (2003) Nat. Biotechnol. 21, 1387–1395
30. Pralle, A., Keller, P., Florin, E. L., Simons, K., and Hörber, J. K. H. (2000) J. Cell Biol. 148, 997–1008
31. Grandbois, M., Dettmann, W., Benoît, M., and Gaub, H. E. (2000) J. Histochem. Cytochem. 48, 719–724
32. Nollert, P., Kiefer, H., and Jähnig, F. (1995) Biophys. J. 69, 1447–1455
33. Schütz, G. J., Schindler, H., and Schmidt, T. (1997) Biophys. J. 73, 1073–1080
34. Raab, A., Han, W., Badt, D., Smith-Gill, S., Lindsay, S. M., Schindler, H., and Hinterdorfer, P. (1999) Nat. Biotechnol. 17, 902–905
35. Alvarez, E., Ruiz-Gutiérrez, V., Santa, M. C., and Machado, A. (1993) Mech. Ageing Dev. 71, 1–12
36. Kröner, E. E., Peskar, B. A., Fischer, H., and Ferber, E. (1981) J. Biol. Chem. 256, 3690–3697
37. Müller, M., Gutsmann, T., Seydel, U., and Schromm, A. B. (2004) Immunobiol. 209, 325
38. Dentener, M. A., Von Asn, J. K. E., Francot, G. J. M., Marra, M. N., and Buurman, W. A. (1993) J. Immunol. 151, 4258–4265
39. Seydel, U., Oikawa, M., Fukase, K., Kussumoto, S., and Brandenburg, K. (2000) Eur. J. Biochem. 267, 3032–3039
40. Tapping, R. I., Orr, S. L., Lawson, E. M., Soldau, K., and Tobias, P. S. (1999) J. Immunol. 162, 5483–5489
41. Post, D., Zhang, D., Eastvold, J. S., Teghanemt, A., Gibson, B. W., and Weiss, J. P. (2005) J. Biol. Chem. 280, 38383–38394
42. Grandbois, M., Beyer, M., Riefl, M., Clauss-Schaumann, H., and Gaub, H. E. (1999) Science 283, 1727–1730