Lipopolysaccharide Interaction with Cell Surface Toll-like Receptor 4-MD-2: Higher Affinity than That with MD-2 or CD14

Sachiko Akashi,1 Shin-ichiroh Saitoh,1 Yasutaka Wakabayashi,1 Takane Kikuchi,1 Noriaki Takamura,1 Yoshinori Nagai,1 Yutaka Kusumoto,1 Koichi Fukase,2 Shoichi Kusumoto,2 Yoshiyuki Adachi,4 Atsushi Kosugi,3,5 and Kensuke Miyake1,5

1Division of Infectious Genetics, The Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan
2Department of Chemistry, Graduate School of Science and 3Department of Medical Technology and Science, Graduate School of Medicine, Osaka University, Osaka 565-0871, Japan
4Laboratory of Immunopharmacology of Microbial Products, School of Pharmacy, Tokyo University of Pharmacy and Life Science, Tokyo 192-0392, Japan
5Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation, Tokyo 101-0062, Japan

Abstract

Toll-like receptors (TLRs) are innate recognition molecules for microbial products, but their direct interactions with corresponding ligands remain unclarified. LPS, a membrane constituent of gram-negative bacteria, is the best-studied TLR ligand and is recognized by TLR4 and MD-2, a molecule associated with the extracellular domain of TLR4. Although TLR4-MD-2 recognizes LPS, little is known about the physical interaction between LPS and TLR4-MD-2. Here, we demonstrate cell surface LPS–TLR4-MD-2 complexes. CD14 greatly enhances the formation of LPS–TLR4-MD-2 complexes, but is not coprecipitated with LPS–TLR4-MD-2 complexes, suggesting a role for CD14 in LPS loading onto TLR4-MD-2 but not in the interaction itself between LPS and TLR4-MD-2. A tentative dissociation constant (K_d) for LPS–TLR4-MD-2 complexes was ~3 mM, which is ~10–20 times lower than the reported K_d for LPS–MD-2 or LPS–CD14. The presence of detergent disrupts LPS interaction with CD14 but not with TLR4-MD-2. E5531, a lipid A antagonist developed for therapeutic intervention of endotoxin shock, blocks LPS interaction with TLR4-MD-2 at a concentration 100 times lower than that required for blocking LPS interaction with CD14. These results reveal direct LPS interaction with cell surface TLR4-MD-2 that is distinct from that with MD-2 or CD14.

Key words: innate immunity • cell surface molecule • activation • macrophage

Introduction

The innate immune response is the first line of defense against microbial pathogens, and plays an important role in activating acquired immunity. Recently, the toll-like receptor (TLR) family has been discovered as specific pathogen recognition molecules in the innate immune system (1). Although TLRs specifically recognize microbial products (2), detection of direct interaction between TLRs and their ligands has been unsuccessful thus far.

LPS is a principal component of gram-negative bacteria that activates the innate immune system, and is one of the best-studied microbial products (3). Great progress has been made recently in the identification of LPS recognition molecules. Core components are CD14 (4), MD-2 (5), and toll-like receptor (TLR) 4 (6, 7). CD14 binds to LPS and augments LPS responses. TLR4-MD-2 is thought to work downstream of this initial binding. TLR4 is a type I membrane protein consisting of extracellular leucine-rich repeats and an intracellular signaling domain. MD-2 is associated with the extracellular leucine-rich repeats of TLR4. Both TLR4 and MD-2 are indispensable for LPS responses because mice lacking either TLR4 or MD-2 do not respond to LPS (8, 9). Despite these evidences for LPS recognition by...
TLR4-MD-2, little is known for LPS interaction with TLR4-MD-2. Although soluble MD-2 was shown to bind to LPS (10), LPS–MD-2 needs to eventually form LPS–TLR4-MD-2 complexes on the cell surface to trigger a signal. However, little is known about LPS–TLR4-MD-2 complexes. Recent works suggested CD14-dependent LPS interaction with TLR4-MD-2 by using chemical cross-linking between LPS and TLR4-MD-2 (11–13). However, these papers showed merely the physical proximity between LPS and TLR4-MD-2, and do not necessarily demonstrate direct LPS interaction with TLR4-MD-2 in physiological condition. Direct interaction between LPS and TLR4-MD-2 still needs to be demonstrated and characterized in responding cells.

Here, we show, with a newly established monoclonal antibody to TLR4-MD-2, LPS–TLR4-MD-2 complexes on the cell surface, and we demonstrate direct LPS interaction with TLR4-MD-2 that is distinct from that with MD-2 or CD14.

**Materials and Methods**

**Reagents.** LPS from *Escherichia coli* 055:B5 and lipid A purified from *Salmonella minnesota* were purchased from Sigma-Aldrich. Synthetic lipid A and 3H-labeled lipid A were described previously (14). E5531 was obtained from Eisai Research Institute. Sources of other microbial products were described previously (8). mAbs to LPS and lipid A were purchased from Hycult Biotechnology. Detergents such as Brij98 and N-octyl-β-d-glucoside were purchased from Sigma-Aldrich and Wako, respectively. Rabbit anti-mouse TLR4 serum was established by immunized rabbits with TLR4 (14). E5531 was obtained from Eisai Research Institute. Sources of other microbial products were described previously (8). mAbs to LPS and lipid A were purchased from Hycult Biotechnology. Detergents such as Brij98 and N-octyl-β-d-glucoside were purchased from Sigma-Aldrich and Wako, respectively. Rabbit anti-mouse TLR4 serum was established by immunized rabbits with TLR4 (14). E5531 was obtained from Eisai Research Institute. Sources of other microbial products were described previously (8). mAbs to LPS and lipid A were purchased from Hycult Biotechnology. Detergents such as Brij98 and N-octyl-β-d-glucoside were purchased from Sigma-Aldrich and Wako, respectively. Rabbit anti-mouse TLR4 serum was established by immunized rabbits with TLR4 (14). E5531 was obtained from Eisai Research Institute. Sources of other microbial products were described previously (8). mAbs to LPS and lipid A were purchased from Hycult Biotechnology. Detergents such as Brij98 and N-octyl-β-d-glucoside were purchased from Sigma-Aldrich and Wako, respectively. Rabbit anti-mouse TLR4 serum was established by immunized rabbits with TLR4 (14). E5531 was obtained from Eisai Research Institute. Sources of other microbial products were described previously (8). mAbs to LPS and lipid A were purchased from Hycult Biotechnology. Detergents such as Brij98 and N-octyl-β-d-glucoside were purchased from Sigma-Aldrich and Wako, respectively. Rabbit anti-mouse TLR4 serum was established by immunized rabbits with TLR4 (14). E5531 was obtained from Eisai Research Institute. Sources of other microbial products were described previously (8). mAbs to LPS and lipid A were purchased from Hycult Biotechnology. Detergents such as Brij98 and N-octyl-β-d-glucoside were purchased from Sigma-Aldrich and Wako, respectively. Rabbit anti-mouse TLR4 serum was established by immunized rabbits with TLR4 (14). E5531 was obtained from Eisai Research Institute. Sources of other microbial products were described previously (8). mAbs to LPS and lipid A were purchased from Hycult Biotechnology. Detergents such as Brij98 and N-octyl-β-d-glucoside were purchased from Sigma-Aldrich and Wako, respectively. Rabbit anti-mouse TLR4 serum was established by immunized rabbits with TLR4 (14). E5531 was obtained from Eisai Research Institute. Sources of other microbial products were described previously (8). mAbs to LPS and lipid A were purchased from Hycult Biotechnology. Detergents such as Brij98 and N-octyl-β-d-glucoside were purchased from Sigma-Aldrich and Wako, respectively. Rabbit anti-mouse TLR4 serum was established by immunized rabbits with TLR4 (14). E5531 was obtained from Eisai Research Institute. Sources of other microbial products were described previously (8). mAbs to LPS and lipid A were purchased from Hycult Biotechnology. Detergents such as Brij98 and N-octyl-β-d-glucoside were purchased from Sigma-Aldrich and Wako, respectively. Rabbit anti-mouse TLR4 serum was established by immunized rabbits with TLR4 (14). E5531 was obtained from Eisai Research Institute. Sources of other microbial products were described previously (8). mAbs to LPS and lipid A were purchased from Hycult Biotechnology. Detergents such as Brij98 and N-octyl-β-d-glucoside were purchased from Sigma-Aldrich and Wako, respectively. Rabbit anti-mouse TLR4 serum was established by immunized rabbits with TLR4 (14). E5531 was obtained from Eisai Research Institute. Sources of other microbial products were described previously (8). mAbs to LPS and lipid A were purchased from Hycult Biotechnology. Detergents such as Brij98 and N-octyl-β-d-glucoside were purchased from Sigma-Aldrich and Wako, respectively. Rabbit anti-mouse TLR4 serum was established by immunized rabbits with TLR4 (14). E5531 was obtained from Eisai Research Institute. Sources of other microbial products were described previously (8). mAbs to LPS and lipid A were purchased from Hycult Biotechnology. Detergents such as Brij98 and N-octyl-β-d-glucoside were purchased from Sigma-Aldrich and Wako, respectively. Rabbit anti-mouse TLR4 serum was established by immunized rabbits with TLR4 (14). E5531 was obtained from Eisai Research Institute. Sources of other microbial products were described previously (8). mAbs to LPS and lipid A were purchased from Hycult Biotechnology. Detergents such as Brij98 and N-oc...
that cell surface expression of TLR4-MD-2 on peritoneal macrophages decreases upon LPS stimulation, as detected by a mAb to TLR4-MD-2, MTS510 (19). We were able to see a similar phenomenon in Ba/F3 transfectants and macrophage line RAW264. The decrease in MTS510 staining is observed with lipid A or LPS stimulation but not with other stimuli, such as peptidoglycan (PGN) or CpG oligodeoxynucleotide (Fig. 2 and not depicted). The down-regulation became apparent with as little as 20 ng/ml in RAW264 and 200 ng/ml in the transfectant, and as early as 10 min after LPS stimulation (unpublished data). Interestingly, lipid A–induced decrease in MTS510 staining was not clear in the absence of membrane CD14 (Fig. 2, left column). In keeping with this, anti-CD14 mAb was able to inhibit LPS-triggered decrease in MTS510 staining (unpublished data).

**Newly Established mAb to TLR4-MD-2 Reveals the LPS-induced Change of TLR4-MD-2.** We asked the cause of the lipid A–dependent decrease in MTS510 staining. We considered three possibilities. First, TLR4-MD-2 may be internalized upon LPS stimulation as described previously (20). Second, MD-2 may be dissociated from TLR4; MTS510 does not bind to TLR4 alone (17). Finally, TLR4-MD-2 remains on the cell surface but LPS stimulation may alter TLR4-MD-2 to a conformation, which MTS510 cannot bind. Biochemical analyses revealed that MTS510 reacted with TLR4-MD-2 before LPS stimulation but not after LPS stimulation. For further study, we established a novel mAb to TLR4-MD-2, Sa15-21, which was able to react with LPS-stimulated TLR4-MD-2, to which MTS510 did not bind. Sa15-21 was similar to MTS510 in that it specifically immunoprecipitated TLR4-MD-2 but not TLR4 alone (Fig. 3 a). However, Sa15-21 recognized an epitope distinct from the MTS510 epitope, because these two mAbs did not crossblock with each other (unpublished data). Sa15-21 remained reactive with LPS- and lipid A–stimulated cells expressing CD14 and TLR4-MD-2 as revealed by flow cytometry staining (Fig. 3 b). Similar results were obtained with RAW264 cells (unpublished data). Biochemical analyses were conducted with Sa15-21 mAb (Fig. 3 c). Ba/F3 cells expressing TLR4-MD-2 and CD14 or RAW264 cells were subjected to LPS stimulation, cell surface biotinylation, and immunoprecipitation with Sa15-21 or MTS510. MTS510 precipitated cell surface TLR4-MD-2 from cells treated with medium alone or a lipid A antagonist, E5531, but not from cells stimulated with LPS. In sharp contrast, Sa15-21 was able to precipitate cell surface TLR4-MD-2 from LPS-stimulated cells. Together, LPS-stimulated TLR4-MD-2 remains on the cell surface, but is likely to undergo the LPS-dependent conformation change, which is not recognized by MTS510 mAb.

**LPS and TLR4-MD-2 Forms Stable Complexes on the Cell Surface.** Next, we hypothesized that the change of TLR4-MD-2 may accompany the direct interaction between LPS and TLR4-MD-2. To address this possibility, we conducted immunoprecipitation of lipid A–stimulated TLR4-MD-2 with Sa15-21, and coprecipitation of lipid A was
We were able to detect lipid A coprecipitation with TLR4-MD-2 by Sa15-21 mAb but not by MTS510 mAb. Interestingly, MTS510 was able to precipitate TLR4-MD-2 from lipid A–stimulated cells as detected by anti-TLR4 polyclonal mAb (Fig. 4 a, bottom). The amount of precipitated TLR4 seems to be smaller than that from cells without stimulation. Precipitated TLR4-MD-2 is likely to be located inside the cells, because we could not detect the precipitation by MTS510 of LPS-stimulated, cell surface TLR4-MD-2 (Fig. 3 c). Lipid A was not present in these MTS510 precipitates containing intracellular TLR4-MD-2 (Fig. 4 a, bottom). We also examined CD14 coprecipitation with lipid A–TLR4-MD-2 complexes to address the possibility that association of lipid A with TLR4-MD-2 is mediated by CD14. We could not detect CD14 in lipid A–TLR4-MD-2 complexes precipitated by Sa15-21, demonstrating the direct interaction between lipid A and TLR4-MD-2 (Fig. 4 a, middle).

Similar results were obtained with LPS and anti-LPS antibody (unpublished data). We also detected LPS–TLR4-MD-2 complexes with native cells such as a macrophage line RAW264 (unpublished data). Thus, the LPS–lipid A–dependent change of TLR4-MD-2 accompanies the formation of LPS–TLR4-MD-2 complexes on the cell surface.

**A Role for Membrane CD14 in LPS Interaction with TLR4-MD-2.** Membrane CD14 was required for the LPS-dependent conformation change of TLR4-MD-2 (Fig. 2), but CD14 was not present in lipid A–TLR4-MD-2 complexes (Fig. 4 a). Next, we addressed a role for membrane CD14 in LPS interaction with TLR4-MD-2 by using Ba/F3 transfectants separately expressing MD-2flag, TLR4flag, CD14, TLR4-MD-2flag, CD14 and TLR4flag, CD14 and MD-2flag, and CD14 and TLR4-MD2flag (Materials and Methods). We used 3H-lipid A to stimulate the transfec-tants. Immunoprecipitation was conducted with MTS510, Sa15-21, anti-flag mAb, or anti-CD14 mAb. Precipitated radioactivity is shown (Fig. 4 b and not depicted). Appre-ciable counts were detected in the precipitate with Sa15-21 from Ba/F3 transfectant expressing CD14 and TLR4-MD-2, but not clearly from that expressing TLR4-MD-2. Lipid A coprecipitation was also observed with anti-flag mAb only from the transfectant expressing CD14 and TLR4-MD-2 (Fig. 4 b). We could not detect lipid A coprecipitation with the anti-flag mAb from cells separately expressing MD-2flag, TLR4flag, TLR4-MD-2flag, CD14 and TLR4flag, or CD14 and MD-2flag (Fig. 4 b and not depicted). We had similar results with LPS, coprecipitation of which was detected by anti-LPS mAb (unpublished data). These results demonstrate an important role for membrane CD14 in facilitating the establishment of the interaction between lipid A and TLR4-MD-2. However, CD14 is not required for the interaction itself, because membrane

---

**Figure 3.** A novel mAb to TLR4-MD-2 reveals the LPS-triggered change of cell surface TLR4-MD-2. (a) Immunoprecipitation with anti-flag (top) or Sa15-21 (bottom) was conducted with Ba/F3 transfectants expressing the indicated molecules (Materials and Methods). The precipitates were probed with rabbit anti–mouse TLR4 sera followed by goat anti-rabbit alkaline phosphatase. Only immature, smaller TLR4 is detected in cells expressing TLR4 alone (top, TLR4f and CD14/TLR4f), because TLR4 without MD-2 cannot reach the cell surface. (b) Ba/F3 transfectants expressing CD14 and TLR4-MD-2 were stimulated with medium alone, 1 μg/ml lipid A, or 1 μg/ml LPS at 37°C for 30 min. Cells were stained with biotinylated MTS510 mAb or Sa15-21 as indicated, followed by streptavidin-PE. Open histograms depict staining with streptavidin-PE alone. (c) Ba/F3 cells expressing TLR4-MD-2 and CD14 (top) or RAW264 (bottom) were stimulated with medium, 2 μg/ml LPS, or 2 μg/ml lipid A antagonist E5531 as indicated at 37°C for 30 min. After washing, cells were subjected to cell surface biotinylation, detergent lysis, probed with anti–lipid A antibody (Fig. 4 a, bottom).
CD14 is not present in lipid A–TLR4-MD-2 complexes (Fig. 4 a). It should be noted that only 1–2% of cell bound lipid A was coprecipitated with TLR4–MD-2 (Fig. 4 b).

Unsurprisingly, lipid A coprecipitation was not detected in the CD14 precipitates with anti-CD14 mAb (Fig. 4, a [right lane]). We prepared cell lysates for immunoprecipitation using lysis buffer containing 1% Triton X-100, which might disrupt LPS interaction with CD14. To address this possibility, soluble CD14–LPS complexes were immunoprecipitated with anti-CD14 in the absence of detergents (Fig. 5). LPS was clearly coprecipitated with soluble CD14. Moreover, LPS coprecipitation was abolished by the presence of detergents such as 1% Triton X-100, 0.5% N-octyl-β-D-glucoside, and 1% Brij98, indicating that these detergents disrupted LPS interaction with CD14. In view of the possibility that interaction between LPS and TLR4–MD-2 can only be observed in the presence of detergent, we conducted immunoprecipitation of sonicated cell membrane in the absence of detergent. The results using mechanically solubilized cell lysate clearly demonstrated physical association between LPS and TLR4–MD-2 (unpublished data). Thus, LPS interaction with TLR4–MD-2 seems to be distinct from and more stable than with CD14.

We could not detect lipid A–MD-2 complexes by immunoprecipitation of MD-2 from cells expressing MD-2flag or CD14 (Fig. 4 b and not depicted). It is possible that lipid A–MD-2 interaction might be disrupted in the presence of detergents such as 1% Triton X-100, 0.5% N-octyl-β-D-glucoside, and 1% Brij98, indicating that these detergents disrupted LPS interaction with CD14. In view of the possibility that interaction between LPS and TLR4–MD-2 can only be observed in the presence of detergent, we conducted immunoprecipitation of sonicated cell membrane in the absence of detergent. The results using mechanically solubilized cell lysate clearly demonstrated physical association between LPS and TLR4–MD-2 (unpublished data). Thus, LPS interaction with TLR4–MD-2 seems to be distinct from and more stable than with CD14.
E5531 was shown to block LPS binding to macrophage cells, suggested concentration of E5531. Soluble CD14 was precipitated with anti-CD14 mAb and LPS coprecipitation was indicated concentration of E5531. Specific binding was obtained by subtracting bound cpm with MTS510 from that with Sa15-21. Bound lipid A (cpm) was plotted against input lipid A (cpm) in panel a. Scatchard plot is shown in panel b. Two independent experiments were conducted and similar results were obtained.

Lipid A Antagonist E5531 Acts on TLR4-MD-2. To further characterize interaction between LPS and TLR4-MD-2, we used E5531, a potent LPS antagonist developed for therapeutic intervention of endotoxin shock (22). E5531 was shown to block LPS binding to macrophage cells, supposedly by antagonizing LPS binding to membrane CD14 (23). However, further analysis demonstrates that E5531 inhibits CD14-independent, TLR4-MD-2 agonists (24). We hypothesized that E5531 acts on TLR4-MD-2 rather than on CD14. Ba/F3 cells expressing CD14 and TLR4-MD-2 were pretreated with graded concentrations of E5531 for 30 min and stimulated with LPS at 3 μg/ml. We added E5531 up to 30 μg/ml, 10 times higher than the concentration of LPS. E5531 was able to block CD14-dependent LPS binding to cells at concentrations higher than 10 μg/ml (Fig. 7 a, right). For further confirmation of the antagonistic effect of E5531, we also conducted LPS coprecipitation with soluble CD14. The supernatant from Ba/F3 cells expressing CD14 was exposed to 3 μg/ml LPS with or without the indicated concentration of E5531. Soluble CD14 was precipitated with anti-CD14 mAb and LPS coprecipitation was probed with anti-LPS. LPS coprecipitation gradually decreased with increased concentrations of E5531, leading to complete inhibition at 10 μg/ml of E5531 (Fig. 7 b).

Next, we examined the effect of E5531 on TLR4-MD-2. E5531 completely antagonized LPS-dependent down-regulation of MTS510 staining at a concentration as low as 0.1 μg/ml (Fig. 7 a, middle). LPS association with TLR4-MD-2 was disrupted with concentrations of E5531 higher than 0.1 μg/ml (Fig. 7 c). Furthermore, E5531 was able to inhibit LPS-stimulated IκBα degradation at 0.1 μg/ml (Fig. 7 d). With regard to sensitivity to E5531 treatment, LPS signaling correlated precisely with LPS interaction with TLR4-MD-2 but not with CD14.

Discussion

A newly established mAb to TLR4-MD-2, Sa15-21, demonstrated lipid A–TLR4-MD-2 complexes on the cell surface by precipitating lipid A–TLR4-MD-2 complexes from cells expressing TLR4-MD-2 and CD14 (Fig. 4). Lipid A interaction with TLR4-MD-2 was dose-dependent and saturable (Fig. 6 a). Cell surface TLR4-MD-2 interacts with lipid A at a tentative K<sub>d</sub> of ∼3 nM, which is ∼10–20 times lower than that of soluble MD-2 (65 nM) or CD14 (74 nM; reference 10). This binding assay uses immunoprecipitation and is different from a conventional ligand binding assay. We could not directly compare, with the binding assay, TLR4-MD-2 with CD14 or MD-2 with regard to lipid A interaction because lipid A was not coprecipitated with CD14 or MD-2, probably due to the presence of detergents (Figs. 4 and 5). However, even in such a rigorous condition where affinity decreased, our binding assay still yielded the K<sub>d</sub> for lipid A–TLR4-MD-2 that is still lower than the reported K<sub>d</sub> for lipid A–CD14 or lipid A–MD-2. In keeping with the lower K<sub>d</sub> LPS–TLR4-MD-2 complexes were disrupted by lipid A antagonist E5531 at ∼100 times lower concentration than LPS–CD14 complexes (Fig. 7). Together, the present results demonstrated LPS–TLR4-MD-2 complexes that interact with each other at higher affinity and are more stable than LPS–MD-2 or LPS–CD14 complexes. The higher affinity for TLR4-MD-2 than that of soluble MD-2 may be due to associated TLR4 that might directly bind to lipid A or strengthen interaction between MD-2 and lipid A.

Although LPS interaction with soluble MD-2 does not require CD14 (10), membrane CD14 was required for LPS interaction with TLR4-MD-2 (Fig. 4 b). Recent papers suggest that LPS triggers CD14 association with TLR4-MD-2 (11, 13). However, we could not detect CD14 association with LPS–TLR4-MD-2 complexes (Fig. 4 a). Even if CD14 associates itself with LPS–TLR4-MD-2 complexes, the association probably plays a minimal role in sustaining the direct interaction between LPS and TLR4-MD-2; LPS interaction with TLR4-MD-2 is higher in affinity and more stable than with CD14. CD14 seems to have a role in loading LPS onto TLR4-MD-2, which by itself showed little binding to LPS (Fig. 1). It should be noted that CD14 is not essential for LPS response. CD14 KO mice still show significant LPS responses (25, 26). A CD14-independent mechanism for loading LPS onto TLR4-MD-2 must be present and is likely to be accelerated by membrane CD14. We believe that a small number of LPS–TLR4-MD-2 complexes are present on cells expressing TLR4-MD-2 without CD14. The number of complexes is probably too small to be detected in the present detection system. Interestingly, only 1–2% of cell-associated lipid A was coprecipitated with TLR4-MD-2, even with membrane CD14 (Fig. 4 b). The CD14-independent lipid A loading mechanism might restrict the amount of lipid A to be loaded onto TLR4-MD-2.

Further study has to focus on molecular mechanisms underlying LPS loading onto TLR4-MD-2.
ter stimulation, demonstrating the ligand-dependent change of TLR4-MD-2 (Figs. 2 and 3). The LPS-triggered change of TLR4-MD-2 correlates perfectly with LPS–TLR4-MD-2 complex formation. It is possible that the LPS-dependent change of TLR4-MD-2 reflects the conformation change of TLR4-MD-2, which leads to dimerization of TLR4-MD-2 and delivering a signal. The LPS–dependent change of TLR4-MD-2 may reveal a link between LPS interaction and LPS signaling. Further studies on the antagonistic action of E5531 may be important to address this issue, because E5531 does not induce the LPS-dependent change and prevents LPS interaction with TLR4-MD-2 (Fig. 7 a).

It has been described previously that E5531 inhibits LPS binding to cells, suggesting competition with LPS in binding to CD14 as an antagonistic mechanism (23). Because CD14 binds other microbial products in addition to LPS (27), E5531 should be able to compete CD14 interaction with these microbial products. However, E5531 is antagonistic to LPS but not to other microbial products (e.g., PGN; reference 23). Moreover, E5531 inhibits CD14-independent TLR4-MD-2 ligands (24). These results suggest that E5531 directly acts on TLR4-MD-2. The present experimental system enabled us to address this question. E5531 inhibited LPS interaction with TLR4-MD-2 at a concentration 100 times lower than that required for inhibiting LPS interaction with CD14 (Fig. 7 g). Given that E5531 acts on TLR4-MD-2, it is reasonable that E5531 antagonizes TLR4-MD-2 ligand other than LPS. Thus, the present paper has revealed a novel mechanism for the action of this lipid A antagonist.

The reported incidence of sepsis syndromes is increasing dramatically in hospitalized patients. Despite aggressive management, many patients die of endotoxin shock. E5531 was developed with an aim to neutralize endotoxin in vivo (22). Here, we showed that E5531 targets TLR4-MD-2, which mediates the adverse effects of endotoxin (8, 9). TLR4-MD-2 is arguably a target for therapeutic interven-
tion of endotoxin shock. The present work provides a theoretical background and assay system for further improvement of lipid A antagonists and for development of other therapeutic interventions for endotoxin shock.

We thank Drs. K. Takatsu and D.R. Liddicoat for reviewing the manuscript, and Ms. K. Tomita and K. Shinoda for technical assistance.

This study was supported by Special Coordination Funds of the Ministry of Education, Culture, Sports, Science and Technology of the Japanese Government; the Uehara Memorial Foundation; the Naito Foundation; the Sankyo Co., and Mitsubishi Pharma.

Submitted: 1 July 2003
Revised: 8 August 2003
Accepted: 8 August 2003

References

1. Janeway, C.A., Jr., and R. Medzhitov. 2002. Innate immune recognition. Annu. Rev. Immunol. 20:197–216.
2. Takeda, K., T. Kaisho, and S. Akira. 2003. Toll-like receptors. Annu. Rev. Immunol. 21:335–376.
3. Ulevitch, R.J., and P.S. Tobias. 1995. Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. Annu. Rev. Immunol. 13:437–457.
4. Wright, S.D., R.A. Ramos, P.S. Tobias, R.J. Ulevitch, and J.C. Mathison. 1990. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science. 249:1431–1433.
5. Shimazu, R., S. Akashi, H. Ogata, Y. Nagai, K. Fukudome, K. Miyake, and M. Kimoto. 1999. MD-2, a molecule that confers lipopolysaccharide responsiveness on toll-like receptor 4. J. Exp. Med. 189:1777–1782.
6. Medzhitov, R., P. Preston-Hurlburt, and C.A. Janeway, Jr. 1997. A human homologue of the Drosophila toll protein signals activation of adaptive immunity. Nature. 388:394–397.
7. Poltorak, A., X. He, I. Smirnova, M.Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, et al. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in toll-like receptor 4 gene. Science. 282:2085–2088.
8. Nagai, Y., S. Akashi, M. Nagafuku, M. Ogata, Y. Iwakura, S. Akira, T. Kitamura, A. Kosugi, M. Kimoto, and K. Miyake. 2002. Essential role of MD-2 in LPS responsiveness and TLR4 distribution. Nat. Immunol. 3:667–672.
9. Hoshino, K., O. Takeuchi, T. Kawai, H. Sanjo, T. Ogawa, Y. Takeda, K. Takeda, and S. Akira. 1999. Cutting edge: toll-like receptor 4 (TLR4)-deficient mice are hypersensitive to lipopolysaccharide: evidence for TLR4 as the Lps receptor. J. Immunol. 162:3749–3752.
10. Vinyakosol, S., P.S. Tobias, R.L. Kitchens, and T.N. Kirkland. 2001. MD-2 binds to bacterial lipopolysaccharide. J. Biol. Chem. 276:38044–38051.
11. da Silva Correia, J., K. Soldau, U. Christen, P.S. Tobias, and R.J. Ulevitch. 2001. Lipopolysaccharide is in close proximity to each of the proteins in its membrane receptor complex. Transfer from CD14 to TLR4 and MD-2. J. Biol. Chem. 276:21129–21135.
12. da Silva Correia, J., and R.J. Ulevitch. 2002. MD-2 and TLR4 N-linked glycosylations are important for a functional lipopolysaccharide receptor. J. Biol. Chem. 277:1845–1854.
13. Muroi, M., T. Ohnishi, and K. Tanamoto. 2002. Regions of the mouse CD14 molecule required for toll-like receptor 2- and 4-mediated activation of NF-kappa B. J. Biol. Chem. 277:42372–42379.
14. Fukase, K., T. Kirikae, F. Kirikae, W.C. Liu, M. Oikawa, Y. Suda, M. Kurosawa, Y. Fukase, H. Yoshizaki, and S. Kusumoto. 2001. Synthesis of [3H]-labeled bioactive lipid A analogs and their use for detection of lipid A-binding proteins on murine macrophages. Bull. Chem. Soc. Jpn. 74:2189–2197.
15. Palacios, R., and M. Steinmetz. 1985. IL-3-dependent mouse clones that express B-220 surface antigen, contain Ig genes in germ-line configuration, and generate B lymphocytes in vivo. Cell. 41:727–734.
16. Kitamura, T. 1998. New experimental approaches in retrovirus-mediated expression screening. Int. J. Hematol. 67:351–359.
17. Akashi, S., R. Shimazu, H. Ogata, Y. Nagai, K. Takeda, M. Kimoto, and K. Miyake. 2000. Cutting edge: cell surface expression and lipopolysaccharide signaling via the toll-like receptor 4–MD-2 complex on mouse peritoneal macrophages. J. Immunol. 164:3471–3475.
18. Tasaka, S., A. Ishizaka, W. Yamada, M. Shimizu, H. Koh, N. Hasegawa, Y. Adachi, and K. Yamaguchi. 2003. Effect of CD14 blockade on endotoxin-induced acute lung injury in mice. Am. J. Respir. Cell Mol. Biol. 29:252–258.
19. Nomura, F., S. Akashi, Y. Sakao, S. Sato, T. Kawai, M. Matsumoto, K. Nakanishi, M. Kimoto, K. Miyake, K. Takeda, and S. Akira. 2000. Cutting edge: endotoxin tolerance in mouse peritoneal macrophages correlates with down-regulation of surface toll-like receptor 4 expression. J. Immunol. 164:3476–3479.
20. Latz, E., A. Visintin, E. Lien, K.A. Fitzgerald, B.G. Monks, E.A. Kurt-Jones, D.T. Golenbock, and T. Espevik. 2002. Lipopolysaccharide rapidly traffics to and from the Golgi apparatus with the toll-like receptor 4–MD-2–CD14 complex in a process that is distinct from the initiation of signal transduction. J. Biol. Chem. 277:47834–47843.
21. Tobias, P.S., K. Soldau, J.A. Gegner, D. Mintz, and R.J. Ulevitch. 1995. Lipopolysaccharide binding protein-mediated complexation of lipopolysaccharide with soluble CD14. J. Biol. Chem. 270:10482–10488.
22. Christ, W.J., O. Asano, A.L. Robidoux, M. Perez, Y. Wang, G.R. Dubuc, W.E. Gavin, L.D. Hawkins, P.D. McGuinness, M.A. Mullarkey, et al. 1995. E5531, a pure endotoxin antagonist of high potency. Science. 268:80–83.
23. Kawata, T., J.R. Bristol, D.P. Rossignol, J.R. Rose, S. Kobayashi, H. Yokohama, A. Ishibashi, W.J. Christ, K. Katayama, I. Yamatsu, and Y. Kishi. 1999. E5531, a synthetic non-toxic lipid A derivative blocks the immunobiological activities of lipopolysaccharide. Br. J. Pharmacol. 127:853–862.
24. Means, T.K., B.W. Jones, A.A. Schromm, B.A. Shurtleff, J.A. Smith, J. Keane, D.T. Golenbock, S.N. Vogel, and M.J. Fenton. 2001. Differential effects of a toll-like receptor antagonist on Mycobacterium tuberculosis-induced macrophage responses. J. Immunol. 166:4074–4082.
25. Haziot, A., E. Ferrero, F. Kontgen, N. Hijiya, S. Yamanoto, J. Silver, C.L. Stewart, and S.M. Goyert. 1996. Resistance to endotoxin shock and reduced dissemination of gram-negative bacteria in CD14–deficient mice. Immunity. 4:407–414.
26. Haziot, A., X.Y. Lin, F. Zhang, and S.M. Goyert. 1998. The induction of acute phase proteins by lipopolysaccharide uses a novel pathway that is CD14-independent. J. Immunol. 160:2570–2572.
27. Pugin, J., I.D. Heumann, A. Tomas, V.V. Kravchenko, Y. Akamatsu, M. Nishijima, M.P. Glauser, P.S. Tobias, and R.J. Ulevitch. 1994. CD14 is a pattern recognition receptor. Immunity. 1:509–516.