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Molecular Basis of Reduced Potency of Underacylated Endotoxins

Athmane Teghanem, DeSheng Zhang, Erika N. Levis, Jerrold P. Weiss, and Theresa L. Gioannini

Potent TLR4-dependent cell activation by Gram-negative bacterial endotoxins depends on sequential endotoxin-protein and protein-protein interactions with LPS-binding protein, CD14, myeloid differentiation protein 2 (MD-2), and TLR4. Previous studies have suggested that reduced agonist potency of underacylated endotoxins (i.e., tetra- or penta- vs hexa-acylated) is determined by post-CD14 interactions. To better define the molecular basis of the differences in agonist potency of endotoxins differing in fatty acid acylation, we compared endotoxins (lipooligosaccharides (LOS)) from hexa-acylated wild-type (wt), penta-acylated mutant msbB meningococcal strains as well as tetra-acylated LOS generated by treatment of wt LOS with the deacylating enzyme, acyloxyacylhydrolase. To facilitate assay of endotoxin:protein and endotoxin:cell interactions, the endotoxins were purified after metabolic labeling with [3H]- or [14C]acetate. All LOS species tested formed monomeric complexes with MD-2 in an LPS-binding protein- and CD14-dependent manner with similar efficiency. However, msbB LOS:MD-2 and acyloxyacylhydrolase-treated LOS:MD-2 were at least 10-fold less potent in inducing TLR4-dependent cell activation than wt LOS:MD-2 and partially antagonized the action of wt LOS:MD-2. These findings suggest that underacylated endotoxins produce decreased TLR4-dependent cell activation by altering the interaction of the endotoxin:MD-2 complex with TLR4 in a way that reduces receptor activation. Differences in potency among these endotoxin species is determined not by different aggregate properties, but by different properties of monomeric endotoxin:MD-2 complexes.

Invading Gram-negative bacteria typically provoke rapid mobilization of host responses through the ability of the innate immune system to detect even minute numbers of bacteria. Recognition of and response to Gram-negative bacterial endotoxin play important roles in this process and are mediated swiftly and sensitively through activation of TLR4 (1, 2). Although initiation of host defense responses at the outset of Gram-negative bacterial invasion is essential, it is equally important for the host immune system to regulate the duration and intensity of these responses to endotoxin. Modulation of host responses is necessary to avoid overexuberant and sustained host inflammatory responses that could provoke severe pathological consequences, even death (3). The work from this laboratory as well as a number of others has contributed to establishing the endotoxin:protein interactions that result in TLR4-dependent activation and subsequent cellular responses (1, 2, 4–8). Efficient and maximal response to endotoxin requires sequential interactions involving LPS-binding protein (LBP), CD14, myeloid differentiation protein 2 (MD-2), and TLR4 (1, 2, 4–6, 8–11). LBP binds endotoxin aggregates (agg) and endotoxin-rich bacterial outer membranes, facilitating extraction of endotoxin by CD14 and formation of a monomeric endotoxin:CD14 complex that activates cells containing endogenous MD-2 and TLR4 (7, 8). Endotoxin is readily transferred from an isolated endotoxin:CD14 complex to soluble, rMD-2 to form a stable monomeric endotoxin:MD-2 complex that can be isolated by gel filtration chromatography (12). This isolated endotoxin:MD-2 complex activates cells in a TLR4-dependent manner at picomolar concentrations, consistent with a system that can initiate potent proinflammatory responses in response to small numbers of invading bacteria. Although the ligand that initiates physiological responses by provoking activation of TLR4 has been shown to be endotoxin:MD-2, the mechanism by which activation through TLR4 occurs has still not been clearly defined (12, 13).

The lipid A portion of endotoxin consists of diphosphorylated β-1,6-linked α-glucosamine disaccharide linked via amide or ester linkages to 3-hydroxy fatty acids that are further substituted by nonhydroxylated fatty acids. This region represents the key component of endotoxin that determines the potency of its agonist properties (14–18). The fatty acids of the lipid A portion of endotoxin, specifically features such as number, length, symmetry, and saturation, have been shown to be a major determinant of the potency of endotoxin in eliciting TLR4-dependent host responses (19–21). It has been demonstrated that underacylated endotoxins exhibit markedly reduced ability to provoke endotoxic responses and can inhibit in a dose-dependent manner the strong endotoxic response elicited by hexa-acylated endotoxins (15–18). Among the underacylated endotoxins that have been studied are naturally occurring underacylated endotoxins, mutant bacterial strains that are unable to produce necessary acyltransferases, synthetic analogues

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of lipid A, and endotoxins that have been deacylated by the enzyme acyloxyacylhydrolase (AOAH). Although these underacylated variants of lipid A have been shown to have reduced agonist and/or antagonistic activities, they appear to interact with CD14 in a manner similar to that observed for hexa-acylated endotoxins (22, 23). Because the ability to activate TLR4 effectively is a reflection of the bioactivity of the endotoxin, the reduced potency of these endotoxins is probably determined by a molecular event that involves interactions with MD-2 and/or TLR4.

To further define the molecular basis of endotoxic potency, we used endotoxins with variations in the fatty acid content of the lipid A structure of Neisseria meningitidis serotype B (NMB lipooligosaccharides (LOS)). The wild-type LOS (LOSwt) lipid A from NMB contains a symmetrical distribution of fatty acids with secondary laurate substitutions on the N-linked 2- and 2’-acyloxy myristates (Fig. 1). We have compared LOSwt to LOSwt from the msbB mutant, and wt msbB NMB treated with AOAH (LOSmsbB; Fig. 1) and LOS from the NMB treated with AOAH (LOSmsbB; Fig. 1) for their ability to interact with LBP, soluble CD14 (sCD14), and soluble rMD-2 to form monomeric endotoxin:MD-2 complexes and provoke TLR4-dependent cell activation. Metabolically labeled endotoxin of high specific radioactivity was used to allow studies with endotoxin at concentrations <1 ng/ml. We now show that hexa-, penta-, and tetra-acylated endotoxins have similar ability to form monomeric complexes with sMD-2 and CD14-dependent manner. However, in contrast to hexa-acylated endotoxin, these monomeric underacylated endotoxin:MD-2 complexes have a reduced ability to evoke TLR4-dependent cell activation. In fact, an underacylated endotoxin:MD-2 complex antagonizes TLR4-dependent activation, presumably by competing with hexa-acylated endotoxin: MD-2 for interaction with TLR4.

**Materials and Methods**

LBP and sCD14 were provided by Xoma (US) LLC. Both parental HEK293 and cells stably transfected with TLR4 (HEK293/TLR4) were provided by Dr. J. Chow (Eisai Research Institute, Andover, MA). HEK293 transfected with CD14, MD-2, and TLR4 were purchased from InvivoGen. Soluble rMD-2 containing a hexapolyhistidine tag on the C-terminal end was prepared as previously described (12). AOAH was a gift from Dr. R. Munford (University of Texas Southwestern, Dallas, TX).

**Preparation of metabolically labeled endotoxins**

\[^{14}C\]LOS (sp. act., 600 cpmp/ng) or \[^{3}H\]LOS (spec. act., 6,000–8, 000 cpmp/ng) was isolated from an acetate auxotroph of NMB after metabolic labeling as previously described (7). \[^{14}C\]- or \[^{3}H\]LOS app (apparent M_r >20 million) and \[^{14}C\]- or \[^{3}H\]LOS:CD14 (M_r ~60,000) was prepared and purified by size exclusion chromatography as previously described (7, 24). An msbB derivative of NMB, NMB11K3cap, was obtained from Dr. M. Apicella (University of Iowa, Iowa City, IA). The strain was grown in medium supplemented with radioactive \[^{14}C\]- or \[^{3}H\]acetate, and LOS (500 or 80 cpmp/ng, respectively) was isolated as described by Post et al. (25). Tetra-acylated LOS (spec. act. of \[^{1}H\], 5000 cpmp/ng; \[^{14}C\], 500 cpmp/ng) from NMBACE1 was prepared by deacylation with AOAH according to the method of Munford and Erwin (26). The specific radioactivities of various LOS preparations were estimated as previously described (7). The \[^{14}C\] fatty acid compositions of \[^{14}C\]-labeled endotoxins (purified agg and protein:endotoxin complexes) were determined by sequential treatment with 4 N HCl and 4 N NaOH at 90°C to release fatty acids. After chloroform/methanol extraction, labeled 3-hydroxy and nonhydroxylated fatty acid recovered in the chloroform phase were analyzed by reverse phase TLC (7). Quantitation of \[^{14}C\]-labeled fatty acids resolved by TLC was performed using the Typhoon (GE Healthcare) image analysis program. Purified LOS app used as the starting material to generate endotoxin:protein complexes, was prepared by either gel sieving chromatography or ultracentrifugation (7).

**HEK293 cell activation assay**

HEK293 cells with or without TLR4 or HEK293/CD14/Ms22/TLR4 have been extensively characterized and were cultured as described previously (27) or according to the recommendations of InvivoGen. For cell activation assays, cells were grown to confluence, then washed twice with warm PBS, pH 7.4 (PBS), and incubated for 4 h at 37°C in 5% CO_2 and 95% humidity in HBSS (HBSS) +0.1% human serum albumin (HSA) with the indicated supplements in 48-well plates. Activation of HEK293 cells was assessed by measuring accumulation of extracellular IL-8 by ELISA (BD Clontech).

**Gel filtration chromatography**

Columns of Sephacryl HR S200 (1.6 × 30 or 70 cm) or Sephacryl HR S500 (1.5 × 18 cm) were equilibrated in HBSS, 10 mM HEPES, or PBS, pH 7.4, with or without 0.1% HSA. Aggregates and complexes were prepared as described using recombinant proteins and either \[^{14}C\]- or \[^{3}H\]LOS app by incubation at 37°C for 30 min before gel filtration chromatography (7, 12). Fractions (1 ml) were collected (flow rate, 0.5 ml/min) at room temperature using GElHealthcare AKTA or AKTA Purifier fast performance liquid chromatography. Aliquots of the collected fractions were analyzed by liquid scintillation spectroscopy with a Beckman LS liquid scintillation counter to detect radioactive LOS. Recoveries of LOS were >70%. All solutions used were pyrogen free and sterile filtered. After chromatography, selected fractions to be used in the bioassays were pooled and passed through sterile syringe filters (0.22-μm pore size) with >90% efficiency.
recovery of radiolabeled material in the sterile filtrate. Fractions were stored under sterile conditions at 4°C until needed. LOSwt and LOSAOAH were stable for at least 1 mo at 4°C, with no detectable changes in chromatographic or functional properties. LOSmsbB was somewhat less stable; generated fractions were used within 1 wk. Sephacryl S200 columns were calibrated with Bio-Rad gel filtration standards that included thyroglobulin, γ-globulin, OVA, myoglobin, vitamin B12, and human serum albumin.

Adsorption of polyhistidine-tagged protein complexes

Cocapture by metal chelation chromatography of radiolabeled LOS associated with sMD-2-His6 was performed as previously described (12). Briefly, samples in PBS/0.1% HSA were incubated with HisLink resin (0.2–1 ng of radiolabeled LOS/100 μl; Promega) at 4°C, batchwise, for 1 h. The resin was spun down, supernatant was removed, and the resin was washed three or four times (500 μl) before elution into 2% SDS. The presence of radiolabeled LOS absorbed to the resin was evaluated by liquid scintillation spectroscopy.

Results

Efficient LBP/sCD14-dependent generation of monomeric endotoxin:MD-2 complexes from agg of hexa-, penta-, and tetra-acylated meningococcal LOS

The potency of endotoxins, including meningococcal endotoxin, has been shown to vary correlating with the extent of lipid A acylation after the rank order: hexa ≥ penta ≥ tetra-acylated (15–18). To better define the molecular basis of the reduced potency of underacylated endotoxins, we chose to compare the properties of hexa-, penta-, and tetra-acylated forms of meningococcal LOS endotoxins. To facilitate these studies and permit the use of low concentrations of endotoxins, the LOS species were metabolically labeled to high specific activity as previously described (7).

Endotoxin with a penta-acylated lipid A (LOSmsbB) was isolated from an isogenic strain of NMB (NMBA11K3capmsbB insertional inactivation of the acyltransferase gene, msbB) confirmed that the lipid A from LOSmsbB contained a decreased amount of lauric acid (C12:0) compared with LOSwt lipid A, consistent with the predicted 50% reduction of C12:0 (Fig. 2A) (25). To obtain a tetra-acylated form of NMB LOS, wt LOS NMBACE1 was treated with the deacylating enzyme AOAH according to the procedure described by Munford and Erwin (26). Fatty acid analysis of the recovered AOAH-treated LOS confirmed the selective and nearly complete loss of the lauric acid residues that are attached to the 3-hydroxymyristoyl chains in the wt lipid A (Fig. 2A). [14C]fatty acid content is illustrated in Fig. 2A, as both the TLC pattern of the [14C]fatty acids recovered after chemical hydrolysis of the various LOS species and a bar graph of the [14C]fatty acid compositional analyses derived from image analysis.

To compare the aggregation state of the isolated wt and underacylated endotoxins, gel filtration chromatography (Sephacryl S500) was used. As shown in Fig. 2B, differences in the acylation state of NMB LOS were associated with differences in aggregation as judged by gel filtration. In contrast to LOSwt (M_r > 20 × 10^6), the majority of the agg of LOSmsbB eluted with an M_r of ~1–2 × 10^6, whereas LOSAOAH agg were even smaller (Fig. 1B).

The reduced activities of penta- and tetra-acylated lipid A species from NMB have been described previously (25, 28). To confirm that the underacylated material used in these experiments also displayed reduced bioactivity, isolated agg of LOSwt, LOSmsbB, and LOSAOAH were compared for their abilities to activate HEK293/CD14/MD-2/TLR4 cells by measuring extracellular accumulation of IL-8. As shown in Fig. 1C, each LOS species produced dose-dependent cell activation. However, compared with LOSwt, the potencies of LOSAOAH and LOSmsbB were reduced at least 10-fold. The residual activities of LOSmsbB and LOSAOAH may be due to residual hexa-acylated LOS in these preparations, more so in LOSAOAH (~10%; Fig. 2A) (26) than in LOSmsbB.

**FIGURE 2.** Structural and functional properties of wt, msbB, and AOAH-treated LOS aggregates. A, TLC of [14C]fatty acids of wt, msbB, and AOAH-treated [14C]LOS released by chemical hydrolysis as described in Materials and Methods. [14C]fatty acids were quantified by image analysis and expressed as a percentage of the total recovered [14C]fatty acids. B, Radiolabeled LOS (1–10 ng/ml) was analyzed by gel filtration chromatography on Sephacryl S500 HR (1.5 × 18 cm) in HBSS, 10 mM HEPES, and 0.1% HSA (representative graphs). Data are expressed as a percentage of the total radiolabeled LOS recovered; total recoveries were ≥70%. C, HEK293/CD14/MD-2/TLR4 cells were incubated for 4 h with increasing amounts of LOS aggregates and LBP (100 ng/ml) in DMEM/0.1% HSA. Extracellular IL-8 accumulated in cell supernatants was determined by ELISA. Results are from one experiment with triplicate samples and are representative of two similar experiments.
(~5%; Fig. 2A) (25), as indicated by the presence of C12:0 remaining in AOAH-treated LOS and the slightly greater than predicted amount of 12:0 remaining in LOS\textsubscript{msbB}. Cell activation by each LOS species was LBP dependent. Little or no activation of HEK293 cells by LOS was observed in the absence of CD14, MD-2, or TLR4.

Previous studies have indicated similar LBP-dependent delivery of wt (hexa-acylated) and AOAH-treated (tetra-acylated) LPS to CD14 (23). Therefore, the differences in activity of hexa- and teta-acylated endotoxin presumably derive from an event after interaction with CD14 (23), e.g., transfer of endotoxin from CD14 to MD-2 and/or activation of TLR4 by endotoxin:MD-2. To test the former possibility, we compared LBP/sCD14-dependent formation of monomeric LOS:MD-2-His\textsubscript{6} complexes from LOS\textsubscript{wt}agg, LOS\textsubscript{msbB}agg, and LOS\textsubscript{AOAH}agg. To promote formation of LOS: sCD14 before addition of sMD-2, the purified LOS\textsubscript{agg} were first treated with LBP and sCD14 for 15 min at 37°C in the presence of albumin (24). After 15 min, recombinant sMD-2-His\textsubscript{6} was added to the incubation mixture and incubated an additional 15 min at 37°C to permit formation of LOS:MD-2. Reaction conditions were chosen that produced ~50% conversion of LOS\textsubscript{wt}agg to the monomeric LOS\textsubscript{wt}:MD-2 complex (latest eluting peak in Fig. 3A) so that either more or less efficient generation of LOS:MD-2 from LOS\textsubscript{msbB}agg and LOS\textsubscript{AOAH}agg could be detected. Remarkably, both the overall elution pattern as well as the apparent degree of formation of LOS:MD-2 from LOS\textsubscript{msbB}agg and LOS\textsubscript{AOAH}agg were virtually identical with that observed with LOS\textsubscript{wt}agg. The latest eluting LOS species (peak) in Fig. 3A is the monomeric LOS: MD-2 complex: 1) the elution volume predicts an M\textsubscript{r} of ~25,000, closely corresponding to the predicted M\textsubscript{r} of a monomeric complex of LOS and MD-2 (12); 2) this peak was only observed after incubation of LOS\textsubscript{agg} with LBP, sCD14, and sMD-12 (12); and 3) the yield of this peak from LOS\textsubscript{msbB}agg LOS\textsubscript{AOAH}agg was increased by addition of a higher concentration of sMD-2 (data not shown). Therefore, these findings demonstrate a close similarity in the efficiency of formation of LOS:MD-2 complexes from LOS\textsubscript{wt}agg, LOS\textsubscript{msbB}agg, and LOS\textsubscript{AOAH}agg. This was observed under both analytical (≤1 ng/ml LOS) and preparative (~500 ng/ml) conditions.

The latest eluting LOS species (M\textsubscript{r} ~25,000), i.e., monomeric LOS:MD-2-His\textsubscript{6} complex formed from LOS\textsubscript{wt}agg, LOS\textsubscript{msbB}agg and LOS\textsubscript{AOAH}agg were also assayed by cocapture of the radiolabeled LOS from these fractions by a metal-chelating resin.

FIGURE 3. LBP/sCD14-dependent formation of LOS:MD-2 complexes from hexa-, penta-, and tetra-acylated LOS aggregates. A, LOS\textsubscript{agg} (500 ng, 100–120 pmol) from wt, msbB, and AOAH-treated LOS were incubated with LBP (0.03 μg, 0.5 pmol) and sCD14 (9 μg, 150 pmol) for 15 min at 37°C before the addition of either insect culture medium (dialyzed against PBS) containing sMD-2-His\textsubscript{6} or partially purified sMD-2-His\textsubscript{6}. Samples were incubated for an additional 15 min at 37°C and then applied to a Sephacryl S200 HR (1.6 × 70 cm) column in PBS. Column fractions were analyzed for [3H]- or [14C]LOS, and results are expressed as a percentage of the total cpm recovered; recoveries were ≥70%. Results shown are from one experiment that is representative of three similar experiments. B, Fractions from the latest eluting major peak (denoted by arrow for LOS\textsubscript{msbB}agg and LOS\textsubscript{AOAH}agg), presumably LOS:MD-2 (M\textsubscript{r} ~25,000), as well as a control (wt LOS:sCD14) were incubated with HisLink (Promega) resin for 1 h at 25°C and washed, and the absorbed material was eluted with 2% SDS and analyzed for the amount of radioactivity. Results are expressed as the percentage of LOS captured; the results shown are from three separate experiments. D, The [14C]fatty acid content of fractions from the ~25,000 M\textsubscript{r} peak (LOS:MD-2) and LOS\textsubscript{msbB}agg and LOS\textsubscript{AOAH}agg was determined as described in Materials and Methods. TLC of the fatty acids isolated from LOS\textsubscript{wt}agg and LOS\textsubscript{AOAH}agg is shown, and data are expressed as described in Fig. 2A.
Comparison of the stability of hexa-, penta-, and tetra-acylated LOS:MD-2 complexes

The findings described above indicate that the differences in potency among LOS\textsuperscript{wt}, LOS\textsuperscript{msbB}, and LOS\textsuperscript{AOAH} are not explained by differences in LBP/CD14-dependent delivery of LOS to MD-2. These findings suggest that differences in bioactivity correspond to differences in TLR4-dependent cell activation by monomeric LOS:MD-2, depending on the acylation state of the LOS complexed to MD-2. However, before testing this prediction directly by comparing activation of HEK293/TLR4 cells by LOS\textsuperscript{wt}:MD-2, LOS\textsuperscript{msbB}:MD-2, and LOS\textsuperscript{AOAH}:MD-2, it was necessary to confirm that each of these complexes had sufficient stability to remain intact during collection at room temperature, storage at 4°C, and incubation at 37°C as needed in cell activation assays. As shown in Fig. 4, LOS\textsuperscript{wt}:MD-2 complexes were virtually unchanged after weeks at 4°C and after 6 h at 37°C. In fact, even overnight incubation at 37°C had little effect on the state of LOS\textsuperscript{wt}:MD-2 (data not shown). LOS\textsuperscript{AOAH}:MD-2 was also stable for at least 1 wk at 4°C, but gradually converted to a higher \(M_t\) species with incubation at 37°C. LOS\textsuperscript{msbB}:MD-2 was the least stable at both 4 and 37°C. These findings demonstrate time- and temperature-dependent differences in the stability of monomeric LOS:MD-2 complexes as a function of the acylation state of LOS. However, the stability of all three species was sufficient to permit direct comparison of their abilities to trigger TLR4-dependent cell activation.

Comparison of the acylation state of LOS with the bioactivity of LOS:MD-2 complexes

To test the abilities of the various LOS:MD-2 complexes to activate cells through TLR4, HEK293/TLR4 cells were incubated with increasing amounts of the three isolated LOS:MD-2 complexes, and the degree of activation was measured by determining the extracellular IL-8 accumulated. We measured extracellular accumulation of IL-8 at 4 h, which reflects the effect of cell activation during the first 2–3 h of incubation (data not shown) at a point in time when each of the LOS:MD-2 complexes showed little or no aggregation at 37°C (Fig. 4; data not shown). Parental HEK293 cells (no TLR4) were not activated by these complexes (data not shown). All three complexes produced dose-dependent TLR4-dependent cell activation (Fig. 5A). However, compared with LOS\textsuperscript{wt}:MD-2, the potencies of LOS\textsuperscript{AOAH}:MD-2 and LOS\textsuperscript{msbB}:MD-2 were reduced at least 10-fold, comparable to the differences in LBP-, CD14-, MD-2-, and TLR4-dependent activity of the LOS\textsuperscript{wt},

shows that, like LOS\textsuperscript{wt}, complexes that contain LOS\textsuperscript{msbB} and LOS\textsuperscript{AOAH} were also specially adsorbed by the resin, consistent with association of each LOS species with sMD-2-His\textsubscript{6}. The reduced capture of LOS\textsuperscript{msbB} by the resin paralleled the reduced stability of the LOS\textsuperscript{msbB}:MD-2 complex compared with LOS\textsuperscript{wt}:MD-2 (see below). The reduced stability may be responsible for the reduced percentage of the LOS remaining bound to MD-2 during the incubations and washing of the metal-chelating resin as well as reduced access of the polyhistidine tag when MD-2 is bound to LOS\textsuperscript{msbB}, possibly due to aggregation of LOS\textsuperscript{msbB}:MD-2 complexes (see Fig. 4).

Because the AOAH-treated LOS almost certainly contains some residual hexa-acylated endotoxin (Fig. 2A) (25, 28), we compared the fatty acid contents of the starting LOS\textsubscript{agg} and the isolated LOS:MD-2 complex formed from AOAH-treated LOS. As shown in Fig. 3C, the fatty acid compositions of the LOS\textsubscript{agg} and LOS:MD-2 complex formed from LOS\textsuperscript{AOAH} were virtually identical, indicating little or no fatty acid selectivity in the sequence of reactions from aggregated LOS to formation of a monomeric LOS:MD-2 complex.

FIGURE 4. Stability of various monomeric LOS:MD-2 complexes. Purified radiolabeled LOS:MD-2 for each type of LOS recovered after Sephadryl S200 chromatography (Fig. 3A) were sterile filtered and stored at 4°C until rechromatography. Samples of 4–6 ng of LOS:MD-2 were either applied directly or preincubated at 37°C for the indicated time before chromatography (Sephadryl S200; 1.6 × 30 cm). Column fractions were analyzed for radioactivity, and results are expressed as a percentage of the total cpm recovered; recoveries were ≥70%. The profiles shown are representative of three separate experiments.

FIGURE 5. Bioactivities of hexa-, penta-, and tetra-acylated LOS:MD-2 complexes. A, Purified LOS:MD-2 complexes from wt (○), msbB (△), and AOAH-treated (□) LOS were incubated with HEK293/TLR4 cells in HBSS\textsuperscript{7}, HEPES, and 0.1% HSA for 4 h. Extracellular IL-8 was measured by ELISA. The x-axis indicates the amount of LOS added. Note that for LOS\textsuperscript{msbB}:MD-2 ≤50% of LOS remains as a monomeric complex (see Fig. 4). B, HEK293/TLR4 cells were incubated with increasing amounts of LOS\textsuperscript{msbB}:MD-2 with (○) or without (△) LOS:MD-2 (0.2 ng/ml) for 4 h. Extracellular IL-8 was determined by ELISA. Data shown represent one experiment that is representative of three experiments, each containing duplicate samples.
Discussion

Variations in endotoxin potency have long been recognized (for review, see Refs. 14–17 and 29). This has stimulated great interest in the structural and molecular bases of these functional differences. This effort was initially driven by pharmacologic interests in generating compounds with either immunostimulatory or immunosuppressive properties. However, it is now apparent that variations in endotoxin structure within the lipid A region among different Gram-negative bacteria are more common than previously appreciated and may play an important role in defining specific features of host interactions with specific Gram-negative bacteria (14).

A prevailing view of the molecular basis of differing agonist/antagonist properties of endotoxin variants has been that variation of endotoxin (lipid A) structure confers differences in the supramolecular organization of aggregates of endotoxins that modify interactions with the LBP/CD14/MD-2/TLR4-dependent pathway of cell activation (for review see Refs. 15, 16, 29, and 30). However, it has also been demonstrated that functional differences between an endotoxin species that is a potent agonist and a partially deacylated derivative that is a potent antagonist involve interactions that occur after CD14 (22, 31, 32). Because, at least in solution, the active form of endotoxin complexed to CD14 is a monomeric protein:endotoxin complex (7, 24), these findings suggest variables that are independent of differences in the aggregate properties of the different endotoxin species. We have previously speculated that, at least for some endotoxin species, decreased acylation would decrease endotoxin agonist properties not by effects on endotoxin aggregate properties, but by altering properties of monomeric protein:endotoxin complexes that regulate delivery of endotoxin to MD-2 and/or activation of TLR4 (8, 12).

Using LOS isolated from the msbB mutant of NMB as a source of penta-acylated endotoxin and LOSMSB deacylated by AOAH as a source of tetra-acylated endotoxin, we have shown that aggregates of both LOSMSB and LOSAOAH, when treated with LBP, sCD14, and sMD-2, generate monomeric LOS:MD-2 complexes in a manner virtually indistinguishable from that seen with LOSwt (Fig. 3). Partial deacylation by AOAH of LOSwt is incomplete (Fig. 2A), making it likely that some residual hexa-acylated (wt) LOS remains in LOSAOAH preparations (26). Nevertheless, LOSAOAH, MD-2 complexes have the identical fatty acid composition as LOSAOAH aggregates, also indicating that LBP, sCD14, and
sMD-2 treatment of endotoxin aggregates to form monomeric endotoxin: MD-2 is equally efficient with tetra-acylated as with hexa-acylated LOS. It should be noted, as shown in Fig. 2, that the aggregate size of LOS\textsuperscript{msbB} and LOS\textsuperscript{AOAH} differs markedly from that of LOS\textsuperscript{wt}. The latter migrates with a $M_c$ of $\geq 20$ million, suggesting $\geq 4000$ molecules of LOS/aggregate. In contrast, the chromatographic behavior of both LOS\textsuperscript{msbB} and LOS\textsuperscript{AOAH} suggest that these species form aggregates of $\geq 200$ LOS/aggregate. In the case of LOS\textsuperscript{AOAH}, exposure to Triton X-100 during AOAH treatment to facilitate enzyme action accounts at least in part for the marked reduction in aggregate size (data not shown). Despite these differences in aggregate size, disaggregation by LBP/sCD14 to form LOS:sCD14 as a prelude to transfer of endotoxin to sMD-2 (12) (A. Teghanemt, unpublished observations) is closely similar (Fig. 3) and, hence, apparently independent of the initial size of the endotoxin aggregate. Thus, at least for these LOS variants, the aggregate properties of these species do not appear to be important determinants of their agonist potencies. Studies are in progress to determine whether, by contrast, the reduced agonist potency of hyperacylated (e.g., hepta-acylated) endotoxin variants or species containing long-chain fatty acids (15, 29) reflects reduced efficiency of LBP/CD14-dependent extraction and transfer of single molecules of endotoxin to CD14, as is generally needed for potent MD-2/TLR4-dependent cell activation.

Our observations contrast with a recent report by Tsuneyoshi et al. (33) that concluded that MD-2 could not bind penta-acylated endotoxin. In these studies, MD-2:LPS complexes could be recovered from *wt Escherichia coli* expressing recombinant MD-2 and hexa-acylated LPS, but not from *IpxM* (equivalent to msbB) *E. coli* that produced MD-2 and penta-acylated LPS. Many differences distinguish the experimental design used in that study from our assays of MD-2:endotoxin interactions. Perhaps most important are the differences in the manner of endotoxin presentation to MD-2. We have previously shown that the reactivity of MD-2 with LOS (or LPS) is markedly enhanced by presentation of endotoxin as a monomeric complex with CD14 (12). This is what physiologically occurs after LBP- and CD14-dependent interactions with endotoxin (8). By contrast, interactions of MD-2 and LPS within *E. coli* or after extraction bear little resemblance to the natural and ordered sequence of interactions that take place in the host. Moreover, the reduced stability we observed for LOS\textsuperscript{msbB}:MD-2 complexes could mean that LPS\textsuperscript{fsbM}:MD-2 complexes from *E. coli*, even if formed, might not withstand the treatment used for coprecipitation analysis.

Because we were able to recover and purify monomeric complexes of LOS\textsuperscript{wt}:MD-2, LOS\textsuperscript{msbB}:MD-2 and LOS\textsuperscript{AOAH}:MD-2, we were able to demonstrate directly that for these meningococcal LOS species, the agonist potency of LOS:MD-2 complexes depends on the acylation state of the endotoxin bound to MD-2 (Fig. 5A). The differences in dose-dependent activation of HEK293/TLR4 cells by these LOS:MD-2 complexes mirrored the differences in LBP-dependent bioactivity of LOS\textsuperscript{wt}, LOS\textsuperscript{msbB}, and LOS\textsuperscript{AOAH} aggregates toward HEK293/mCD14/MD-2/TLR4 cells, further supporting the conclusion that the differences in activity of different LOS aggregates are due to differences conferred after extraction and delivery of endotoxin monomers to MD-2. Although the reduced stability of the LOS:MD-2 complexes containing underacylated endotoxins could contribute to their reduced activity, we assayed cell activation within a time frame (4 h) in which an appreciable percentage of the complexes remained in monomeric form (Fig. 4). Aggregates that formed during storage had no effect on bioactivity. We cannot fully exclude the possibility that complexes of MD-2 with underacylated endotoxins bind less avidly to TLR4, leading to less potent TLR4-dependent cell activation. However, the ability of the LOS\textsuperscript{msbB}:MD-2 complex to inhibit TLR4-dependent cell activation by LOS\textsuperscript{wt}:MD-2 seems most compatible with a model (Fig. 6) in which the occupancy of MD-2 by an underacylated endotoxin species leads to occupation of TLR4 by MD-2 in a form that cannot efficiently trigger receptor activation (e.g., oligomerization) (34).

Finally, it should be noted that the agonist potencies of the various LOS:MD-2 complexes we studied were directly related to the stability of these complexes (i.e., LOS\textsuperscript{wt}:MD-2 $> LO S_{msbB}$:MD-2 $> LO S_{AOAH}$:MD-2; Figs. 4 and 5A). Thus, unless binding of TLR4 by MD-2 markedly alters the stability of endotoxin- MD-2 interactions, our findings suggest that TLR4 activation is not dependent upon a transfer of endotoxin from MD-2 to TLR4. We propose, instead, that endotoxin is an allosteric regulator of MD-2, inducing conformational changes in MD-2 that are needed to induce changes in TLR4 resulting in receptor activation (Fig. 6). The computational model of MD-2 suggested by Ichikawa et al. (35) supports this view. Accordingly, we predict that underacylated endotoxins function as antagonists by occupying MD-2 without inducing the conformational changes needed to trigger TLR4 activation and oligomerization (36–38) (Fig. 6). This is consistent with the findings of Akashi et al. (39), who showed that CD14-dependent binding of the lipid A antagonist E5531 does not induce the changes in mAb reactivity of MD-2/TLR4 that accompany cell binding and activation by an agonist. Studies are in progress using the wt and underacylated endotoxin:MD-2 complexes described in this study to test this hypothesis.

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**Disclosures**

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