MD-2, a glycoprotein that is essential for the innate response to lipopolysaccharide (LPS), binds to both LPS and the extracellular domain of Toll-like receptor 4 (TLR4). Following synthesis, MD-2 is either secreted directly into the medium as a soluble, active protein, or binds directly to TLR4 in the endoplasmic reticulum before migrating to the cell surface. Here we investigate the function of the secreted form of MD-2. We show that secreted MD-2 irreversibly loses activity over a 24-h period at physiological temperature. LPS, but not lipid A, prevents this loss in activity by forming a stable complex with MD-2, in a CD14-dependent process. Once formed, the stable MD-2-LPS complex activates TLR4 in the absence of CD14 or free LPS indicating that the activating ligand of TLR4 is the MD-2-LPS complex. Finally we show that the MD-2-LPS complex, but not LPS alone, induces epithelial cells, which express TLR4 but not MD-2, to secrete interleukin-6 and interleukin-8. We propose that the soluble MD-2-LPS complex plays a crucial role in the LPS response by activating epithelial and other TLR4+/MD-2− cells in the inflammatory microenvironment.

Lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, stimulates an exceptionally potent innate immune response in mammals that can result in septic shock and death (1). The LPS response is mediated by four proteins (2): LPS binding protein extracts single molecules from LPS micelles and transfers them to CD14, a glycosylphosphatidylinositol-anchored cell surface receptor that also exists as a serum protein. In turn, the CD14-LPS complex activates Toll-like receptor 4 (TLR4) and MD-2 (3–6). The activation of TLR4 is a type I integral membrane glycoprotein and is one of the LPS response, Toll-like receptor 4 (TLR4) and MD-2 (3–6). TLR4 is a type I integral membrane glycoprotein and is one of 10 TLR paralogs that activate NF-κB, mitogen-activated protein kinases, and other transducers of inflammatory signals in response to pathogen-specific structural motifs. MD-2, a small cysteine-rich glycoprotein, binds to the ectodomain of TLR4 (7) in the endoplasmic reticulum and then transits to the cell surface in an active TLR4-MD-2 complex. However, MD-2 is also secreted into the medium as a soluble, active protein (sMD-2) by primary cells such as immature dendritic cells (iDC), and by MD-2-transfected cell lines (8). The activity of sMD-2 was shown by its ability to bind to TLR4 and confer LPS responsiveness to cells that express TLR4 but lack MD-2 (8, 9). Forward genetic and gene deletion studies have demonstrated that both MD-2 and TLR4 are required for normal responsiveness to LPS in vitro and in vivo (3–5, 10).

Analyses of species specificity differences for various forms of LPS provide strong evidence that LPS interacts directly with the TLR4-MD-2 complex (11–16). However, the molecular events leading to LPS binding and TLR4 activation are only partially understood. Photoaffinity labeling (17) and binding (9, 18, 19) studies have shown that LPS binds directly to MD-2 and TLR4, and that binding of LPS to TLR4 requires MD-2 (17–19). In addition, it has been shown that CD14 is required for LPS binding to MD-2 (18) or the TLR4-MD-2 complex (17, 19). Taken together, these data have suggested a current model in which CD14 delivers LPS to a complex between TLR4 and MD-2, and that this interaction promotes signal transduction (18–20).

The purpose of the current investigation is to define the role of soluble MD-2 in the LPS response. We show that sMD-2 is a labile protein that loses most of its activity over a 24-h period at physiological temperature. Treatment with LPS stabilizes sMD-2 in a process that requires CD14, and when purified, the stable MD-2-LPS complex, in the absence of CD14 or free LPS, directly activates TLR4. Finally, we show that the MD-2-LPS complex has the capacity to trigger the release of cytokines by epithelial cells, which express TLR4 but not MD-2, and therefore fail to respond to LPS alone. We conclude that the activating ligand of TLR4 is a complex between LPS and MD-2, and that the soluble form of this complex can play a role in regulating the innate response to LPS.

**EXPERIMENTAL PROCEDURES**

*Reagents—*The following antibodies were used in this study: anti-His6-horseradish peroxidase mAb (BD Biosciences, Clontech, Palo Alto, CA), anti-FLAG M2 mAb, anti-FLAG M2 mAb peroxidase conjugate, and anti-biotin agarose (Sigma), alkaline phosphocyanin-conjugated goat anti-mouse IgG (Caltag, Burlingame, CA), anti-bovine CD14 mAb, CAM36A (VMRD, Inc., Pullman, WA), and anti-TLR4 mAb (HTA1216, a gift from Dr. Kensuke Miyake, University of Tokyo, Tokyo, Japan). Phenol-extracted Salmonella minnesota Re 595 LPS and diphosphoryl lipid A from Escherichia coli F583 (Rd mutant), were from Sigma, and biotin LPS was a gift from Dr. Alberto Visintin (University of Massachusetts, Worcester, MA). Recombinant human CD14 (rCD14) was purchased from R&D Systems (Minneapolis, MN).

*Cells—*The human embryonic kidney (HEK) 293T cell line was obtained from the American Type Culture Collection (Manassas, VA). TLR4 reporter cells, HEK293 cells stably expressing human TLR4 and
the NF-κB reporter plasmid, ELAM-1-luciferase, were a gift from Dr. Jesse Chow (Eisai Research Institute, Andover, MA). HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% low endotoxin fetal bovine serum (FBS), 2 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. The protein-free medium HyQ® FF 293 (Hyclone, Logan UT) was used for serum-free conditions. Human conjunctival epithelial cells (HCEC) (21) were cultured in Earle’s minimal essential medium containing L-glutamine, 10% FBS, 2 mM glutamine, 50 μg/ml streptomycin. The protein-free medium containing 100 ng/ml LPS. Medium HyQ® was added (100 μg/ml per well) and incubated for 1 h at room temperature. After 3 washes with phosphate-buffered saline, 0.05% Tween 20, 100 μl of TMB substrate solution (Bio-Rad Systems) was added. The reaction was stopped using 1 x sulfuric acid (50 μl per well) and absorbance values were measured at 450 nm.

MD-2 Activity Assay—TLR4 reporter cells (5 × 10⁴ cells per well) were allowed to adhere in a 96-well tissue culture plate. To initiate the assay, medium was removed and 100 μl of MD-2 supernatant and, where required, 100 ng/ml LPS were added to each well. The cells were incubated 8–16 h at 37 °C, lysed in Reporter Lysis Buffer (Promega, Madison, WI), and luciferase activities were determined by using a Luciferase Chemiluminescent assay kit (Promega). Each sample was measured in triplicate, and results are reported as average luciferase units (×10⁻⁶) ± S.D. In one experiment, HCEC cells (8 × 10⁴ cells per well in a 48-well plate) were allowed to adhere 2 days, then incubated for 24 h with either LPS (100 ng/ml) or MD-2-LPS complex (1:4 dilution of purified sample) after which IL-6 and IL-8 were determined by ELISA (23).

MD-2 Preincubation Conditions—In some experiments, sMD-2 was either preincubated with 100 ng/ml LPS for 24 h, or produced in medium containing 100 ng/ml LPS, and in these cases, no additional LPS was added at the beginning of the assay. In other experiments, MD-2 samples were preincubated at 37 °C in the absence of LPS, and in these cases 100 ng/ml LPS was added at the beginning of the assay. For background stimulation, LPS was omitted altogether.

Fig. 1. sMD-2 is unstable at physiological temperature. A, supernatants containing sMD-2 were incubated at 37 °C for the indicated times, then added with LPS to TLR4 reporter cells. The solid square represents NF-κB activity in the absence of LPS. The time 0 sample was kept at 4 °C during the entire incubation. Data are representative of three separate experiments. B, sMD-2 was preincubated at 37 °C (lanes 3 and 4) or maintained at 4 °C (lanes 1 and 2) for 24 h, then incubated with (lanes 2 and 4) or without (lanes 1 and 3) biotinylated LPS for 8 h at 37 °C. Upper panel, 10-ml samples were immunoprecipitated (IP) with anti-biotin-agarose. Lower panel, 1-ml samples were immunoprecipitated with anti-FLAG, which binds sMD-2. Immunoprecipitates were resolved by SDS-PAGE (13%) under reducing conditions and immunoblotted (IB) with an anti-FLAG mAb. C, FACS profiles of cell-bound MD-2. sMD-2 was maintained at 4 °C (solid lines) or preincubated for 24 h at 37 °C (dashed lines) and tested for binding to cells expressing TLR4-GFP (thick lines) or TLR9-GFP (thin lines). Cells were labeled with anti-FLAG (or an isotype control) and stained with an allophycocyanin-conjugated secondary antibody. Shown are histograms gated on GFP⁺ (transfected) cells. Surface expression of TLR4 was similar in all samples (data not shown).
**A Complex of MD-2 and LPS Is the Activating Ligand of TLR4**

**RESULTS**

MD-2 Is Unstable at Physiological Temperature—MD-2, an essential component of the LPS signaling pathway, is secreted as a soluble form (sMD-2) that confers LPS responsiveness to cells expressing TLR4 (8, 9). However, we found that preincubation of sMD-2 at 37 °C prior to addition to TLR4 reporter cells resulted in a dramatic loss in activity. As seen in Fig. 1A, after 24 h at 37 °C the capacity of sMD-2 to confer LPS responsiveness to TLR4, as measured by NF-κB activation, decreased by ~90% when compared with sMD-2 that had been kept at 4 °C (zero time point). During this same period, the amount of sMD-2 in solution remained constant as determined by ELISA and there was no change in apparent molecular weight by SDS-PAGE (data not shown), suggesting that the loss in activity was because of a non-covalent structural transition rather than proteolytic degradation or precipitation. We next asked whether sMD-2 lost the ability to bind either LPS or TLR4 at 37 °C. To examine LPS binding, we incubated sMD-2 with biotin-labeled LPS and immunoprecipitated with anti-biotin;
LPS-bound MD-2 was detected by SDS-PAGE and immunoblotting. Fig. 1B (upper panel) shows that sMD-2 maintained at 4 °C binds LPS, confirming previous results (9, 18) (lane 2). By contrast, sMD-2 that had been preincubated at 37 °C failed to bind LPS (lane 4). Immunoprecipitations with anti-FLAG confirmed that similar amounts of sMD-2 protein were present in each sample (Fig. 1B, lower panel). To determine whether the ability of sMD-2 to bind TLR4 was also lost at 37 °C, TLR4 expressing cells were incubated with sMD-2, and cell-bound MD-2 was detected by flow cytometry. As a negative control, sMD-2 was also incubated with cells expressing TLR9. Fig. 1C shows that sMD-2 maintained at 4 °C stained the TLR4 transfectants brightly, but gave weak staining on the TLR9 transfectants, as expected. However, after a 24-h preincubation at 37 °C the sMD-2 exhibited a substantial (85%) decrease in TLR4 binding. Thus, at 37 °C sMD-2 rapidly loses the ability to confer LPS responsiveness to TLR4 reporter cells, accompanied by a loss in LPS and TLR4 binding capacities.

**LPS Stabilizes MD-2**—Because ligand binding often protects proteins from denaturation, we asked whether LPS would stabilize sMD-2 against loss of activity at 37 °C. Accordingly, we incubated sMD-2 for 24 h at 37 °C with LPS and then assayed the samples for activity. As a control, sMD-2 was preincubated for 24 h without LPS, and LPS was added at the beginning of the activity assay. As seen in Fig. 2A, sMD-2 that had been preincubated with LPS was at least 10-fold more active than the control sample preincubated without LPS, and was as active as sMD-2 that had been kept at 4 °C. Treatment of inactivated (24 h, 37 °C) sMD-2 with LPS for 24 h at 37 °C failed to restore activity, indicating that inactivation at 37 °C is irreversible (data not shown). To compare recombinant sMD-2 with its endogenously expressed counterpart, we collected conditioned medium from human iDC, a potent source of sMD-2-like activity (8, 22). Fig. 2B shows that iDC supernatants lost activity at 37 °C and addition of LPS prevented this loss. Thus, recombinant sMD-2 closely resembles the endogenously produced protein. We also asked whether lipid A, the toxic portion of LPS (1), would stabilize sMD-2. Interestingly, lipid A activated TLR4 when added with sMD-2 to reporter cells but failed to stabilize sMD-2 during a 24-h preincubation at 37 °C (Fig. 2C). This observation suggests that the lipid A portion of LPS is responsible for TLR4 activation, whereas the carbohydrate portion mediates MD-2 stabilization.

Because sMD-2 is derived from cells cultured for 24 h at 37 °C, a substantial amount of activity must be lost during production. To prevent this loss in activity, LPS was added to the culture medium of MD-2-transfected cells, and after 24 h supernatants were collected and assayed for activity. Parallel cultures not containing LPS were treated in the same way. The left two clusters in Fig. 3A show that immediately after culture, sMD-2 from the LPS containing supernatant was about 4–8-fold more active than sMD-2 produced in the absence of LPS, as estimated from sMD-2 dilutions giving similar activities. Conversely, these same two samples bound TLR4 to similar extents (Fig. 3B, left 2 clusters), indicating that sMD-2 can bind to TLR4 regardless of whether or not it has associated with LPS. The same sMD-2 samples were then incubated an additional 48 h at 37 °C. Following this incubation, the activity of the sMD-2 produced in LPS had decreased only slightly, whereas sMD-2 cultured in medium lacking LPS was inactive (Fig. 3A, right 2 clusters). The TLR4 binding capacity of sMD-2 also decreased after 48 h incubation at 37 °C, and LPS, in part, prevented this loss (Fig. 3B, right 2 clusters). From these results we can conclude that interaction with LPS prevents the loss of sMD-2 function that would otherwise occur at 37 °C.

**The MD-2-LPS Complex Triggers TLR4**—In view of our finding that LPS binds and stabilizes sMD-2, we asked whether the sMD-2-LPS complex, by itself, would activate TLR4. Accordingly, sMD-2 produced in LPS-containing cultures was purified by metal affinity chromatography and added in graded concentrations to TLR4 reporter cells. As seen in Fig. 4A, the purified sMD-2-LPS complex activated TLR4 at nanomolar concentrations in a dose-dependent manner, with the same activity as the sample prior to purification. As expected, purified sMD-2 from a culture not containing LPS failed to activate the TLR4 reporter cells, although this sample did activate TLR4 when LPS was added at the time of the assay (data not shown). To control for the possibility that our purification procedure failed to remove free LPS, we subjected supernatants from mock transfected cultures containing LPS (mock/LPS) to the same purification procedure. The purified mock sample was added to TLR4 reporter cells along with supernatants that contained unpurified sMD-2, which was shown to be active in the presence of LPS (Fig. 4B, right bar). If free LPS contaminated the purified mock sample, then combining it with sMD-2 would result in TLR4 activation. However, no activation was observed (Fig. 4B, middle bar), indicating that the amount of LPS re-
Thus, the MD-2/H18528LPS complex activates TLR4 in the absence of free LPS. To demonstrate a physiological relevance for this finding, we treated HCEC with either LPS alone or with the MD-2/H18528LPS complex and measured IL-6 and IL-8 secretion. Similar to other types of epithelial cells, the HCEC express TLR4, but not MD-2 (21, 24). Fig. 4C shows that HCEC in fact, respond to the sMD-2/H18528LPS complex but not to LPS alone, indicating that the complex is essential for LPS recognition in some cell types.

**CD14 Is Required for MD-2 Stabilization, but Not for TLR4 Activation by MD-2-LPS**—The studies described above were performed in medium containing FBS, which contains soluble CD14. CD14 is known to be required for LPS responsiveness at the low LPS levels used in the current study (25). To examine the role of CD14 in the stabilization of sMD-2 by LPS, sMD-2, produced in serum-free medium, was preincubated with LPS for 24 h at 37 °C. Recombinant human CD14 (rCD14) was added either before or after the preincubation. As seen in Fig. 5A, sMD-2 displayed activity only when rCD14 was present during the preincubation with LPS. To provide further evidence for the involvement of CD14, we used an anti-bovine CD14 (anti-bCD14) mAb to neutralize the CD14 in serum-containing sMD-2 supernatants (Fig. 5B). When the anti-bCD14 was added before the preincubation with LPS, the stabilization of sMD-2 was inhibited. However, if the anti-bCD14 was added after the preincubation with LPS, there was no change in activity. Taken together, these results suggest that CD14 is required for the interaction between LPS and MD-2, as previously reported (17, 18), but is not required for the activation of TLR4 by the sMD-2/LPS complex (20). To provide direct evidence for the lack of involvement of CD14 in the interaction between the sMD-2-LPS complex and TLR4, anti-bCD14 mAb was added to purified sMD-2-LPS complex prior to transfer to TLR4 reporter cells. As seen in Fig. 4C, anti-bCD14 failed to block activation, ruling out the possibility that the purified complex contained a CD14 contaminant that was required for the activation of TLR4 by sMD-2-LPS.

**DISCUSSION**

In the current study we demonstrate that the interaction of LPS with sMD-2 produces a stable, activating ligand for TLR4. Our results, summarized in Fig. 6, show that MD-2 is secreted as a labile molecule that, over a relatively short period of time at 37 °C, irreversibly decays into an inactive form (1) that is unable to bind either LPS (2) or TLR4 (3). If, however, freshly
FIG. 6. Model for the activation of TLR4 by sMD-2/LPS. MD-2 is secreted as a labile protein (sMD-2) that will normally decay within a 24-h period (1) into an inactive form, incapable of binding LPS (2) or TLR4 (3). However, if LPS is present, signaling a bacterial infection, sMD-2 binds the LPS in a CD14 dependent manner (4), forming a stable MD-2/LPS complex. This complex directly activates TLR4, thereby initiating an inflammatory response (5).
synthesized sMD-2 is exposed to LPS and CD14, it converts to a stable MD-2-LPS complex (4) that is capable of activating TLR4 (5). Purified MD-2-LPS complex activates TLR4 in the absence of free LPS or CD14. Thus, the MD-2-LPS complex by itself is competent to activate TLR4, and the principal role of CD14 is to aid in the formation of the MD-2-LPS complex, and not to facilitate the transfer of MD-2-LPS to TLR4, or to transfer LPS directly to TLR4. Of interest, a recent report indicates that sMD-2 produced by insect cells also binds LPS and activates TLR4 (20).

Both freshly secreted sMD-2 and sMD-2-LPS complex bind to TLR4, but only the complex triggers a response. What, then, is different about the complex that permits it to activate TLR4? One possibility is that LPS induces a conformational change in sMD-2 and this conformation, rather than LPS itself, triggers TLR4. An important prediction for these findings, we prefer a model in which MD-2 forms a stable MD-2/LPS complex, and that this complex activates TLR4 (20).

Although many cells, such as monocytes, dendritic cells, and B cells express both TLR4 and MD-2, other cells, such as epithelial cells express TLR4 but little or no MD-2 (21, 27). As we have shown, these cells depend upon sMD-2 for the generation of an inflammatory response to LPS. Thus, in microenvironments that are populated with appreciable numbers of TLR4+/MD-2- cells, sMD-2 would play a crucial role in shaping the inflammatory response. Based upon our results with the HCEC cells, we would expect, for example, that MD-2-LPS would induce epithelial cells to secrete IL-8, a chemokine that initiates potent anti-microbial responses by recruiting neutrophils to the site of inflammation. An important prediction for tissues that contain TLR4+/MD-2- cells is that the concentration of active sMD-2 in the interstitial fluids controls their responses to LPS. Presumably sMD-2 concentrations are maintained at low basal levels to guard against overwhelming inflammatory responses to Gram-negative bacteria, and the instability of sMD-2 at physiological temperature may provide a means for limiting its activity. However, at sites of inflammation local concentrations of sMD-2 may be increased, for example, by the recruitment of cells such as immature dendritic cells that secrete high amounts of sMD-2.

Although we have dealt with the soluble form of MD-2 in this study, many types of cells co-express TLR4 and MD-2, and in these cells a portion of the MD-2 reaches the cell surface bound to TLR4. An important question posed by our studies is whether the soluble and TLR4-bound forms of MD-2 are similar in structure and function. Specifically, is the TLR4-associated form of MD-2, like its soluble counterpart, unstable at physiological temperature? In addition, does the sMD-2-LPS complex activate cells that express both MD-2 and TLR4? If so, is this mediated by an exchange of the entire MD-2-LPS complex, or of the LPS alone? Studies currently underway to answer these questions should provide important new insights into the biology of the innate response to LPS.

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