

MD-2, a Novel Accessory Molecule, Is Involved in Species-Specific Actions of *Salmonella* Lipid A

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***Salmonella* lipid A is inactive in human macrophages despite being potently active in murine macrophages. We investigated the molecular basis for this species-specific action of *Salmonella* lipid A. When murine CD14 (mCD14), mTLR4, and mMD-2 were all expressed in human monocytic THP-1 cells, these cells were capable of responding to *Salmonella* lipid A. Expressing each of these proteins separately did not impart such responsiveness. Expression of mTLR4 plus mMD-2, but not mCD14 plus mTLR4 or mCD14 plus mMD-2, conferred this responsiveness. In THP-1 cells expressing mCD14, mTLR4, and mMD-2, replacing mCD14 with human CD14 had no effect on responsiveness to *Salmonella* lipid A or synthetic *Salmonella*-type lipid A (compound 516). When mTLR4 was replaced with human TLR4, the responses to these lipid A preparations were decreased to half, and the replacement of mMD-2 decreased responsiveness to one-third, although the responses to *Escherichia coli* lipid A or synthetic *E. coli*-type lipid A (compound 506) were not affected. These results indicate that both TLR4 and MD-2 participate in the species-specific action of *Salmonella* lipid A.**

Bacterial lipopolysaccharide (LPS) is a constituent of the outer membrane of the cell wall of gram-negative bacteria and plays a major role in septic shock in humans (24, 29). The lipid A portion has been identified as the active center responsible for most of the LPS-induced biological effects (17, 24). Lipid A species from *Escherichia coli* and *Salmonella* are well characterized in terms of their structures and biological activities (3, 4, 6, 10, 13, 14). Both *E. coli*-type (compounds called 506) and *Salmonella*-type (compounds called 516) lipid A molecules have been synthesized chemically (8, 9, 15) and have been demonstrated to be highly active in all test systems examined, although the activity of *Salmonella*-type lipid A was slightly less than that of *E. coli*-type lipid A.

CD14 has been recognized as a receptor for LPS (5, 31). However, since CD14 does not possess an intracellular domain, functional receptors transmitting LPS signaling into intracellular components have been sought, and the Toll-like receptor (TLR) 4 (1, 28) was found to confer LPS responsiveness. Toll-like receptors are mammalian homologues of the *Drosophila* Toll protein (18). TLR4 was initially recognized as a molecule which increases constitutive NF- κ B but not LPS-inducible activity (19). However, the finding of a novel accessory molecule, MD-2 (25), which confers LPS responsiveness on TLR4, and analyses of TLR4-deficient mice (7, 20, 22, 26, 30) have provided strong evidence for involvement of the TLR4/MD-2 complex in LPS signaling. Although MD-2 is reported to associate with TLR4 (25), the molecular mechanism by which MD-2 participates in LPS signaling via TLR4 has not been clarified.

We previously reported that lipid A preparations from various *Salmonella* strains and synthetic *Salmonella*-type lipid A (compound 516) possess very little stimulatory activity in hu-

man macrophages, despite being potently active in murine macrophages (27). On the other hand, lipid A preparations from *E. coli* and synthetic *E. coli*-type lipid A (compound 506) were equally active in human and murine macrophages (27). The only structural difference between the lipid A molecules typically found in these lipid A preparations is the hexadecanoyl acid attached to the hydroxy residue of 3-hydroxy tetradecanoic acid bound to position 2 of the reducing glucosamine (Fig. 1). Thus, human monocytes/macrophages appear capable of discriminating this fine structural difference. To explore the mechanism of this discriminating recognition, we investigated in the present study which molecule of the aforementioned LPS signal transducers participates in this species-specific action of *Salmonella* lipid A and found that the acquisition of responsiveness is associated with the murine TLR4/MD-2 complex.

MATERIALS AND METHODS

Cell culture and reagents. The human monocyte-like cell line THP-1 (obtained from the Human Science Research Resources Bank, Tokyo, Japan) was grown in RPMI 1640 (Gibco BRL, Rockville, Md.) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (FCS; Gibco BRL), penicillin (100 U/ml), and streptomycin (100 μ g/ml). Diphosphoryl lipid A from *E. coli* F-583 was obtained from Sigma (St. Louis, Mo.). *Salmonella* lipid A was prepared from *Salmonella enterica* serovar Abortus-equi as described previously (27). We confirmed the absence of TLR2-stimulatory activity in both lipid A preparations by checking NF- κ B-dependent reporter activity in 293 cells transfected with CD14 and TLR2. Synthetic *E. coli*-type lipid A (compound 506) and *Salmonella*-type lipid A (compound 516) were kind gifts of Daiichi Kagaku (Tokyo, Japan).

Expression plasmids. Plasmids containing human CD14 (hCD14) and mouse CD14 (mCD14) cDNAs were provided by Shunsuke Yamamoto (Medical College of Oita, Oita, Japan). The coding regions of hTLR4, mTLR4, and hMD-2 were amplified by reverse transcription-PCR from total RNA prepared from human spleen (OriGene Technologies, Rockville, Md.), murine fibroblast L929 cells, and THP-1 cells, respectively. The coding region of mMD-2 was amplified from a mouse embryo cDNA library (Clontech, Palo Alto, Calif.). Each PCR product was cloned into a mammalian expression vector, pcDNA3 (Invitrogen, Carlsbad, Calif.). The coding regions of all constructs described above minus their respective signal peptide sequences were subcloned into the downstream region of a modified pcDNA3 vector, in which the preprotrypsin signal peptide sequence precedes the NH₂-terminal EIAV-tag epitope (amino acid sequence, ADRRIPGTAEE). A similar LPS

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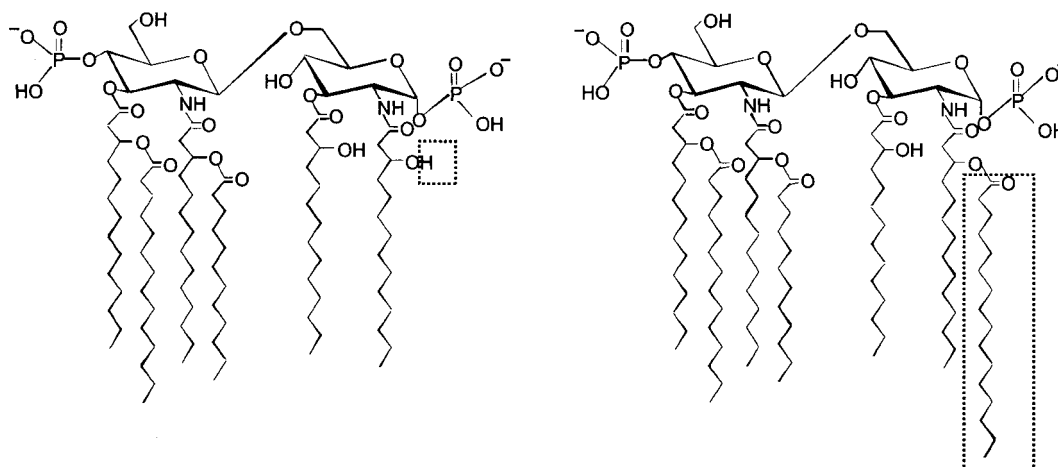


FIG. 1. Chemical structures of lipid A molecules. Structures of *E. coli*-type (compound 506) (left) and *Salmonella*-type (compound 516) (right) lipid A molecules are shown. The only difference between the structures of 506 and 516 is indicated by the dotted rectangles.

response was observed in 293 cells transfected with either tagged or untagged versions of the plasmids described above. The PCR primers used for constructing the plasmids are listed in Table 1. An NF- κ B-dependent luciferase reporter plasmid, pELAM-L, was constructed by inserting the PCR fragment (-730 to +52) of the E-selectin (ELAM-1) promoter (23) into the *SacI-HindIII* site of the pGL3-Basic vector (Promega, Madison, Wis.).

NF- κ B reporter assay. THP-1 cells (2×10^6 /well) were plated in six-well dishes and induced to differentiate by 100 ng of phorbol myristate acetate (Sigma)/ml plus 100 nM 1,25-dihydroxy vitamin D₃ (Wako Pure Chemical Industries, Tokyo, Japan) for 3 to 4 days. Following differentiation, the cells were transfected in 1 ml of the culture medium using 3 μ l of FuGene 6 transfection reagent (Roche Molecular Laboratories, Burlington, N.C.) according to the manufacturer's instructions with 0.1 μ g (each) of the indicated expression plasmids, 1 μ g of pELAM-L, and 0.1 μ g of pRL-TK (Promega) for normalization. After 24 h, the cells were stimulated in the culture medium containing 10% FCS for 6 h, and reporter gene activity was measured according to the manufacturer's instructions.

RESULTS

Expression of mCD14, mTLR4, and mMD-2 imparts responsiveness to *Salmonella* lipid A. Lipid A preparations from *Salmonella* induce I κ B- α degradation in mouse macrophages

but not in human macrophages (27). To explore the molecular basis for this species-specific action of *Salmonella* lipid A, we first transfected human macrophage THP-1 cells with an NF- κ B-dependent luciferase reporter plasmid and then stimulated them with either an *E. coli* or a *Salmonella* lipid A preparation. Stimulation with *E. coli* lipid A caused an approximately sevenfold increase in reporter activity, whereas stimulation with *Salmonella* lipid A did not increase this activity (Fig. 2). When mCD14, mTLR4, and mMD-2 were all expressed in THP-1 cells, not only was the response to *E. coli* lipid A increased, but the cells also became responsive to *Salmonella* lipid A (Fig. 2). Expression of these human proteins also increased the response to *E. coli* lipid A, whereas the response to *Salmonella* lipid A was only slightly increased (Fig. 2).

Expression of mCD14, mTLR4, or mMD-2 alone does not impart responsiveness to *Salmonella* lipid A. To determine which molecule of mCD14, mTLR4, and mMD-2 is involved in the acquisition of the responsiveness to *Salmonella* lipid A, we expressed each of these molecules in THP-1 cells and then

TABLE 1. Primers used for PCR

Gene	Orientation ^a	Sequence (5' to 3') ^b
hTLR4	upper-1	AT <i>GGA TCC ACC</i> ATG ATG TCT GCC TCG CGC CTG
	upper-2	AC <i>CGA AGC TTG TCC TGC GTG AGA CCA GAA AGC TG</i>
	lower	TAA <i>ATT CTC GAG TCA GAT AGA TGT TGC TTC CTG CCA</i>
mTLR4	upper-1	GAA <i>GGA TCC ACC</i> ATG ATG CCT CCC TGG CTC CTG
	upper-2	GGG <i>GTA CCG TCC TGC CTG ACA CCA GGA AGC TTG</i>
	lower	C <i>AGA GTT TGC GGC CGC TCA GGT CCA AGT TGC CGT TTC TTG</i>
hMD-2	upper-1	G <i>GAA TTC ACC</i> ATG TTA CCA TTT CTG TTT TTT TCC ACC
	upper-2	ACC <i>GAA GCT TTG GAA GCT CAG AAG CAG TAT TGG GTC</i>
	lower	GC <i>TCT AGA CTA ATT TGA ATT AGG TTG GTG TAG GAT</i>
mMD-2	upper-1	G <i>GAA TTC ACC</i> ATG TTG CCA TTT ATT CTC TTT TCG ACG
	upper-2	AT <i>TAA AGC TTG GAA TCT GAG AAG CAA CAG TGG</i>
	lower	CCG <i>CTC TAG ATT TTT TTT TTT TTT TTT T^c</i>

^a The upper-1 and upper-2 primers were used to amplify the entire coding region and the coding region minus the signal peptide sequence, respectively.

^b Sequences in italics and boldface indicate restriction sites created and the respective coding regions, respectively.

^c Since the 3' portion of the mMD-2 sequence had not been determined at the time of creation, an oligo(dT) primer was used.

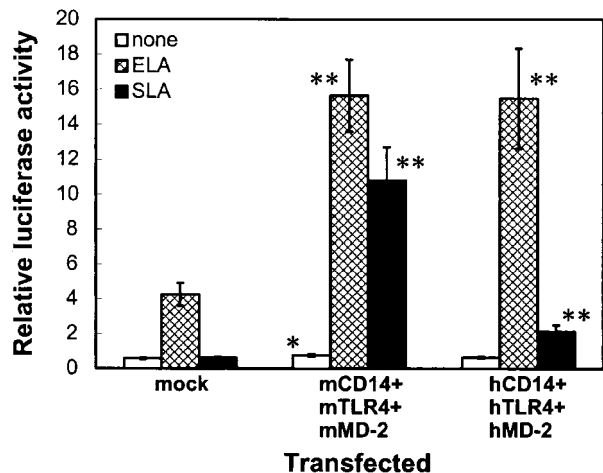


FIG. 2. Expression of mCD14, mTLR4, and mMD-2 imparts responsiveness to *Salmonella* lipid A. THP-1 cells were transiently transfected with either a control vector alone (0.3 μ g) (mock) or plasmids for either mCD14, mTLR4, and mMD-2 or hCD14, hTLR4, and hMD-2 (0.1 μ g each) together with the pELAM-L luciferase reporter plasmid. After 24 h, the cells were stimulated for 6 h without (none) or with 10 ng of *E. coli* lipid A/ml (ELA) or 100 ng of *Salmonella* lipid A/ml (SLA) in the presence of 10% (vol/vol) FCS, and the luciferase activities were measured. The values are means \pm standard errors of the means from at least 17 independent experiments. *, $P < 0.05$; **, $P < 0.01$ (compared with the respective responses in mock-transfected cells by two-tailed Student's *t* test).

stimulated them with an *E. coli* or a *Salmonella* lipid A preparation (Fig. 3). Expression of mCD14 or mMD-2 did not significantly affect responses to the two lipid A preparations. Expression of mTLR4 clearly increased the response to *E. coli*

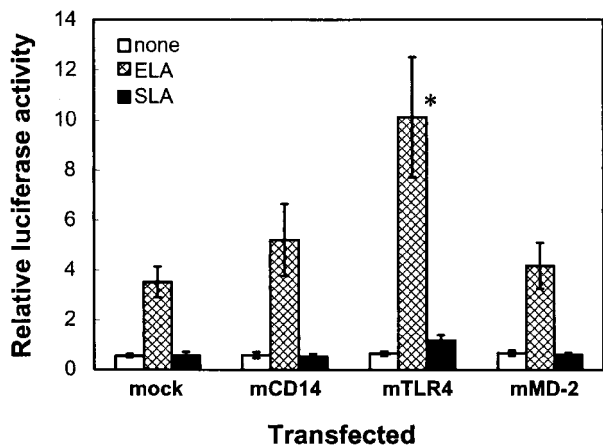


FIG. 3. Expression of mCD14, mTLR4, or mMD-2 alone does not impart responsiveness to *Salmonella* lipid A. THP-1 cells were transiently transfected with either a control vector (mock) or a plasmid for mCD14, mTLR4, or mMD-2 (all 0.1 μ g) together with the pELAM-L luciferase reporter plasmid. After 24 h, the cells were stimulated for 6 h without (none) or with 10 ng of *E. coli* lipid A/ml (ELA) or 100 ng of *Salmonella* lipid A/ml (SLA) in the presence of 10% (vol/vol) FCS, and the luciferase activities were measured. The values are means \pm standard errors of the means from seven independent experiments. *, $P < 0.05$ compared with the respective responses in mock-transfected cells by two-tailed Student's *t* test.

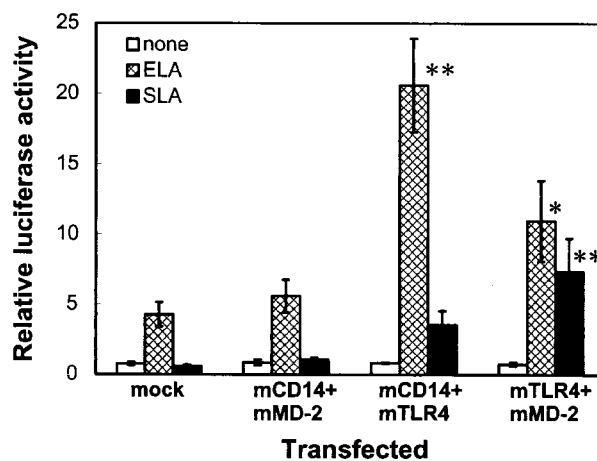


FIG. 4. Expression of both mTLR4 and mMD-2 imparts responsiveness to *Salmonella* lipid A. THP-1 cells were transiently transfected with either a control vector alone (mock) or plasmids for mCD14 and mMD-2, mCD14 and mTLR4, or mTLR4 and mMD-2 (0.1 μ g each) together with the pELAM-L luciferase reporter plasmid. After 24 h, the cells were stimulated for 6 h without (none) or with 10 ng of *E. coli* lipid A/ml (ELA) or 100 ng of *Salmonella* lipid A/ml (SLA) in the presence of 10% (vol/vol) FCS, and the luciferase activities were measured. The values are means \pm standard errors of the means from at least four independent experiments. *, $P < 0.05$; **, $P < 0.01$ (compared with the respective responses in mock-transfected cells by two-tailed Student's *t* test).

lipid A, whereas the response to *Salmonella* lipid A was not significantly increased. This indicates that the acquisition of responsiveness to *Salmonella* lipid A cannot be explained by a single molecule.

Expression of both mTLR4 and mMD-2 imparts responsiveness to *Salmonella* lipid A. Since mCD14, mTLR4, or mMD-2 alone did not impart responsiveness to *Salmonella* lipid A, we expressed two of these proteins in THP-1 cells, and the reporter activity in response to each lipid A preparation was examined (Fig. 4). Expression of mCD14 plus mMD-2 did not affect responses to either of the lipid A preparations. Expression of mCD14 plus mTLR4 markedly enhanced the response to *E. coli* lipid A, and these cells were capable of responding to *Salmonella* lipid A to some extent. In contrast, expression of mTLR4 plus mMD-2 greatly increased the response to *Salmonella* lipid A, whereas only a twofold increase was observed in the response to *E. coli* lipid A. This indicates that both mTLR4 and mMD-2 are required to impart responsiveness to *Salmonella* lipid A.

Replacement of mMD-2 with hMD-2 severely impairs responsiveness to *Salmonella* lipid A. To further explore whether mTLR4 or mMD-2 is essential for responsiveness to *Salmonella* lipid A, mCD14, mTLR4, or mMD-2 was replaced with the respective human protein instead of expressing all three mouse proteins, and reporter activities in response to these lipid A preparations, as well as synthetic *E. coli*-type lipid A (compound 506) and *Salmonella*-type lipid A (compound 516), were examined in THP-1 cells (Fig. 5). Upon expression of mCD14, mTLR4, and mMD-2 in THP-1 cells, responses to both the *E. coli* and the *Salmonella* lipid A preparations were greatly increased, as shown in Fig. 2. Expression of hCD14

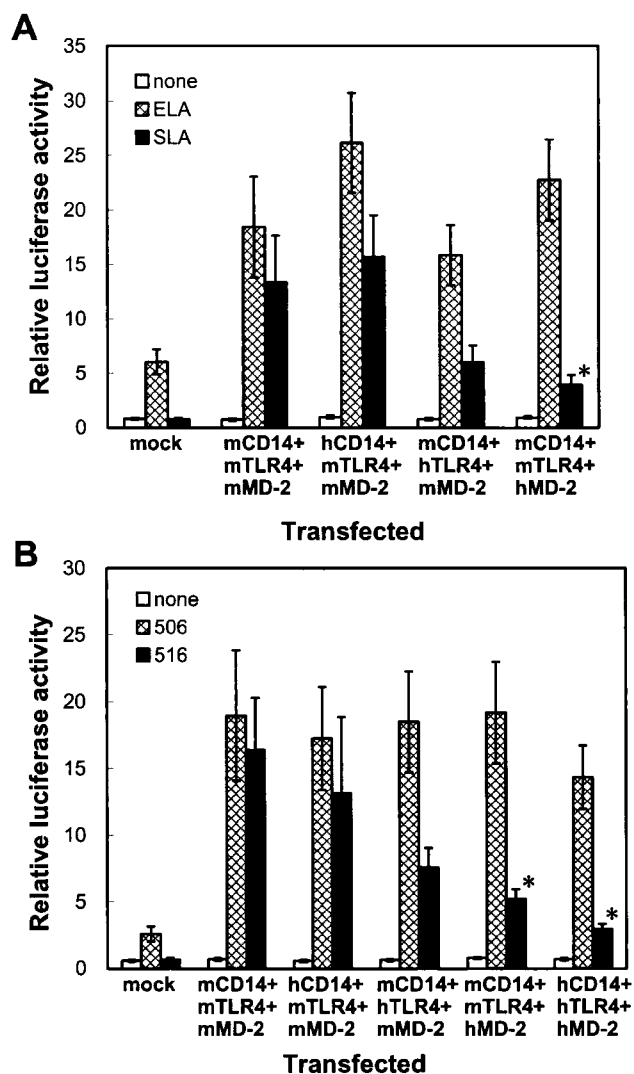


FIG. 5. Replacement of mMD-2 with hMD-2 severely impaired responsiveness to *Salmonella* lipid A. THP-1 cells were transiently transfected with either a control vector alone (mock) or plasmids for mCD14, mTLR4, and mMD-2; hCD14, mTLR4, and mMD-2; mCD14, hTLR4, and mMD-2; mCD14, mTLR4, and hMD-2; or hCD14, hTLR4, and hMD-2 (0.1 μ g each) together with the pELAM-L luciferase reporter plasmid. After 24 h, the cells were stimulated for 6 h without (none) or with 10 ng of *E. coli* lipid A/ml (ELA) or 100 ng of *Salmonella* lipid A/ml (SLA) (A) or 3 ng of synthetic *E. coli*-type lipid A/ml (compound 506) or 30 ng of synthetic *Salmonella*-type lipid A/ml (compound 516) (B) in the presence of 10% (vol/vol) FCS, and the luciferase activities were measured. The values are means \pm standard errors of the means from at least five independent experiments. *, $P < 0.05$ compared with the respective responses in cells transfected with plasmids for mCD14, mTLR4, and mMD-2 by two-tailed Student's *t* test (comparison of responses between mock-transfected cells and cells transfected with plasmids for mCD14, mTLR4, and mMD-2 was not performed).

instead of mCD14 in THP-1 cells transfected with mTLR4 and mMD-2 did not affect the response to either lipid A preparation. When mTLR4 was replaced with hTLR4, the response to *Salmonella* lipid A was decreased to some extent, and the response to *E. coli* lipid A was unaffected. The replacement of

mMD-2 with hMD-2 greatly reduced the response to *Salmonella* lipid A, although the response to *E. coli* lipid A was not affected (Fig. 5A). Similar results were obtained by using synthetic lipid A compounds instead of lipid A preparations of bacterial origin (Fig. 5B). Taken together, these results indicate that both TLR4 and MD-2 participate in the species-specific action of *Salmonella* lipid A.

DISCUSSION

The structures of lipid A molecules found in *E. coli* and *Salmonella* lipid A preparations have been characterized chemically (27). The only structural difference between the lipid A molecules typically found in these lipid A preparations is the hexadecanoyl acid attached to the hydroxy residue of 3-hydroxy tetradecanoic acid bound to position 2 of the reducing glucosamine (Fig. 1). Typical *E. coli*-type and *Salmonella*-type lipid A molecules possess six and seven fatty acids, respectively (27). In contrast to this slight structural difference, the activities of these lipid A molecules are clearly different in human macrophages. We previously demonstrated that lipid A preparations from various *Salmonella* strains and synthetic *Salmonella*-type lipid A (compound 516) possess very little stimulatory activity in human macrophages, while they are potently active in murine macrophages. Furthermore, lipid A preparations from *E. coli* and synthetic *E. coli*-type lipid A (compound 506) were equally active in both human and murine macrophages (27). Similar species-specific actions were also reported with a tetraacylated lipid A precursor (also called compound 406 or lipid IVa) and *Rhodobacter sphaeroides* lipid A (2, 16, 21). In these studies, Poltorak et al. (21), and Lien et al. (16) investigated species specificity by transfecting TLR4 proteins from different species. They found that expression of only mTLR4 in immortalized C3H/HeJ macrophages or THP-1 cells was enough to confer responsiveness to 406 and concluded that TLR4 was responsible for species specificity, although they did not examine the involvement of MD-2. In our present study, expression of only mTLR4 in THP-1 was not enough to confer responsiveness to *Salmonella* lipid A, and mMD-2 with the aid of mTLR4 was required for the acquisition of responsiveness. Therefore, the species-specific action of *Salmonella* lipid A is clearly different from that of 406.

Taxol has also been reported to exhibit species-specific actions. Taxol has been shown to possess LPS-mimetic activities in mouse macrophages but not in human macrophages (11). Kawasaki et al. (12) showed Gln²² of mMD-2 to be essential for taxol signaling but not for LPS signaling. We also examined the effects of *E. coli* and *Salmonella* lipid A preparations on THP-1 cells expressing an mMD-2 mutant, in which Gln²² was replaced with Tyr, together with mCD14 and mTLR4 and found that these cells still responded to *Salmonella* lipid A (data not shown). In addition, it has been reported (11) that CD14, which is required for lipid A responsiveness, was not required for taxol responsiveness. Therefore, the site of action of *Salmonella* lipid A is clearly different from that of taxol. We are attempting to narrow the region in MD-2 responsible for the species-specific recognition by using human-mouse chimeric MD-2.

CD14 is known to recognize LPS and lipid A molecules on the macrophage surface, and this is widely believed to be the

first step in the LPS signal transduction pathway. However, the species dependency of responsiveness to lipid A molecules is not explained by interspecies differences in CD14 structure because our data and those of others (2) indicate that CD14 is not involved in species dependency. Therefore, species-dependent discrimination of lipid A structures apparently takes place after the binding of lipid A to CD14 has occurred. Poltorak et al. (21) and Lien et al. (16) found that TLR4 is involved in species-specific actions of 406 and *R. sphaeroides* lipid A and explained this species-dependent discrimination in two ways: TLR4 is capable of discriminating between lipid A structures, or TLR4 discriminates between CD14 molecule conformations induced by the binding of these lipid A molecules. In the present study, we showed MD-2 to be involved in species-dependent discrimination between *E. coli* and *Salmonella* lipid A molecules. This suggests that MD-2 is also capable of discriminating among lipid A structures or CD14 molecule conformations induced by these lipid A molecules. *Salmonella* lipid A may serve as a useful tool for understanding the signaling mechanism which operates between the lipid A/CD14 complex and the TLR4/MD-2 complex.

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