A Novel Synthetic Acyclic Lipid A-like Agonist Activates Cells via the Lipopolysaccharide/Toll-like Receptor 4 Signaling Pathway*

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‡ The abbreviations used are: LPS, lipopolysaccharide; Toll-like receptor; LBP, LPS-binding protein; sCD14, soluble CD14; GPI, glycosylphosphatidylinositol; CHO, Chinese hamster ovary; EMSA, electrophoretic mobility shift assay; NF-κB, nuclear factor κB; TNF, tumor necrosis factor; IL-1β, interleukin-1β; FMA, phorbol myristate acetate; FACS, fluorescence-activated cell scan; FITC, fluorescein isothiocyanate; DMEK, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; PBS, phosphate-buffered saline.

ER-112022 is a novel synthetic acyclic lipid A analog that contains six symmetrically organized fatty acids on a noncarbohydrate backbone. Chinese hamster ovary (CHO)-K1 fibroblasts and U373 human astrocytoma cells do not respond to lipopolysaccharide (LPS) in the absence of CD14. In contrast, exposure to ER-112022 effectively induced activation of CHO and U373 cells under serum-free conditions. Expression of CD14 was not necessary for cells to respond to ER-112022, although the presence of soluble CD14 enhanced the sensitivity of the response. Several lines of evidence suggested that ER-112022 stimulates cells via the LPS signal transduction pathway. First, the diglucosamine-based LPS antagonists E5564 and E5531 blocked ER-112022-induced stimulation of CHO-K1, U373, and RAW264.7 cells. Second, ER-112022 was unable to activate C3H/HeJ mouse peritoneal macrophages, containing a mutation in Toll-like receptor (TLR) 4, as well as HEK293 cells, an epithelial cell line that does not express TLR4. Third, ER-112022 activated NF-κB in HEK293 cells transfected with TLR4/MD-2. Finally, tumor necrosis factor release from primary human monocytes exposed to ER-112022 was blocked by TLR4 antibodies but not by TLR2 antibodies. Our results suggest that ER-112022 and the family of lipid A-like LPS antagonists can functionally associate with TLR4 in the absence of CD14. Synthetic molecules like ER-112022 may prove to be valuable tools to characterize elements in the LPS receptor complex, as well as to activate or inhibit the TLR4 signaling pathway for therapeutic purposes.

Lipopolysaccharide (LPS, endotoxin) is the major constituent of the outer envelope of Gram-negative bacteria. LPS is thought to be a major cause of the widespread cellular activation observed in patients with Gram-negative septic shock. The lipid A portion of LPS consists of a backbone of two phosphorolylated glycosamine molecules acylated with fatty acids and contains the pro-inflammatory toxicity associated with LPS (1, 2). LPS activation of mammalian cells is mediated by its interaction with membrane CD14, a 55-kDa glycosylphosphatidylinositol (GPI)-anchored protein primarily expressed on phagocytes (3, 4). CD14 appears to be the highest affinity receptor for LPS, but other LPS-binding molecules involved in proinflammatory responses by phagocytic leukocytes have been described, such as the β2 integrin family members CD11/CD18 (5). However, neither CD14, which does not have a transmembrane domain (6–8), nor CD11/CD18 (5) appear to transduce a signal directly. Members of the family of Toll-like receptors (TLR), especially TLR4, have recently been suggested as LPS signal transducers (9–12). In addition, a small protein, known as MD-2, enhances the ability of LPS to activate cells via TLR4 (13) and appears to be a part of a TLR4 complex (14). The Toll family member RP105 may also associate with TLR4 and mediate LPS responsiveness in B cells (15). In addition to the membrane-bound receptors, blood contains several factors, including soluble CD14 (sCD14) (16) and LPS-binding protein (LBP) (17), that are important modulators of LPS activity.

Several lines of evidence suggest that TLR4 is the main signal-transducing molecule in the LPS receptor complex. First, animals that lack a functional TLR4, but not those without a functional TLR2, are markedly hyporesponsive to LPS (11, 18–22). Second, LPS induces activation of HEK293 cells transfected with TLR4 cDNA, and this activation is blocked by a lipid A-based LPS antagonist (12). Third, TLR4 is responsible for the fine discriminatory ability of the LPS receptor. For example, the unique species-specific pharmacology associated with small alterations in lipid A structure can be attributed to differences in the primary structure of TLR4 (23, 24). In contrast, TLR2 appears to be involved in responses to a variety of bacterial compounds, such as bacterial lipoproteins, peptidoglycan, and lipoarabinomannan, as well as whole bacteria and yeast particles (21, 22, 25–34). It is still unclear whether TLR2 can mediate lipid A signals (35), although it is possible that TLR2 represents an LPS receptor involved in the recognition of non-enteric endotoxins.

An important approach to understanding how any receptor system functions is to define its pharmacology. Partial lipid A structures have been important in investigating mechanisms of LPS binding and cell activation (36). These compounds include several naturally occurring, bacterially derived LPS antagonists such as deacylated LPS, lipid IVα, and Rhodobacter sphaeroides lipid A (37–39) as well as several synthetic lipid A-like
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structures (40–42) that do not exist in nature. One advantage of the synthetic compounds is that they are less likely to be contaminated with minor, but potent, bacterial products that may mislead the investigator, as has been an occasional confounding problem with LPS (35, 43).

In an attempt to dissect further the LPS-signaling pathway, we utilized a newly developed lipid A agonist, compound ER-112022. This compound is a symmetric phospholipid dimer connected by a noncarbohydrate backbone. We observed that under serum-free conditions, ER-112022 was able to stimulate cells lacking membrane CD14. Furthermore, ER-112022 appeared to mediate stimulatory effects through the TLR4 and MD-2 pathway. We suggest that ER-112022 interacts directly with the LPS-signaling receptor, obviating the need for CD14.

**EXPERIMENTAL PROCEDURES**

Reagents—PBS, trypsin-EDTA, DMEM (high glucose), and Ham's F12 medium were obtained from BioWhittaker (Walkersville, MD); Ex-Cell 301 serum-free medium was from JRH Biosciences (Lenexa, KS); and Hank's balanced salt solution, glutamine, and RPMI 1640 were from Life Technologies, Inc. Fetal calf serum (FCS) was from Summit Biotechnologies (Greeley, CO). Human serum was collected in Vacutainer SST gel and clot activator tubes (Becton Dickinson, San Jose, CA), separated from cellular elements by centrifugation at 4 °C, and stored at −70 °C. Ciprofloxacin was a gift from Miles; penicillin/streptomycin was obtained from Sigma; hygromycin B was from Calbiochem; and geneticin (G418) from Life Technologies, Inc. LPS (Re595 LPS from Salmonella minnesota) was a gift from Drs. N. Qureshi and K. Takayama (University of Wisconsin, Madison, WI); alternatively, smooth LPS from S. minnesota or E. coli (from Sigma, Zymed Laboratories, Cambridge, MA) was used. Murine recombinant tumor necrosis factor-α (TNF) and human interleukin (IL)-1β were from Genzyme (Cambridge, MA). Phorbol myristate acetate (PMA) was a gift from Sigma. Recombinant human sCD14 and LBP were gifts from H. Lichtenstein (Amgen, Thousand Oaks, CA); alternatively, sCD14 was purified by affinity chromatography as described (12). The 47L synthetic lipopeptide was based on the N-terminal structure of the 47-kDa major membrane lipoprotein (1). Tumor necrosis factor-α (TNF) was a gift from T. Sellati and J. D. Radolf, University of Connecticut. Antibodies (FITC anti-CD25/Tac and isotype controls) for flow cytometry (FACS) experiments were purchased from Becton Dickinson. Antibodies for inhibition experiments were purified from hybridoma supernatant on Sepharose goat anti-mouse IgG as described by the manufacturer (Zymed Laboratories Inc., San Francisco, CA) and were stored as frozen stock solutions in sterile PBS.

Synthetic lipid A-like compounds used in this study, ER-112022 (53), and the LPS antagonists E5564 (also known as B1287) (41, 42) and E5531 (40) were synthesized at the Eisai Research Institute (Andover, MA). All lipids (including LPS) were stored as frozen stock suspensions in PBS at 1 mg/ml. Lipids were thawed and sonicated 1–3 min before use. The activity of compound ER-112022 in the Limulus amebocyte lysate assay (Chromogenix, Molndal, Sweden) was less than 0.3 pg/μg.

**Cells and Cell Culture Conditions**—The Chinese hamster ovary (CHO)-K1 fibroblast cell line, the mouse macrophage cell line RAW264.7, and the human astrocytoma U373 cell line were obtained from the American Type Culture Collection (Manassas, VA). CHO cells (CHO-K1 fibroblast cell line, the mouse macrophage cell line RAW264.7, and the human astrocytoma U373 cell line were obtained from the American Type Culture Collection (Manassas, VA). CHO cells were grown in medium containing 0.5 mg of hygromycin B/ml. Lipids were thawed and sonicated 1–3 min before use. The activity of compound ER-112022 in the Limulus amebocyte lysate assay (Chromogenix, Molndal, Sweden) was less than 0.3 pg/μg.

**Cells and Cell Culture Conditions**—The Chinese hamster ovary (CHO)-K1 fibroblast cell line, the mouse macrophage cell line RAW264.7, and the human astrocytoma U373 cell line were obtained from the American Type Culture Collection (Manassas, VA). CHO cells were grown in Ham's F12 medium containing 10% fetal bovine serum and ciprofloxacin (10 μg/ml), whereas RAW and U373 were maintained in DMEM containing 10% FCS, L-glutamine, and penicillin/streptomycin (Life Technologies, Inc.). The stably transfected cell lines CHO/CD14 and CHO/Neo (44) were grown in medium containing 0.5 mg of G418/ml (Life Technologies, Inc.). The reporter cell lines CHO/CD14/ElamTac (clone 3E10) (45) and CHO/ElamTac were cultured in complete medium supplemented with 400 units of hygromycin B/ml (Calbiochem).

The NF-κB reporter cell line CHO/ELAM.Tac was engineered by stable co-transfection of the plasmid pCEP4 (Invitrogen, Carlsbad, CA) and the NF-κB reporter plasmid pELAM.Tac (45), using calcium phosphate precipitation as described previously (21). A clonal reporter cell line was isolated from nonclonal “bulk” transfected cells that survived hygromycin selection by stimulating these cells overnight with ER-112022 (300 ng/ml). Cells were subsequently positively selected for a functional reporter transgene by cell sorting for the induced surface expression of Tac (CD25) expression (Epics Elite cell sorter, Coulter, Hialeah, FL) by staining the stimulated transfectedants with anti-Tac monoclonal antibody (Becton Dickinson). After expansion of the enriched cells, the sort was repeated. Finally, to minimize the background expression of the transgene, unstimulated cells were sorted once for low CD25 expression. A clonal cell line (designated clone EL1) was obtained by limiting dilution.

The day before each stimulation experiment, CHO cells were washed once with PBS, harvested using trypsin-EDTA, and plated in 6-well dishes (Costar) at densities of 0.5 × 10^6 cells/dish (for preparation of nuclear extracts) or 1 × 10^6 cells/dish (for flow cytomtery analysis) in 1 ml of fresh culture medium. Immediately prior to stimulation, cells were washed once with PBS, and the indicated medium was added. When analyzing the effect of the presence of serum or serum components, cells were stimulated in Ex-Cell serum-free medium with or without additives (human serum, sCD14, and LBP).

U373 cells were seeded in 96-well dishes at a density of 2 × 10^4 cells/well. The following day, the cells were washed three times with Hank's balanced salt solution (Life Technologies, Inc.), and the indicated medium was added. Cells were stimulated for 18–22 h, and supernatants were analyzed for IL-6 content by ELISA (Endogen, Woburn, MA).

RAW264.7 cells were plated at a density of 2 × 10^5 cells/well in 96-well plates. The following day, the cells were washed three times with serum-free DMEM. The day before the experiment, the cells were plated at a density of 3 × 10^5 cells/well in 24-well dishes. Cells were transfected by the calcium phosphate method (CalPhos, CLONTECH, Palo Alto, CA) with the following plasmids: TLR4 in the vector pcDNA3 (a gift from R. Medzhitov and C. Janeway, Yale University), MD-2 in the vector pEF-BOS (13), or the empty vector pcDNA3 (Invitrogen, Carlsbad, CA). The total amount of plasmid was kept at a constant 90 ng by the addition of empty vector. In addition, all cells were co-transfected with 50 ng of the NF-κB-dependent luciferase reporter plasmid pCMV-βgal (Promega, Madison, WI). After 18 h in DMEM, 1% FCS, the cells were washed and exposed to medium alone, sCD14, E. coli LPS, or ER-112022 plus sCD14 for 6 h. Cells were harvested in lysis buffer and analyzed for luciferase activity using reagents from Promega (Dual Luciferase Reporter System), by a Wallach 1450 MicroBetaTrilux counter. Firefly luciferase activity in samples was normalized for transfection efficiency by specific Renilla luciferase activity per the manufacturer's instructions.

Peritoneal Macrophages—Peritoneal macrophages from C3H/HeN and C3H/HeJ mice (Harlan Ltd., Oxon, UK) were collected by lavage 4 days after intraperitoneal injection of 3% thioglycollate (3 ml, Difco). Cells (5 × 10^6 cells) in 24-well dishes were adhered for 2 h, and nonadherent cells were removed by washing three times with PBS. Macrophages were stimulated in RPMI, 10% FCS for 8 h in a total volume of 0.25 ml. Cell-free supernatants were analyzed for TNF by the WEHI 164 clone 13 bioassay (47).

Flow Cytometry Analysis (FACS) of NF-κB Activation in CHO Reporter Cell Lines—Cells were stimulated for 18–22 h as indicated, washed once with PBS, and harvested with trypsin-EDTA (TLR2, TLR4, CD25, and CD14 are all resistant to the effects of trypsin [data not shown]). All subsequent steps were performed on melting ice. A cell pellet was resuspended in PBS, 1% FCS, and the cells were labeled with a 1:30 dilution of the FITC-labeled anti-CD25 or isotype control antibodies (total labeling volume of 30 μl) for 30 min. Then, the cells were washed once with 2 ml of PBS/FCS and resuspended in 0.3 ml of PBS/FCS containing 1 μg/ml propidium iodine (Sigma) to identify dead cells. The cells were analyzed using a FACSscan microfluorimeter (Becton Dickinson) as described previously (48).

Preparation of Nuclear Extracts and NF-κB Electrophoretic Mobility Shift Assay (EMSA)—Preparation of nuclear extracts and NF-κB EMSA were performed as described earlier (41). The binding pattern of

2 B. Naume and T. Espevik, unpublished results.
nuclear proteins to the 32P-labeled NF-κB consensus oligonucleotide was visualized by separation on a 4% nondenaturating polyacrylamide (Protogel, National Diagnostics, Atlanta, GA) gel, and dried gels were exposed to Biomax MS x-ray film (Eastman Kodak Co.). In some experiments, a 100-fold excess of unlabeled probe was used for specific blocking of NF-κB binding to the radiolabeled oligonucleotide.

RESULTS

Structure of Compound ER-112022—Several recent advances in the chemical synthesis of compounds are notable because they provide tools for characterizing the LPS signal transduction system. Both E5531 (40) and E5564 (41, 42) (Fig. 1) are potent LPS antagonists that are under development as therapeutic compounds for the treatment of sepsis. In contrast, compound ER-112022 (Fig. 1) has no LPS antagonistic activity but rather is an LPS mimetic. ER-112022 is a phospholipid dimer connected via an acyclic backbone. Each monomeric unit contains three unique fat groups that are bound indirectly to a phosphate diester. The fat groups include a 10-carbon ether chain, an unsaturated 12-carbon, acyloxy chain bound to the ether chain, and a 14-carbon, β-oxo-amide chain linked closer to the phosphodiester. Thus, the compound has three unique features compared with naturally occurring lipid A from E. coli (Fig. 1). First, ER-112022 is devoid of a cyclic carbohydrate backbone. Second, the phosphates are phosphodiesters incorporated within the confines of the structural backbone, unlike the phosphoesters on E. coli lipid A, and finally, the structure is symmetrical (Fig. 1).

ER-112022 Stimulates CHO Cells Bypassing CD14 in a Serum-independent Fashion—We added ER-112022 to CHO fibroblasts transfected with human CD14 (CHO/CD14) and measured nuclear translocation of NF-κB by EMSA. ER-112022, LPS, and TNF stimulated the nuclear translocation of NF-κB in CHO/CD14, both in the absence and presence of 1% human serum (Fig. 2). This LBP-independent response is presumably due to the extremely high levels of CD14 expression in CHO/CD14 (to observe serum or LBP-dependent LPS stimulation of CHO/CD14, it is necessary to test these cells with pg/ml concentrations of LPS, data not shown). Unlike many cells that do not express membrane CD14, mock-transfected CHO/Neo (or wild-type CHO-K1) cells are not enabled to respond to LPS by the presence of soluble CD14 (Fig. 2). The basis of this resistance to LPS stimulation is not known but is

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**Fig. 1. Structure of compounds ER-112022, E5564, E5531, and E. coli lipid A.** The structure of the LPS agonist ER-112022 (empirical formula of the disodium salt: C₈₈H₁₆₂N₄Na₂O₂₀P₂, Mr 1704.19) is compared with the E. coli lipid A and the LPS antagonists E5564 and E5531. Note that ER-112022 does not contain the diglucosamine backbone found in lipid A, E5564, and E5531.
Soluble CD14, but Not LBP, Enhances the Activity of ER-112022—To identify the serum proteins that enhanced the stimulatory effects of ER-112022 on CHO cells, CHO/Neo and CHO/CD14 cells were plated at a density of $5 \times 10^5$/well in 6-well dishes. The following day, the cells were washed three times with PBS and stimulated with ER-112022, TNF, or ReLPS in Ex-Cell serum-free medium in the absence or presence of 1% human serum. After 1 h, the induced nuclear translocation of NF-κB was assessed by EMSA as described under “Experimental Procedures.” 0 means unstimulated cells. No extr. represents $^{32}$P-labeled NF-κB probe analyzed by EMSA in the absence of nuclear extracts. CTR refers to extracts from cells stimulated with 100 ng/ml ER-112022 that were analyzed in the presence of a 100-fold excess of unlabeled probe. All concentrations given are in nanograms of stimulus per ml. Shown is one experiment out of four.

Presumed to be due to the failure of LPS to interact with TLRs expressed on the surface of CHO cells. Surprisingly, ER-112022 was able to stimulate control CHO/Neo cells under serum-free conditions (Fig. 2), although the cells remained refractory to the effects of LPS under identical conditions. Extensive washing of the cells did not alter the CD14-independent effect of ER-112022 on NF-κB translocation in CHO/Neo cells. However, when the CHO/Neo cells were activated in the presence of 1% human serum, which contains both sCD14 and LBP, the potency of ER-112022 was increased (Fig. 2). Thus, the effect of ER-112022 is markedly different from LPS, which fails to activate CHO cells unless they are engineered to express LPS-binding proteins, such as membrane-anchored CD14 (44), CD11/CD18 (5, 49), or GPI chimeras of LBP or BPI (50), even in the presence of soluble LBP and sCD14.

Soluble CD14, but Not LBP, Enhances the Activity of ER-112022—To identify the serum proteins that enhanced the stimulatory effects of ER-112022 on CHO cells, CHO/Elam.Tac NF-κB reporter cells were exposed to ER-112022 in the absence or presence of recombinant sCD14 and recombinant LBP. The CHO reporter cell line responded to ER-112022, TNF, and IL-1β under serum-free conditions by up-regulating membrane CD25 (Fig. 3A). Although CD14 was not necessary for a robust response to ER-112022, the presence of sCD14 enhanced ER-112022-induced NF-κB activation (Fig. 3B), whereas recombinant LBP had no significant effect (data not shown). No synergistic effects were observed when both sCD14 and LBP were added together with ER-112022 (not shown). Similar results were obtained when NF-κB nuclear translocation in CHO-K1 and CHO/Neo was evaluated by EMSA (not shown). The reporter cell line was hyporesponsive to LPS both in the absence or in the presence of serum components (not shown), as previously reported (21).

We further analyzed the effect of serum and sCD14 on the cellular activation induced by ER-112022 in other cell lines, such as U373 astrocytoma cells and RAW264.7 monocytes/macrophages. Both of these lines differ from CHO cells in their requirements for serum proteins to respond to LPS; for example, the CD14-negative U373 cell line is enabled to respond to LPS in the presence of sCD14 (16), and the RAW cell line expresses GPI-linked CD14. U373 cells responded to LPS to release IL-6 only in the presence of serum or sCD14 (Fig. 4A). In contrast to LPS, ER-112022 induced IL-6 release from U373 cells under serum-free conditions, and this effect was enhanced by the presence of serum or sCD14 (Fig. 4B). Hence, despite their different responses to LPS in the absence of serum access proteins, both CHO cells and U373 cells responded to ER-112022 in the absence of serum, and the response was enhanced by serum or sCD14. Similar results were obtained with the endothelial cell line HMEC-1 (data not shown). RAW cells had a different pattern of response. These cells responded to LPS in the absence of serum, but serum enhanced the TNF release induced by LPS (Fig. 4C). However, the ability of ER-112022 to activate these monocyte/macrophage cells was not affected by serum (Fig. 4D). Hence, serum/sCD14 may have different effects on ER-112022-mediated activation of nonmyeloid and myeloid cells. Our results indicate that in serum-free medium, low doses of ER-112022 activate cells such as CHO-K1 and U373 that do not normally respond to LPS under these conditions. When serum is added to U373 cells, the potency of LPS and ER-112022 is comparable. Finally, upon stimulation of RAW and CHO/CD14 cells in the presence of serum, ER-112022 is considerably less potent than LPS on a mass basis in inducing cellular activation.

The LPS Antagonists E5564 and E5531 Inhibit ER-112022 Activation of Cells—To determine whether LPS and ER-112022 interact with a common recognition element in cells, a pharmacological strategy was applied, using the synthetic LPS antagonists E5564 (41) and E5531 (40) (Fig. 1). This type of drug is thought to block interactions between LPS and TLR4 (12,

**Fig. 2**. ER-112022 activates CHO cells in the absence of CD14 expression. CHO/Neo and CHO/CD14 cells were plated at a density of $5 \times 10^5$/well in 6-well dishes. The following day, cells were washed three times with PBS and stimulated with ER-112022, TNF, or ReLPS in Ex-Cell serum-free medium in the absence or presence of 1% human serum. After 1 h, the induced nuclear translocation of NF-κB was assessed by EMSA as described under “Experimental Procedures.” 0 means unstimulated cells. No extr. represents $^{32}$P-labeled NF-κB probe analyzed by EMSA in the absence of nuclear extracts. CTR refers to extracts from cells stimulated with 100 ng/ml ER-112022 that were analyzed in the presence of a 100-fold excess of unlabeled probe. All concentrations given are in nanograms of stimulus per ml. Shown is one experiment out of four.

**Fig. 3**. Soluble CD14 enhances the ER-112022 stimulatory effect toward CHO reporter cells, whereas LBP does not. CHO/Elam.Tac cells express CD25 as a consequence of NF-κB activation (Fig. 3A). Hence, despite their different responses to LPS in the absence of serum access proteins, both CHO cells and U373 cells responded to ER-112022 in the absence of serum, and the response was enhanced by serum or sCD14. Similar results were obtained with the endothelial cell line HMEC-1 (data not shown). RAW cells had a different pattern of response. These cells responded to LPS in the absence of serum, but serum enhanced the TNF release induced by LPS (Fig. 4C). However, the ability of ER-112022 to activate these monocyte/macrophage cells was not affected by serum (Fig. 4D). Hence, serum/sCD14 may have different effects on ER-112022-mediated activation of nonmyeloid and myeloid cells. Our results indicate that in serum-free medium, low doses of ER-112022 activate cells such as CHO-K1 and U373 that do not normally respond to LPS under these conditions. When serum is added to U373 cells, the potency of LPS and ER-112022 is comparable. Finally, upon stimulation of RAW and CHO/CD14 cells in the presence of serum, ER-112022 is considerably less potent than LPS on a mass basis in inducing cellular activation.
Acyclic LPS Agonist Activates Cells via TLR4

ER-112022 Activates HEK293 Cells That Have Been Co-transfected with TLR4 Plus MD-2—To test directly if the LPS-mimetic effects of ER-112022 were due to its interaction with the LPS receptor, we transiently transfected TLR4 and MD-2 into LPS nonresponder HEK293 cells. These cells do not express either TLR4 or MD-2 as assessed by reverse transcriptase-polymerase chain reaction (data not shown and Ref. (14)). Transfectants were subsequently stimulated with ER-112022 or LPS. Neither compound activated HEK293 cells in the absence of gene transfection or activated cells transfected with MD-2 alone (Fig. 6). In contrast, both ER-112022 and LPS activated the NF-κB reporter in cells that co-expressed TLR4 and MD-2 (Fig. 6).

ER-112022 Activates Primary Monocytic Cells through TLR4—To investigate the role of TLRs in ER-112022 activation of monocytes, we exposed freshly isolated adherent human monocytes to ER-112022, in the presence or absence of antibodies to TLR2 or TLR4, and we measured the release of TNF. Treatment with ER-112022 induced release of TNF, although the efficacy was substantially less than that of LPS (Fig. 7A). As one might have predicted from the cellular transfection data, the TLR4 antibody HTA125 completely blocked the TNF-inducing ability of ER-112022 and most of the cellular activation induced by LPS (Fig. 7A). In contrast, the TLR2 antibody TL2.1 did not have any effect on the ER-112022 activation of primary monocytes, although TL2.1 inhibited ~70% of the TNF release mediated by the synthetic lipohexapeptide 47L, which we have previously demonstrated to be a ligand for TLR2 (21). The activation induced by 47L was not affected by the TLR4 antibody. Thus, ER-112022 and 47L appear to mediate TNF-inducing ability in monocytes through two different receptors. By using synthetic chemistry, it may be possible to determine precisely the molecular structures necessary to activate either TLR2 or TLR4.

C3H/HeJ mice are hyporesponsive to LPS due to a dominant negative mutation in tlr4 gene that results in a proline to histidine substitution at position 712 (11, 18, 19). We exposed peritoneal macrophages from C3H/HeJ and the LPS-responsive C3H/HeN mice to ER-112022 and LPS. Both of these compounds induced TNF release from the HeN macrophages; however, the HeJ macrophages did not respond to LPS or ER-112022 (Fig. 7B). The lipopeptide 47L activated macrophages from the two mouse strains equally (data not shown).

**DISCUSSION**

The signaling events leading to the activation of cells by LPS are not fully understood, although recent reports strongly implicate TLR4 and MD-2 as CD14-associated signaling components of an LPS receptor complex. CD14 is the best characterized portion of the LPS receptor, having been shown to actually bind LPS. However, multiple lines of evidence suggest that the GPI-anchored CD14 is not an actual signal transducer and that it does not account for the specificity of biological responses to endotoxins. When dissecting the mechanisms of signaling in the LPS receptor system, the use of both agonists as well as antagonists may help increase the understanding of the com-

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**FIG. 4.** ER-112022 induces cytokine release from U373 and RAW264.7 cells in a serum-independent but serum-enhancing manner. A and B, U373 cells were plated at a density of 2 × 10⁴ cells/well in 96-well dishes. The following day, cells were washed three times with Hanks’ balanced salt solution and stimulated with E. coli LPS in serum-free DMEM with (open circles) or without (closed circles) 1% human serum or sCD14 (150 ng/ml) (A). Cells were stimulated with increasing amounts of ER-112022 in serum-free DMEM (closed circles) or DMEM containing 1% human serum (triangles) or sCD14 (open circles, 150 ng/ml) (B). After 16–18 h of incubation, supernatant was harvested and assessed for IL-6 concentration by ELISA. Shown are the mean of triplicate determinations ± S.D.
plex interactions between ligands and receptor molecules. Whereas neither pharmacological nor molecular genetic approaches can replace biophysical and biochemical analysis of receptor function, both approaches can add substantially to our understanding of receptor mechanisms.

In this study, we describe ER-112022, a unique new acyclic and symmetrical LPS mimetic. ER-112022 activates cells through the LPS-signaling pathway via TLR4 and MD-2 but does not require membrane or soluble CD14. This observation suggests that the role of CD14 in LPS signaling may be limited to the simple transfer of LPS to TLRs and may not involve the formation of a ternary complex involving TLR4/MD-2 and CD14. The symmetrical organization of ER-112022 further suggests that compounds of the correct size, shape, and hydrophobicity may cross-link transmembrane signaling molecules, perhaps resulting in the formation of a TLR4 homodimer, and thus the initiation of a proinflammatory signal.

A great deal of effort has been focused on the role of LPS receptor cofactors present as soluble molecules in blood that augment the potency of LPS in vitro and to a lesser (but important) extent in vivo (51, 52). The data presented in this work suggest a series of events that begin with LPS binding to serum LPS-enhancing proteins that alter the conformation of the LPS molecule itself. Transfer of LPS from soluble mediators like LBP and sCD14 to membrane CD14, as well as transfer of LPS from membrane CD14 to TLR4, presumably occurs because of slight alterations in LPS conformation that facilitate Toll receptor binding. ER-112022 seems to have a structural conformation that enables it to bypass all of these LPS-facilitating proteins in binding to TLRs and thus to interact directly with the LPS signal transducer. The structural basis of this remarkable physical property, when elucidated, should shed light on the nature of the microenvironment of the putative LPS-binding pocket on TLR4.

The essential components necessary for activation of the LPS-signaling pathway are present in both nonmyeloid and myeloid cells, and the data to date suggest that in vitro work involving transfection of nonmyeloid cells with genes encoding
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LPS receptors can be highly informative about the nature of phagocyte LPS receptors. The unique structure of the novel acyclic LPS-like agonist ER-112022 allows the compound to interact directly with this pathway in a CD14-independent manner. ER-112022 and derivatives may directly bind to the TLR4-MD-2 complex. Because of its defined synthetic basis, as well as its independence from CD14 and LBP, ER-112022 should be useful for simplifying both the binding and co-crystallization studies with TLR4 that will be necessary for identifying the region or regions of TLR4 that directly contact endotoxin. Hence, these studies may help to design specific drugs for endotoxin-related diseases, including Gram-negative bacterial septic shock.

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