Isolation of an endotoxin–MD-2 complex that produces Toll-like receptor 4-dependent cell activation at picomolar concentrations

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Host proinflammatory responses to minute amounts of endotoxins derived from many Gram-negative bacteria require the interaction of lipopolysaccharide-binding protein (LBP), CD14, Toll-like receptor 4 (TLR4) and MD-2. Optimal sensitivity to endotoxin requires an ordered series of endotoxin–protein and protein–protein interactions. At substoichiometric concentrations, LBP facilitates delivery of endotoxin aggregates to soluble CD14 (sCD14) to form monomeric endotoxin–sCD14 complexes. Subsequent interactions of endotoxin–sCD14 with TLR4 and/or MD-2 have not been specifically defined. This study reports the purification of a stable, monomeric, bioactive endotoxin–MD-2 complex generated by treatment of endotoxin–sCD14 with recombinant MD-2. Efficient generation of this complex occurred at picomolar concentrations of endotoxin and nanogram per milliliter doses of MD-2 and required presentation of endotoxin to MD-2 as a monomeric endotoxin–CD14 complex. TLR4-dependent delivery of endotoxin to human embryonic kidney (HEK) cells and cell activation at picomolar concentrations of endotoxin occurred with the purified endotoxin–MD-2 complex, but not with purified endotoxin aggregates with or without LBP and/or sCD14. The presence of excess MD-2 inhibited delivery of endotoxin–MD-2 to HEK/TLR4 cells and cell activation. These findings demonstrate that TLR4-dependent activation of host cells by picomolar concentrations of endotoxin occurs by sequential interaction and transfer of endotoxin to LBP, CD14, and MD-2 and simultaneous engagement of endotoxin and TLR4 by MD-2.

Potent proinflammatory cellular responses to endotoxin are mediated through activation of Toll-like receptor 4 (TLR4), a member of the Toll-like receptor family of proteins (1–3). TLR4 contains a leucine-rich extracellular domain involved in ligand recognition, a transmembrane region, and an intracellular domain responsible for triggering signaling pathways that results in activation of genes of the innate immune defense system (4, 5). TLR4 requires MD-2 for CD14-dependent cellular response to low concentrations of endotoxin, but neither the precise nature of the ligand that binds to TLR4 or the role of MD-2 has been defined. MD-2, either endogenously expressed or exogenously added, associates with TLR4 on the cell surface (6–11), and its endogenous expression is needed for optimal surface expression of TLR4. This finding suggests that MD-2 may act as a "chaperone," promoting surface expression of TLR4 and, indirectly, surface recognition of endotoxin (10, 12–14). TLR4 responsiveness to endotoxin is disrupted by point mutations of MD-2 (7, 15–18) (e.g., Cys-95, Lys-128, and Lys-132) despite surface expression of TLR4–MD-2 complexes, implying other roles for MD-2 in TLR4-dependent cell activation by endotoxin. A more direct role of MD-2 in recognition and discrimination of TLR4 ligands has been suggested (14, 18–20). However, direct interactions of MD-2 with endotoxin that have been demonstrated have not yet been linked directly to cell activation or observed at very low concentrations of endotoxin and MD-2 normally sufficient for potent TLR4-dependent cell activation (6, 11).

Maximal potency of TLR4-dependent cell activation by endotoxin requires four different extracellular and cell surface host proteins: lipopolysaccharide-binding protein (LBP), CD14, MD-2, and TLR4 (1, 14, 21). We have speculated that these complex cofactor requirements reflect a need for sequential interactions of endotoxin with each of these proteins for optimal molecular recognition (22–24). In support of this hypothesis, sensitive endotoxin recognition by CD14 requires prior interaction of endotoxin aggregates with LBP (22, 23, 25–29). Moreover, potent activation of cells containing TLR4 and MD-2 but not CD14 requires presentation of endotoxin as a monomeric complex with CD14, achieved by prior interaction of endotoxin aggregates with LBP and soluble CD14 (sCD14) (22, 24, 29, 30). The recognition of these molecular requirements for host cell activation by endotoxin has been greatly facilitated by the use of bacterial acetate auxotrophs to metabolically label endotoxin to high specific radioactivity that permits assay of protein–endotoxin and host cell–endotoxin interactions at physiologically relevant endotoxin concentrations. In this study, we have extended this approach to address two hypotheses: (*i*) MD-2 has a direct role in recognition of endotoxin–CD14 complexes necessary for TLR4-dependent cell activation, and (*ii*) cell activation is triggered by simultaneous engagement by MD-2 of endotoxin and TLR4 without CD14. Our findings show that MD-2 interacts directly with endotoxin–sCD14 complexes to generate an endotoxin–MD-2 complex that produces TLR4-dependent cell stimulation at concentrations consistent with the ability of the innate immune system to detect and respond to minute amounts of endotoxin. Thus, endotoxin-bearing MD-2, rather than endotoxin itself, may be the ligand triggering TLR4 receptor activation.

Materials and Methods

LBP and sCD14 were provided by Xoma (Berkeley, CA). Both parental human embryonic kidney 293 (HEK293) cells and cells stably transfected with TLR4 (HEK/TLR4) were provided by J. Chow (Eisai Research Institute, Andover, MA). Chromatography matrices and electrophoresis supplies were purchased from Amersham Pharmacia Biosciences. Human serum albumin (HSA) was obtained as an endotoxin-free, 25% stock solution

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Abbreviations: LPS, lipopolysaccharide; LBP, LPS-binding protein; LOS, lipooligosaccharide; HEK, human embryonic kidney; sCD14, soluble CD14; TLR4, Toll-like receptor 4; HBSS, Hanks' balanced salt solution; HSA, human serum albumin; wt, wild type, "MD-2," conditioned insect cell culture medium containing MD-2.

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(Baxter Health Care, Glendale, CA). ^{14}C-lipooligosaccharide (^{14}C-LOS) or ^{3}H-LOS was isolated from an acetate auxotroph of *Neisseria meningitidis* serogroup B after metabolic labeling and isolated as described (29). ^{14}C- or ^{3}H-LOS$_{agg}$ (apparent $M_r > 20$ million) and ^{14}C- or ^{3}H-LOS:CD14 ($M_r \approx 60{,}000$) were purified as described (22, 29). ^{3}H-lipopolysaccharide (^{3}H-LPS) from *Escherichia coli* LCD25 was purchased from List Biological Laboratories (Campbell, CA) and processed as described (23).

Preparation of Recombinant MD-2. MD-2 cDNA was isolated, linearized, and inserted, by using *Nco*I- and *Xho*I-sensitive restriction sites, into the baculovirus transfection vector pBAC11 (Novagen) that provides a six-residue polyhistidine (His-6) tag at the carboxyl-terminal end of MD-2 and a 5′ flanking signal sequence (gp64) to promote secretion of the expressed protein. DNA encoding each desired product was sequenced in both directions to confirm fidelity of the product. Production and amplification of recombinant viruses were undertaken in collaboration with the Diabetes and Endocrinology Research Center at the Veterans Affairs Medical Center (Iowa City, IA). Sf9 cells were transfected with linear baculovirus DNA and the pBAC11 vector with Bacfectin according to a procedure described by Clontech. For production of recombinant protein, HiFive cells (Invitrogen) were incubated in serum-free medium and inoculated at an appropriate virus titer. Supernatants were collected and dialyzed either against Hepes-buffered (10 mM, pH 7.4) Hanks' balanced salt solution (HBSS) with divalent cations (HBSS$^+$, pH 7.4) or 50 mM phosphate/150 mM NaCl (pH 7.4, PBS). To absorb the expressed polyhistidine-tagged protein, nickel-charged agarose resin (HisBind, Novagen) was incubated batchwise with culture medium predialyzed against PBS containing 5 mM imidazole. After extensive washing with this same buffer, adsorbed material was eluted with 200 mM imidazole. Flow-through and eluate fractions were analyzed by immunoblotting as described below. The presence of ^{14}C-LOS was evaluated by liquid scintillation spectroscopy.

Immunoblotting. To detect polyhistidine-labeled wild-type (wt) and C95Y MD-2, an anti-polyhistidine antibody (Tetra-His antibody, Qiagen, Valencia, CA) was used. Samples were electrophoresed by using an Amersham Pharmacia Biosciences PhastGel System (10–15% gradient acrylamide gel) and transferred to nitrocellulose by semidry transfer. The nitrocellulose was washed with Tris-buffered saline (TBS, pH 7.5), containing 0.05% Tween 20 and 0.2% Triton X-100 (TBSTT), blocked to reduce nonspecific background with 3% BSA in TBSTT for 1 h at 25°C and incubated with the anti-His-4 antibody in TBSTT overnight. After washing with TBSTT, the blot was incubated with donkey anti-mouse IgG conjugated to horseradish peroxidase (Bio-Rad) for 1 h at 25°C in TBS containing 3% goat serum and washed with TBSTT exhaustively. Blots were developed by using the Pierce SuperSignal substrate system.

HEK Cell Activation Assay. HEK cells with or without TLR4 have been extensively characterized and were cultured as described (31). For cell activation assays, cells were grown to confluency in 48-well plates. Cell monolayers were washed two times with warm PBS and incubated overnight at 37°C, 5% CO_2, and 95% humidity in HBSS$^+$/0.1% HSA with the supplements indicated in the legends to Figs. 1–3 and Table 1. Activation of HEK cells was assessed by measuring the accumulation of extracellular IL-8 by ELISA as described (32).

Chromatography. Columns of Sephacryl HR S200 (1.6 × 30 cm) or S100 (1.0 × 60 cm) were preequilibrated in 10 mM Hepes, HBSS$^+$ with or without 0.1% HSA. Aliquots containing ^{14}C-LOS$_{agg}$ with or without LBP, sCD14, or dialyzed conditioned insect cell medium or ^{14}C-LOS:sCD14 with or without dialyzed conditioned insect cell medium or ^{14}C-LOS:MD-2 were incubated at 37°C, 30 min before gel filtration chromatography. Fractions (1 ml) were collected (flow rate, 0.5 ml/min) at room temperature by using an Amersham Pharmacia Biosciences AKTA FPLC. Samples for chromatography contained from 2 to 200 ng of ^{14}C-LOS$_{agg}$, ^{14}C-LOS:sCD14, or ^{14}C-LOS:MD-2 in 1 ml of column buffer with or without 0.1% HSA. Aliquots of the collected fractions were analyzed by liquid scintillation spectroscopy by using a Beckman LS liquid scintillation counter to detect ^{14}C-LOS. Recoveries of ^{14}C-LOS were >70% with or without albumin. All solutions used were pyrogen-free and sterile-filtered. After chromatography, selected fractions to be used in bioassays were pooled and passed through sterile syringe filters (0.22 μm) with >90% recovery of radiolabeled material in the sterile filtrate. Fractions were stored under sterile conditions at 4°C for >3 months with no detectable changes in chromatographic or functional properties. Columns were calibrated with Bio-Rad gel filtration standards that included thyroglobulin (V_o), γ-globulin, ovalbumin, myoglobin, vitamin B12 (V_i), and HSA. Note that experiments using ^{3}H-LOS and ^{3}H-LPS were carried out by the same procedure.

Cell Association of Various Forms of Endotoxin. HEK or HEK/TLR4 cells were grown to confluency in six-well plates and washed twice with warm PBS, and ^{3}H-LOS aggregates or ^{3}H-LOS–protein complexes with or without indicated supplements were incubated overnight at 37°C, 5% CO_2, and 95% humidity in DMEM, and 0.1% HSA with the supplements indicated in the legends to Figs. 3 and 4. After the incubation, supernatants (extracellular media) were collected, cells were washed twice with cold PBS, and cells were lysed and solubilized with RNeasy lysis buffer (Qiagen). The amount of radioactivity associated with the cells was determined by liquid scintillation spectroscopy. Total recovery of radioactivity was >90%.

Results

Expression and Function of Recombinant MD-2 Secreted by Infected Insect Cells. To further define the mechanism by which ^{14}C-LOS:sCD14 promotes cell activation and the role of MD-2, we generated conditioned insect cell culture medium containing soluble, polyhistidine-tagged recombinant wt or C95Y mutant MD-2 according to the method of Viriyakosol *et al.* (6). A HEK cell line (HEK293) that stably expresses TLR4 (HEK/TLR4), but lacks both CD14 and MD-2 (31), was used to evaluate the effect of MD-2 on the ability of LOS to interact with TLR4 and promote activation.

Conditioned medium from insect cells inoculated with baculovirus encoding either wt or mutant C95Y MD-2, but not conditioned control medium, contained a polyhistidine-tagged protein that migrated with a size appropriate to that reported for MD-2 ($M_r \approx 20{,}000$; ref. 33 and Fig. 1 *A* and *B*). In the absence of added conditioned medium, HEK/TLR4 cells were not activated by ^{14}C-LOS aggregates with or without LBP and sCD14 or by the isolated ^{14}C-LOS:sCD14 complex (Fig. 1*C*). However, addition of dialyzed conditioned medium from cells expressing wt MD-2 ("MD-2") with ^{14}C-LOS$_{agg}$ plus LBP and sCD14 or with purified ^{14}C-LOS:sCD14 alone resulted in robust activation of HEK/TLR4 (Fig. 1*C*). Little or no activation of these cells occurred when "MD-2" was added with LOS$_{agg}$ with or without LBP but without sCD14. Parental HEK cells (TLR4−) were not activated by endotoxin under any of the conditions tested (data not shown). Thus, activation of HEK293 cells by LOS requires the concerted action of LBP, sCD14 (to produce LOS:sCD14), MD-2, and TLR4. The effects of the conditioned medium containing wt MD-2 were not seen with control-conditioned medium (not shown) or medium containing C95Y MD-2 (Fig. 1*C*) even when added at 100-fold greater amounts (Fig. 1*D*).

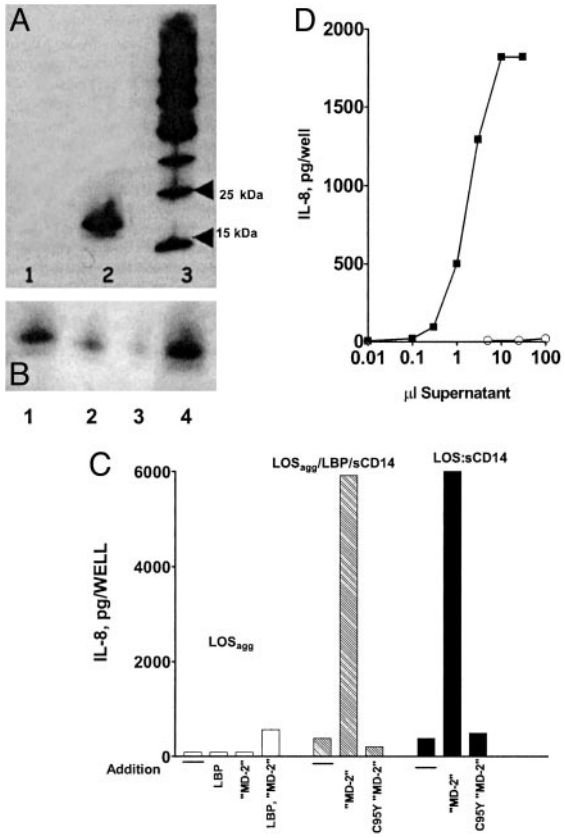

Fig. 1. Expression and bioactivity of recombinant MD-2-His-6. (*A* and *B*) SDS/PAGE immunoblots of control culture medium (*A*, lane 1) or medium from HiFive cells infected with recombinant baculovirus encoding wt (*A*, lane 2, and *B*, lanes 1–3) or C95Y MD-2 (*B*, lane 4). MD-2 was detected by using anti-$(His)_4$ antibody. All samples represent 1 μl of culture medium except *B*, lanes 2 and 3, which represent 0.3 and 0.1 μl, and *A*, lane 3, which represents molecular mass markers. (*C*) HEK/TLR4 cells were incubated in Hepes-buffered $HBSS^+$/0.1% albumin with ^{14}C-LOS$_{agg}$ (3 ng/ml) with or without LBP (30 ng/ml) and/or 60 μl of culture medium containing wt MD-2 (''MD-2,'' open bars), LOS$_{agg}$ plus LBP and sCD14 (250 ng/ml) with or without wt or C95Y ''MD-2'' (striped bars), or ^{14}C-LOS:sCD14 (2 ng of LOS per ml) with or without wt or C95Y ''MD-2'' (filled bars). After overnight incubation, extracellular IL-8 was assayed by ELISA. (*D*) HEK/TLR4 cells were incubated with increasing amounts of wt (■) or C95Y (○) ''MD-2'' plus ^{14}C-LOS:sCD14 (2 ng/ml), and the cell activation was measured. Results shown are from one experiment (duplicate samples) representative of four independent experiments.

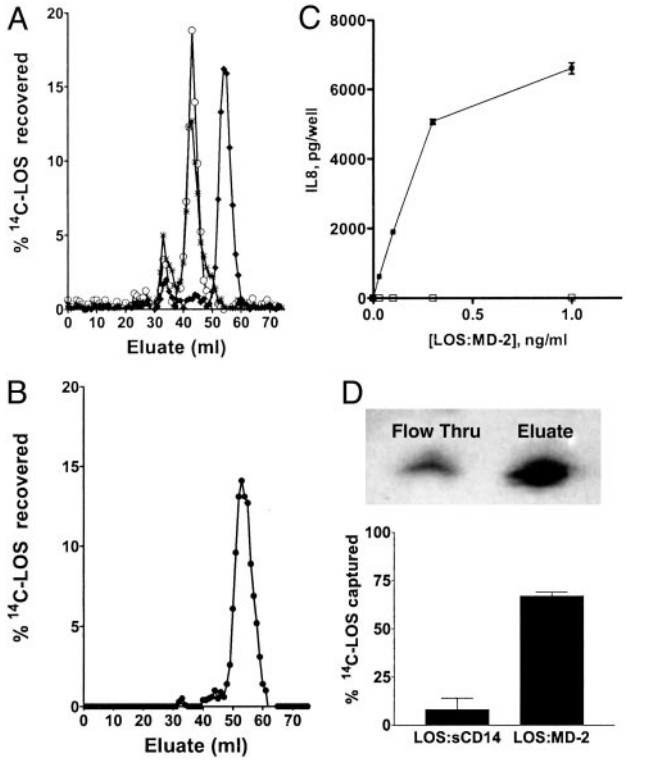

Fig. 2. A bioactive complex ($M_r \approx$ 25,000) containing MD-2 and ^{14}C-LOS is formed by incubation of ^{14}C-LOS:sCD14 with wt but not C95Y MD-2. (*A*) Dialyzed control insect cell medium (○) or medium containing wt (♦) or C95Y (*) MD-2 was incubated for 30 min, at 37°C with ^{14}C-LOS:sCD14 (1:1 vol/vol) in $HBSS^+$/10 mM Hepes and chromatographed on Sephacryl S100. Column fractions were analyzed for ^{14}C-LOS. Identical results were obtained in analytical (5 ng of ^{14}C-LOS per ml plus 200 μl of culture medium) or more preparative runs (reagents concentrated ×20). (*B*) Peak fractions ($M_r \approx$ 25,000) from treatment of ^{14}C-LOS:sCD14 with wt ''MD-2'' (*A*) were rechromatographed on S100 in $HBSS^+$/10 mM Hepes without HSA; recovery of ^{14}C-LOS was >80%. (*C*) HEK (□) or HEK/TLR4 (■) cells were incubated overnight with the indicated amounts of LOS added as purified $M_r \approx$ 25,000 (LOS:MD-2) complex. Cell activation was measured by IL-8 accumulation. Results shown correspond to one experiment, in duplicate, representative of three similar experiments. (*D* and *E*) Adsorption and elution of bioactive $M_r \approx$ 25,000 complex to HisBind resin. Peak fractions of the purified complex (*B*; 10 ng of ^{14}C-LOS) were dialyzed against PBS and incubated with HisBind resin (0.125 ml) for 1 h at 25°C and processed as described in *Materials and Methods*. (*D*) Nonadsorbed (FlowThru) and adsorbed material eluted with 200 mM imidazole were precipitated with trichloroacetic acid to concentrate the sample for SDS/PAGE immunoblot analysis. (*E*) Alternatively, absorbed material was eluted with 2% SDS and counted by liquid scintillation spectroscopy. Adsorption of ^{14}C-LOS:sCD14 was tested as a negative control. Overall recovery of ^{14}C-LOS was >90%. Results shown are the mean or representative of two closely similar experiments.

Maximum cell activation was produced with as little as 30 ng of wt MD-2 per ml added.

Formation and Function of an Endotoxin–MD-2 Complex. We have demonstrated a close correlation between the bioactivity of endotoxin and changes in the physical state of endotoxin induced by reversible protein associations (22, 23, 29). Because incubation of "MD-2" with ^{14}C-LOS:sCD14 is necessary for activation of HEK/TLR4 cells (Fig. 1 *C* and *D*), we examined by gel filtration the result of incubation of "MD-2" with ^{14}C-LOS:sCD14 at concentrations of MD-2 and LOS similar to the concentrations used in the bioassays (Fig. 1). Treatment of ^{14}C-LOS:sCD14 with "MD-2" efficiently generated a new ^{14}C-LOS-containing complex that eluted as $M_r \approx$ 25,000 on Sephacryl S100 (Fig. 2*A*). In contrast, treatment of ^{14}C-LOS:sCD14 with the nonfunctional C95Y MD-2 produced no change in the chromatographic behavior of ^{14}C-LOS:sCD14 (Fig. 2*A*). Rechromatography of the peak fraction(s) from preparative generation of this ^{14}C-LOS-containing complex (Fig. 2*B*) yielded a single, symmetrical peak (recovery >90% with or without albumin, Fig. 2*B*). This ^{14}C-LOS-containing complex is resolved from albumin and any residual ^{14}C-LOS:sCD14 or sCD14 released from LOS:sCD14 during formation of the complex, as judged by gel filtration chromatography (Fig. 2*B*) and immunoassay for CD14 and LOS:sCD14 (data not shown). The isolated $M_r \approx$ 25,000 complex activated HEK/TLR4 cells in a potent, dose- and TLR4-dependent manner (Fig. 2*C*); half-maximal activation occurred at ≈150 pg of ^{14}C-LOS per ml (30 pM). Cell activation did not require addition of sCD14 or albumin.

The apparent size of this active complex, as judged by gel-sieving chromatography, was consistent with a monomeric complex of LOS:MD-2. To determine whether the ^{14}C-LOS in this

Table 1. Ability of various forms of ^{14}C-LOS with or without proteins to form LOS:MD-2 and activate HEK/TLR4

Materials	LOS:MD-2	Activation
LOS_{agg} + LBP	−−−	−−−
LOS_{agg} + LBP, MD-2	−−−	−−−
LOS_{agg}, LBP, sCD14	−−−	−−−
LOS_{agg}, LBP, sCD14, MD-2	+++	+++
LOS:sCD14	−−−	−−−
LOS:sCD14 + MD-2	+++	+++
LOS:sCD14 + conditioned culture media (no MD-2)	−−−	−−−

Purified ^{14}C-LOS_{agg} or ^{14}C-LOS:sCD14 (3 ng of LOS per ml) with or without indicated proteins (30 ng/ml LBP, 250 ng/ml sCD14, and 60 μl of culture medium) were incubated at 37°C for 30 min. After this incubation, samples were analyzed by gel filtration chromatography to monitor formation of ^{14}C-LOS:MD-2 and by incubation with HEK/TLR4 cells to measure cell activation.

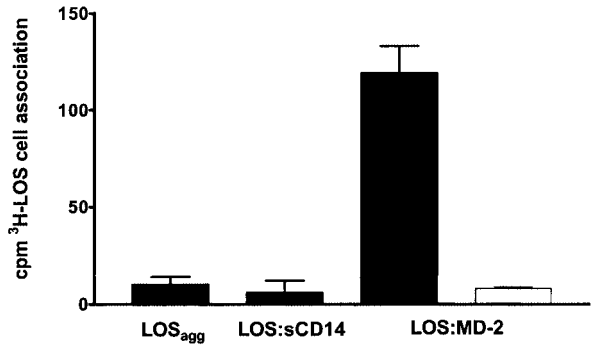

Fig. 3. Delivery of ^{3}H-LOS:MD-2 but not ^{3}H-LOS_{agg} or ^{3}H-LOS:sCD14 to HEK/TLR4. HEK (□) or HEK/TLR4 (■) cells were incubated with ^{3}H-LOS (0.75 ng/ml) in the form of LOS_{agg}, LOS:sCD14, or LOS:MD-2. After overnight incubation at 37°C, cells were washed and lysed as described in *Materials and Methods*. The amount of ^{3}H-LOS associated with the cells was measured by liquid scintillation spectroscopy. Results are from one experiment in duplicate, which is representative of three similar experiments.

active complex was linked to MD-2, we examined the ability of nickel-charged agarose resin (HisBind) to cocapture polyhistidine-tagged MD-2 and ^{14}C-LOS. Both MD-2 and ^{14}C-LOS adsorbed to the HisBind resin and were eluted with 200 mM imidazole (Fig. 2 *D* and *E*). The low adsorption of ^{14}C-LOS:sCD14 confirmed that the binding to the HisBind resin of ^{14}C-LOS in the bioactive $M_r \approx 25{,}000$ complex was specific and reflected its association with MD-2. Thus, treatment of ^{14}C-LOS:sCD14 with soluble MD-2 generated an apparently monomeric ^{14}C-LOS:MD-2 complex that activated HEK/TLR4 cells in a potent dose (pg/ml)- and TLR4-dependent manner independent of CD14. We have also generated a ^{3}H-LPS:MD-2 complex from ^{3}H-LPS purified from *E. coli* LCD25 (34) with chromatographic and functional properties virtually identical with ^{14}C-LOS:MD-2 (data not shown).

Efficient Formation of Bioactive Endotoxin–MD-2 Complex Requires Monomeric Endotoxin–CD14 Complex. We have speculated that the LBP and sCD14 dependence of potent cell activation by endotoxin reflects the preference of the TLR4/MD-2-containing receptor complex for interaction with endotoxin complexed to CD14 (24). The demonstration that ^{14}C-LOS:sCD14 could activate HEK/TLR4 cells by first transferring ^{14}C-LOS to MD-2 suggested that it was this step that was facilitated by presentation of endotoxin as a monomeric complex with CD14. We compared various presentations of ^{14}C-LOS (i.e., ^{14}C-LOS_{agg} with or without LBP and with or without sCD14) for their ability to react with MD-2 to form the LOS:MD-2 complex (assessed by gel filtration chromatography) and subsequently activate HEK/TLR4 cells. Only ^{14}C-LOS:sCD14 (either purified or generated during incubation of ^{14}C-LOS_{agg} with LBP and sCD14) was able to react with MD-2 to produce ^{14}C-LOS:MD-2 and activate HEK/TLR4 cells (Table 1). These findings directly demonstrate the role of CD14 (i.e., endotoxin–CD14) in the delivery of endotoxin to MD-2 and demonstrate that CD14 is not part of the complex that directly activates TLR4.

Molecular Requirements for MD-2-Dependent Delivery of Endotoxin to Host Cells and Cell Activation. Table 1 also suggests that endotoxin must be presented in the form of a monomeric endotoxin–MD-2 complex to activate HEK/TLR4 cells. We speculated that this reflected a unique ability of MD-2 to deliver endotoxin to TLR4. To test this hypothesis, we compared cell association of purified LOS_{agg}, LOS:sCD14 or LOS:MD-2 complexes with parental and HEK/TLR4 cells. Initial experiments with ^{14}C-LOS did not reveal significant cell association of radiolabeled LOS under any condition. We reasoned that these negative results could simply reflect the limited amount of surface TLR4 available and needed to engage LOS:MD-2 for cell activation. To address this, we isolated LOS after metabolic labeling with [^{3}H]acetate to achieve nearly 10-fold higher specific radioactivity (≈4,000 cpm/ng) and generated ^{3}H-LOS_{agg} and protein:^{3}H-LOS complexes. Using the ^{3}H-LOS, we readily detected specific TLR4-dependent cell association of ^{3}H-LOS:MD-2 only, with virtually no TLR4-independent cell association of LOS:MD-2 (Fig. 3). In addition, no cell association of either ^{3}H-LOS_{agg} or ^{3}H-LOS:sCD14 to HEK cells with or with TLR4 was detected (Fig. 3).

In conjunction with earlier observations (8, 10, 11, 15, 16, 35), these findings suggest a bifunctional role for MD-2, coupling endotoxin recognition to TLR4 activation. If simultaneous engagement of endotoxin and TLR4 by MD-2 is required for TLR4-dependent cell activation by endotoxin, the presence of a stoichiometric excess of MD-2 relative to TLR4 should inhibit cell activation by endotoxin. To test this hypothesis, we examined the effect of adding varied amounts of conditioned insect cell culture medium containing wt, C95Y, or no MD-2. Addition of medium containing wt MD-2, but not control medium, produced a dose-dependent inhibition of the activation of HEK/TLR4 by ^{14}C-LOS:MD-2 (Fig. 4*A*). Medium containing C95Y MD-2 had an intermediate inhibitory effect consistent with the (partial) retention of TLR4 binding by this mutant MD-2 species (7, 11, 18). Inhibitory effects of added MD-2 had no direct effect on LOS:MD-2 (Fig. 4*B*, no change in chromatographic behavior) but blocked TLR4-dependent cell association of ^{3}H-LOS:MD-2 (Fig. 4*C*). This finding is consistent with a need for simultaneous engagement of endotoxin and TLR4 by individual molecules of MD-2 for TLR4-dependent cell activation. Thus, depending on levels of expression, MD-2, like LBP (24, 36) and CD14 (37), can have inhibitory and stimulatory effects on TLR4-dependent cell activation by endotoxin. Our results extend earlier observations showing that addition of excess soluble MD-2 inhibited TLR4-dependent responses in cell types containing endogenous TLR4/MD-2 (6).

Discussion

This study describes the formation and isolation of a bioactive and apparently monomeric endotoxin–MD-2 complex. Previous studies have demonstrated endotoxin–MD-2 interactions by using relatively high concentrations (μg/ml) of both endotoxin and MD-2 (6, 8–10, 18, 38). Neither the bioactivity nor the composition of the product of this interaction was completely defined. This study has described the generation of a defined endotoxin–MD-2 complex at very low concentrations (pM) of

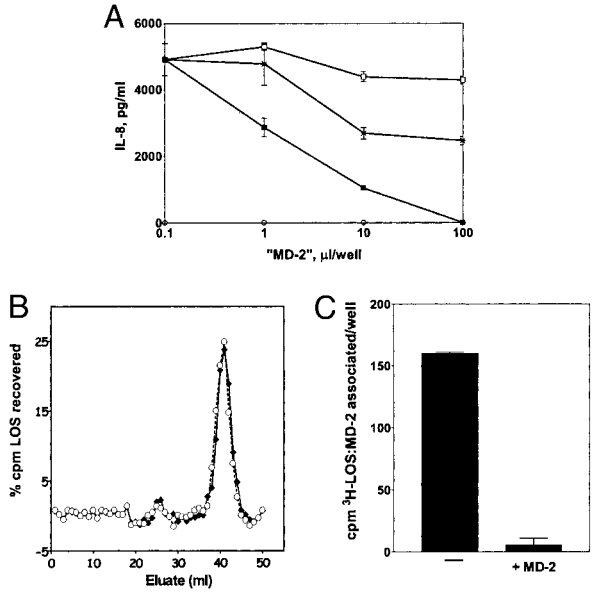

Fig. 4. Effect of added MD-2 on activation of HEK/TLR4 cells by LOS:MD-2 and delivery of ^{3}H-LOS:MD-2 to HEK/TLR4 cells. (*A*) Cells were incubated in HBSS^{+}/10 mM Hepes/0.1% albumin with ^{14}C-LOS:MD-2 (0.3 ng/ml) and increasing amounts of wt (■) and C95Y (*) MD-2 or negative control medium (□) and with wt MD-2 but no ^{14}C-LOS:MD-2 (○). After overnight incubation, extracellular accumulation of IL-8 was measured. The concentrated and dialyzed conditioned media contained ≈10 ng of MD-2 (wt or C95Y) per μl. Results are from one experiment in duplicate, which is representative of three similar experiments. (*B*) Purified ^{14}C-LOS:MD-2 (1 ng/ml) was preincubated with (●) or without (○) an amount of MD-2 that completely inhibited activation (40 μl of 20-fold concentrated and dialyzed conditioned medium) for 30 min, at 37°C in HBSS^{+}/10 mM Hepes before chromatography on Sephacryl S200. Column fractions were analyzed for ^{14}C-LOS by liquid scintillation spectroscopy. (*C*) ^{3}H-LOS:MD-2 (0.75 ng/ml; ≈3,000 cpm) with or without excess MD-2 (as indicated in *B*) was incubated with HEK/TLR4 cells overnight at 37°C as described in *Materials and Methods*. After supernatants were removed, cells were washed and then lysed as described in *Materials and Methods*. The amount of radioactivity associated with the cells was determined by liquid scintillation spectroscopy. No radioactivity was associated with parental cells.

endotoxin and soluble MD-2 and demonstrated that this complex, at pg/ml concentrations, activates cells in a TLR4-dependent fashion without the inclusion of other host or bacterial factors. We have made essentially the same observations with meningococcal LOS and *E. coli* LPS, supporting the generality of these findings at least with respect to "conventional" endotoxin species that display potent TLR4-dependent proinflammatory activity.

Our success in achieving formation of a bioactive endotoxin–MD-2 complex at such low concentrations of endotoxin and MD-2 reflects the importance of presenting endotoxin to MD-2 after endotoxin has been first modified by LBP and CD14. As interactions of CD14 with endotoxin are greatly enhanced by prior interaction of endotoxin with LBP (22, 23, 25–29), our findings indicate that MD-2–endotoxin interactions leading to the generation of the bioactive endotoxin–MD-2 complex are greatly enhanced by presentation of endotoxin as a monomeric complex with CD14. Whether this enhancement reflects a greater reactivity of MD-2 for disaggregated vs. aggregated forms of endotoxin or the need for an additional protein–protein interaction between CD14 and MD-2 remains to be determined. Whatever the precise molecular basis of the high affinity and reactivity of endotoxin–CD14 complexes with MD-2, our findings support a direct role of MD-2 in endotoxin recognition and delivery of endotoxin to host cells containing TLR4 (Figs. 2*C* and 3), not requiring prior association of MD-2 with TLR4. These findings also support the contention that the key role of LBP and CD14 in enhancing cell responses on exposure to minute amounts of endotoxin is to transform aggregates of endotoxin to monomeric endotoxin–CD14 complexes that react preferentially with MD-2 (Table 1). Conversely, the remarkably potent activity of the purified endotoxin–MD-2 complex toward HEK/TLR4 cells provides the strongest evidence to date that CD14 is not needed as part of a more complex heterooligomeric receptor, as suggested (14, 15, 29, 34, 37)

An essential feature of TLR4-dependent cell activation by endotoxin is its extraordinary sensitivity, permitting timely host responses to small numbers of invading Gram-negative bacteria, essential for efficient host defense (1–3). The reaction pathway we describe, in which endotoxin molecules in purified aggregates (or membranes) containing thousands to millions of endotoxin molecules per particle are extracted and transferred to first CD14 and then MD-2, provides a unique physicochemical mechanism to attain the potency that is needed. The ability to generate a homogeneous protein–endotoxin complex that alone potently triggers TLR4-dependent cell activation, interacts with host cells in an almost exclusively TLR4-dependent fashion (Fig. 3), and can be metabolically labeled to sufficient specific radioactivity to monitor interactions at picomolar concentrations should make it possible to measure host cell–endotoxin interactions that are directly relevant to TLR4-dependent cell activation.

Many endotoxin-responsive cells contain membrane-associated CD14 and MD-2 (associated with TLR4) (2, 14, 39). However, we have recently demonstrated that resting airway epithelial cells, like HEK/TLR4 cells, express TLR4 without MD-2 and respond to endotoxin only if LBP, sCD14, and soluble MD-2 are added.‖ Each of these proteins is likely to be present in biological fluids at the concentrations needed to drive endotoxin-dependent TLR4 activation, especially in view of the very low extracellular MD-2 concentrations demonstrated in this study to be sufficient (Fig. 1*D*). An anti-CD14 monoclonal antibody we have used to identify and immunocapture endotoxin–sCD14 complexes blocks cell activation mediated by soluble MD-2, membrane TLR4, and membrane TLR4/MD-2 complexes (e.g., endothelial cells; refs. 22 and 29). Hence, the reaction pathway we have defined is likely to be relevant at the cell surface when TLR4/MD-2 complexes are endogenously present and also when only TLR4 is present at the cell surface and MD-2, which has been produced and secreted by neighboring cells, is present in the extracellular medium.

Accumulating evidence favors the view that MD-2 is a bifunctional protein, coupling endotoxin recognition to TLR4 activation. Mutagenesis studies have suggested distinct structural determinants within MD-2 for endotoxin (CD14?) and TLR4 interactions (7, 11, 15, 16, 18). Our findings provide the most convincing evidence that MD-2 can engage both endotoxin and TLR4 and that simultaneous interaction of MD-2 with endotoxin and TLR4 is crucial for TLR4-dependent cell activation by endotoxin. We predict, therefore, that binding sites within MD-2 for TLR4 and endotoxin are topologically and structurally distinct, permitting engagement of endotoxin–MD-2 complexes with TLR4, as our findings suggest (Fig. 3), and interaction and transfer of endotoxin from endotoxin–CD14 complexes to MD-2 already associated with TLR4 (Fig. 5). The complete lack of reactivity of the C95Y MD-2 mutant with endotoxin–sCD14 (Fig. 1*D*) explains the complete absence of activity in this mutant protein (Fig. 2*B* and refs. 7, 11, and 18) despite a partial retention of reactivity with TLR4 (Fig. 4*A* and refs. 7, 11, and 18).

‖Jia, H. P., Kline, J. N., Penisten, A., Apicella, M. A., Gioannini, T., Weiss, J. & McCray, P. B., Jr., manuscript submitted for publication.

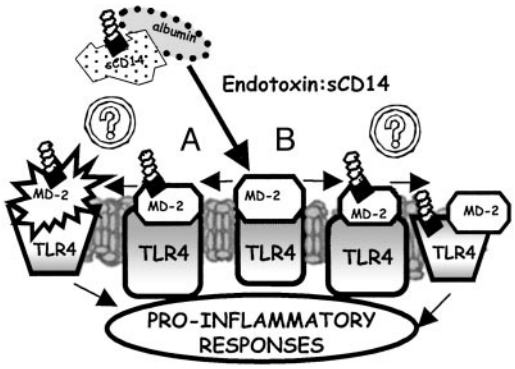

Fig. 5. Possible mechanism of action of MD-2 in endotoxin-dependent activation of TLR4. TLR4 activation may involve either conformational changes in MD-2 that follow the interaction of MD-2 with endotoxin and TLR4 (*A*) or transfer of endotoxin from MD-2 to TLR4 (*B*).

Finally, how does the binding of both endotoxin and TLR4 by MD-2 result in TLR4 activation? In contrast to ^{14}C-LOS:sCD14, the "stability/solubility" of LOS:MD-2 in aqueous buffer and its bioactivity do not require albumin (data not shown). We have speculated that the requirement for albumin in the transfer of endotoxin from endotoxin–LBP aggregates to sCD14 and from endotoxin–sCD14 to MD-2 may reflect the need to shield the lipid A region of endotoxin from the surrounding aqueous environment during transfer from one endotoxin-binding protein to another (22). That albumin is no longer required once the endotoxin–MD-2 complex is formed suggests that a (deep) hydrophobic site in MD-2 accommodates and shields the hydrophobic lipid A region of the bound endotoxin making subsequent transfer to TLR4 less likely. We favor the hypothesis that binding of endotoxin to MD-2 induces conformational changes in MD-2 that lead to TLR4 activation (Fig. 5).

Several studies have suggested that MD-2 has the ability to discriminate between TLR4 agonists and antagonists (10, 20, 40). Agonists and antagonists may differ in their ability to form a complex with MD-2 or in the structural and functional properties of the (endotoxin–MD-2) complex that is formed. Perhaps, only endotoxins that are TLR4 agonists are transferred from CD14 to MD-2 or, within the endotoxin–MD-2 complex, trigger changes in MD-2 conformation or protein–protein contacts between TLR4 and MD-2 needed for TLR4 activation. Therefore, rather than transferring the buried endotoxin molecule to TLR4, MD-2 may function in a manner analogous to that observed with Toll receptors in *Drosophila* where a modified protein, Spaetzle, is the ligand that initiates the cytoplasmic signaling pathway (41). Studies are needed to decipher the nature of the interaction of endotoxin–MD-2 with TLR4.

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