CD14-mediated translocation of nuclear factor-κB induced by lipopolysaccharide does not require tyrosine kinase activity

Article *in* Journal of Biological Chemistry · October 1994

Source: PubMed

CITATIONS
111

READS
39

7 authors, including:

Russ Delude
Lake Sumter Community College
119 PUBLICATIONS **5,002** CITATIONS
SEE PROFILE

Stefanie N Vogel
University of Maryland, Baltimore
433 PUBLICATIONS **27,637** CITATIONS
SEE PROFILE

Douglas Golenbock
University of Massachusetts Medical School
359 PUBLICATIONS **50,123** CITATIONS
SEE PROFILE

THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 269, No. 35, Issue of September 2, pp. 22253–22260, 1994
Printed in U.S.A.

CD14-mediated Translocation of Nuclear Factor-κB Induced by Lipopolysaccharide Does Not Require Tyrosine Kinase Activity*

(Received for publication, March 22, 1994, and in revised form, June 21, 1994)

Russell L. Delude‡§¶‖, Matthew J. Fenton¶, Ricardo Savedra, Jr.‡, Pin-Yu Perera, Stefanie N. Vogel**, Rolf Thieringer‡‡, and Douglas T. Golenbock‡¶¶**

*From the ‡Maxwell Finland Laboratories for Infectious Diseases, Boston City Hospital, the ¶Research Immunology Section and the §Department of Medicine, Boston University School of Medicine, Boston, Massachusetts 02118, the **Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814, and the ‡‡Department of Biochemistry, Merck Research Laboratories, Rahway, New Jersey 07065*

During the course of serious bacterial infections, lipopolysaccharide (LPS) is believed to interact with macrophage receptors, resulting in the generation of inflammatory mediators and systemic symptoms including hemodynamic instability and shock. CD14, a glycosylphosphatidylinositol-linked antigen, functions as an LPS signaling receptor. A critical issue concerns the mechanism by which CD14, which has no transmembrane domain, transduces its signal following LPS binding. Recently, investigators have hypothesized that CD14-mediated signaling is effected through a receptor-associated tyrosine kinase (TK), suggesting a multicomponent receptor model of LPS signaling.

Wild-type Chinese hamster ovary (CHO)-K1 cells can be activated by endotoxin to release arachidonate following transfection with human CD14 (CHO/CD14). Nuclear translocation of cytosolic NF-κB is correlated with a number of LPS-inducible responses. We sought to determine if this pathway were present in CHO/CD14 cells and to elucidate the relationship of NF-κB activation to the CD14 receptor system. LPS-stimulated translocation of NF-κB in CHO/CD14 cells resembled the same response in the murine macrophage-like cell line RAW 264.7. Protein synthesis inhibitors and corticosteroids, which suppress arachidonate release and the synthesis of proinflammatory cytokines, had no effect on translocation of NF-κB in CHO/CD14 or RAW 264.7 cells, demonstrating that NF-κB translocation is an early event. Although TK activity was consistently observed by immunoblotting extracts from activated RAW 264.7 cells, LPS-induced phosphotyrosine residues were not observed from similarly treated CHO/CD14 cells. Furthermore, the TK inhibitors herbimycin A and genistein failed to inhibit translocation of NF-κB in CHO/CD14 or RAW 264.7 cells, although both of these agents inhibited LPS-induced TK activity in RAW 264.7 cells. These results imply that TK activity is not obligatory for CD14-mediated signal transduction to occur in response to LPS.

Gram-negative bacterial sepsis is believed to be initiated by interactions between lipopolysaccharide (LPS,[1] endotoxin), a bacterial membrane constituent, and specific receptors on mononuclear phagocytes, resulting in the release of cytokines and other inflammatory mediators that are responsible for the hemodynamic instability and shock that are characteristic of the syndrome. CD14, the only molecule that has been shown definitively to be involved in LPS-initiated cellular activation, is a 53-kDa glycosylphosphatidylinositol-linked antigen present on macrophages and neutrophils (1). Serum proteins (2–4), such as LPS-binding protein (LBP), act to facilitate binding of LPS to CD14. Both 53- and 48-kDa forms of CD14 are present as soluble LPS receptors in human serum and have been shown to enable LPS responsiveness in certain cell types *in vitro* (5–12). A central question in endotoxin research is the mechanism by which CD14 induces a signal following ligand binding in the absence of a transmembrane domain.

Kitchens *et al.* (11) demonstrated that LPS receptor antagonists, such as lipid IVa, could effectively block signaling by human monocytes in response to LPS when used at nanomolar concentrations. Yet, when LPS binding to the cells was assessed under identical conditions, micromolar concentrations of antagonist were required to displace LPS from CD14. The observation that a significant quantity of LPS remained associated with membrane-bound CD14, in the presence of inhibitory concentrations of antagonist, suggested that these agents function at a step distal to LPS binding to CD14. The authors hypothesized that a membrane-associated receptor distinct from CD14 is present in limiting quantities on the cell surface. This putative receptor might function as a signal transducer following LPS binding to CD14. Alternatively, the role of CD14 may be to act as a transferase, delivering LPS to a downstream molecule which then effects the signal across the plasma membrane.

Several reports have described endotoxin-induced TK activity in mononuclear phagocytes (13–15) and CD14-transfected 70Z/3 pre-B cells (16). LPS stimulation resulted in prominent phosphorylation of tyrosine residues in various proteins with molecular masses ranging from 38 to 48 kDa concomitant with dephosphorylation of several protein species (17). Furthermore, Stefanova *et al.* (18) demonstrated that the nonreceptor protein tyrosine kinases $p56^{lyn}$, $p58^{hck}$, and $p59^{c\text{-}fgr}$ were transiently activated in human monocytes following exposure of the cells to

* This work was supported in part by National Institutes of Health Grants GM47127 and AI33087 (to D. T. G.), AI18797 (to S. N. V.), and AI29088 (to M. J. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‖ Evans Foundation postdoctoral fellow.

¶¶ Recipient of an American Cancer Society Junior Faculty Award. To whom correspondence should be addressed: Maxwell Finland Laboratory for Infectious Diseases, 774 Albany St., Boston MA, 02118. Tel.: 617-534-7965; Fax: 617-534-5280.

[1] The abbreviations used are: LPS, lipopolysaccharide; LBP, lipopolysaccharide-binding protein; TK, tyrosine kinase; [^{3}H]20:4, [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid; CHO-K1, Chinese hamster ovary fibroblast-K1; NF-κB, nuclear factor-κB; EMSA, electrophoretic mobility shift assay; rsCD14, recombinant soluble CD14; PBS, phosphate-buffered saline; mAb, monoclonal antibody; CNTF, ciliary neurotrophic factor; gp, glycoprotein.

endotoxin. Pharmacologic studies with TK inhibitors supported the concept that TK activity is essential for the earliest LPS-inducible events. For example, treatment of LPS-responsive cells with the TK antagonist herbimycin A blocked induction of phosphotyrosine residues in cellular lysates (16) and inhibited induction of $p56^{lyn}$, $p58^{hck}$, and $p59^{c\text{-}fgr}$ activity (18). Herbimycin A also blocked physiologic responses of mononuclear phagocytes following exposure to LPS, including the release of [^{3}H]arachidonic acid ([^{3}H]20:4 (13)) and the production of cytokines (18). Collectively, the observations outlined above suggested that TK activity is essential, at least in part, for the LPS-induced signal transduction. One model of signaling proposes that CD14 comprises a portion of an LPS-specific signaling complex which includes a transmembrane signal transducer with TK activity.

Wild-type hamster CHO-K1 cells are not responsive to LPS. The construction of a CHO cell line, CHO/CD14, which expresses membrane-bound human CD14, has been reported previously (9). Stimulation of CHO/CD14 cells with as little as 1 ng of LPS/ml resulted in the release of [^{3}H]20:4 into the culture supernatant, whereas wild-type CHO-K1 or a mock-transfected CHO cell line (designated CHO/NEO) failed to release [^{3}H]20:4 at concentrations as high as 100 μg of LPS/ml. This CD14-mediated response closely resembled [^{3}H]20:4 release from the murine macrophage-like cell line, RAW 264.7, which expresses CD14. Another macrophage response to LPS-stimulation is the translocation of the transcription factor NF-κB into the nucleus (19). NF-κB activation appears to be a sentinel event in LPS-stimulated macrophages, heralding the activation of proinflammatory cytokines (20–22) as well as the production of human immunodeficiency virus, type 1 virions in latently infected mononuclear phagocytes (19). We used the electrophoretic mobility shift assay (EMSA) to examine LPS-induced NF-κB activation in CHO/CD14 cells in response to LPS, to determine if this response resembles LPS-induced NF-κB activation in murine macrophages. Expression of CD14 in CHO-K1 cells imparted LPS-inducible NF-κB translocation. Both wild-type CHO-K1 or CHO/NEO cells failed to respond to LPS, confirming our previous observation that we were able to isolate a CD14-specific pathway of LPS activation in transfected CHO cells. Recombinant soluble CD14 (rsCD14), when added simultaneously with LPS to wild-type CHO cells, failed to enable responses to LPS. Surprisingly, LPS-inducible TK activity was not apparent in CHO/CD14 cells following exposure to LPS, and pharmacologic inhibitors of TK failed to prevent LPS-induced activation of NF-κB in either CHO/CD14 or RAW 264.7 cells. When taken together, these data suggest that TK activity is not required for CD14-dependent LPS-induced NF-κB translocation.

MATERIALS AND METHODS

Reagents—Unless otherwise indicated, reagents were obtained from Sigma. Ham's F-12 medium with L-glutamine, and PBS were purchased from M.A. Bioproducts (Walkersville, MD). Macrophage serum-free medium was purchased from Life Technologies, Inc. Fetal bovine serum (LPS < 0.02 ng/ml) was from HyClone Laboratories (Logan, UT). Pyrogen-free water was obtained from Baxter Healthcare Corp. (Deerfield, IL). Erbstatin analog, herbimycin A, and genistein were obtained from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA).

ReLPS was a gift from Dr. Nilofor Qureshi (Middleton Veterans Administration Hospital, Madison, WI) and was extracted with phenol from *Salmonella minnesota* R595. LPS solutions were stored in baked glass tubes frozen at −20 °C and were sonicated in an 80-watt sonicator bath (model G112SP1G; Lab Supply Co., Hicksville, NY) for 2 min immediately prior to use. rsCD14 was provided by Dr. Henri Lichenstein (Amgen, Inc., Thousand Oaks, CA). MY4 (anti-human CD14) mAb was obtained from Coulter (Hialeah, FL). Anti-phosphotyrosine mAb was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-human p65 (1226) mAb was a gift from Dr. Nancy Rice (NCI, FCRF, Frederick, MD). Poly(dI·dC) was purchased from Pharmacia Biotech. Inc. Prestained protein molecular mass markers were obtained from Bio-Rad.

Cell Culture and Cell Stimulation Conditions—CHO-K1 (ATCC CCL61) and RAW 264.7 (ATCC TIB71) cells were obtained from the American Type Culture Collection. CHO/CD14 and CHO/NEO cell lines were transfected with plasmids pcDNA I/CD14 and pKONEO, or pKONEO alone, respectively, as described in (9). All cell lines were cultured in Ham's F-12 medium containing 10% fetal bovine serum and 10 μg of ciprofloxacin/ml in a 5% CO_2 atmosphere at 37 °C. The day before each experiment, 2×10^6 cells were plated/well in a six-well tissue culture dish (Costar, Cambridge, MA) in fresh medium. At the time of stimulation, the culture medium was aspirated and replaced with 800 μl of Ham's F-12 medium containing 2% heat-inactivated human serum, 10 μg of ciprofloxacin/ml, and the appropriate stimulant or vehicle. Culture dishes were then returned to a 37 °C 5% CO_2 environment for 0–240 min and then placed on ice just prior to harvesting.

Nuclear Extracts—The procedure used is a modification of previously published procedures by Dignam *et al.* (23) and Schreiber *et al.* (24). All steps were performed on ice or at 4 °C. Cells were harvested mechanically using a rubber policeman, then transferred to a 1.5-ml tube, and pelleted in a microcentrifuge (Beckman Microfuge 11) at 5,400 × *g* (speed 6) for 10 s at 4 °C. The cells were washed with 1 ml of PBS containing 2% fetal bovine serum and then pelleted by centrifugation as above. The cell pellet was resuspended in 400 μl of buffer I (10 mM Tris·HCl, pH 7.8, 5 mM $MgCl_2$, 10 mM KCl, 0.3 mM EGTA, 0.5 mM dithiothreitol, 0.3 M sucrose, 1 mM phenylmethylsulfonyl fluoride, 10 mM β-glycerol phosphate, and 1.0 μg/ml each of the following protease inhibitors: aprotinin, antipain, leupeptin, chymostatin, and pepstatin A). The tubes were placed on ice for 15 min to allow the cells to swell and facilitate lysis. Nonidet P-40 was added to 0.5% (25 μl of a 10% (v/v) stock), and the tubes were vortexed at full speed for 10 s. Nuclei were harvested by centrifugation at 7,200 × *g* (speed 8) for 10 s at 4 °C. The supernatant was aspirated, and the nuclei were resuspended in 100 μl of buffer II (20 mM Tris·HCl, pH 7.8, 5 mM $MgCl_2$, 320 mM KCl, 0.2 mM EGTA, 0.5 mM dithiothreitol, and the mixture of protease inhibitors described above). Nuclear proteins were extracted for 15 min on ice followed by centrifugation at 13,500 × *g* (full speed) for 15 min. The protein concentration of the nuclear extracts was determined using the Bio-Rad protein concentration reagent per the manufacturer's instructions. Nuclear extracts were stored at −80 °C until analyzed by EMSA.

EMSA—Two synthetic oligonucleotides (Oligos Etc., Guilford, CT) containing the NF-κB binding sequence from the murine immunoglobulin κ light chain gene enhancer:

```
5′ AGCTCAGAGGGGACTTTCCGAGAG     3′
3′     GTCTCCCCTGAAAGGCTCTCTCGA 5′
```

SEQUENCE 1

were annealed in buffer (10 mM Tris·HCl, pH 7.9, 50 mM NaCl, 10 mM $MgCl_2$ and 1 mM dithiothreitol). Fifty ng of this material was end labeled using Klenow fragment (New England Biolabs, Beverly, MA) in the presence of 100 μCi each of [α-^{32}P]dATP and [α-^{32}P]dCTP (3,000 Ci/mmol, DuPont NEN) and unlabeled dGTP and dTTP at 100 μM in a final volume of 50 μl as described in (25). Unincorporated nucleotides were removed using a G-25 (Pharmacia) spin column. Two-tenths ng of DNA probe (20,000 cpm) was used in a DNA binding reaction containing 4 μg of crude nuclear extract. The DNA binding reaction also contained 50 μg of poly(dI·dC)/ml, 5% glycerol, and 1 × band shift buffer (10 mM Tris·HCl, pH 7.8, 1 mM EDTA, 40 mM KCl, and 1 mM dithiothreitol). Reactions were incubated at room temperature for 30 min and size fractionated by electrophoresis in 4% native polyacrylamide (Protogel brand acrylamide:bisacrylamide (v/v, 30:0.8), National Diagnostics, Atlanta, GA) gels containing 0.25 × TBE (1 × TBE: 89 mM Tris borate, 89 mM boric acid, and 2 mM EDTA) which had been preelectrophoresed for 30 min at 100 volts. Gels were run at 10 volts/cm for 45 min at room temperature. Following electrophoresis, gels were transferred to 3MM paper (Whatman Ltd.) and dried in a gel dryer under vacuum at 80 °C. The dried gels were used to expose XAR-2 x-ray film (Eastman Kodak) at −80 °C using an intensifying screen. Competition analysis was performed by including a 25-molar excess of unlabeled double-stranded DNA oligonucleotides in the binding reaction. Nuclear extracts were added to the binding reactions last and then processed as described above. Unlabeled NF-κB probe DNA was used as a specific competitor. Nonspecific competitor DNA contained the NF-βA-binding element from the interleukin-1β gene promoter (25). The sequence of the syn-

thetic double-stranded DNA molecule is presented below:

```
5′ GATCTACTTCTGCTTTTG      3′
3′      ATGAAGACGAAAACCTAG 5′
```

SEQUENCE 2

Anti-phosphotyrosine Immunoblotting—Following stimulation of the cells (1–2 × 10^6), culture medium was aspirated, and 60 μl of lysis buffer (100 mM Tris·HCl, pH 8.0 (22 °C), 137 mM NaCl, 2 mM EDTA, 1% glycerol (v/v), 1% Triton X-100, 1 mM sodium vanadate, 50 mM sodium fluoride, 100 μM quercitin, 100 μM 1-tosylamido-2-phenylethyl chloromethyl ketone, 0.5 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin, 0.14 unit/ml aprotinin) was added to each well. The plates were then incubated for 30 min on ice. Cell lysates were collected and cleared by centrifugation at 4 °C for 15 min in a microcentrifuge (13,500 × *g*), and protein concentrations were determined. Equal amounts of protein (10 μg) were mixed with 4 × Laemmli sample buffer (62 mM Tris·HCl, pH 6.8, 2% glycerol, 5% SDS, 0.05% β-mercaptoethanol, and 0.05% bromphenol blue), boiled for 5 min, and frozen at −80 °C until analysis by immunoblotting. Samples were electrophoresed on a 10% SDS-polyacrylamide gel. Following electrophoresis, proteins were blotted onto Immobilon-P membranes (Millipore Corp., Bedford, MA) and then probed with anti-phosphotyrosine mAb using a horseradish peroxidase-based chemiluminescence system as described in (17).

Immunoprecipitation and Kinase Assay—For immunoprecipitation assays, CHO/CD14 and CHO/NEO cells were grown on 100-mm tissue culture dishes to approximately 80% confluence (10^7 cells) in Ham's F-12 medium containing L-glutamine supplemented with 10% (v/v) fetal bovine serum, penicillin G (100 units/ml), and streptomycin (100 mg/ml) in a humidified atmosphere containing 5% CO_2. The growth medium was aspirated and replaced with 5 ml of fresh medium containing 1 μg/ml LPS when required. After the stimulation period, the medium was aspirated, the cells were washed with 5 ml of ice-cold PBS containing 1 mM phenylmethylsulfonyl fluoride and 1 mM sodium vanadate (PBSPV), and lysed in ice-cold lysis buffer (10 mM Tris·HCl, pH 8.2, 140 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 15 milliunits/ml aprotinin, 20 mM leupeptin, 1 mM sodium vanadate, and 0.1 mM 1-chloro-3-tosylamido-7-amino-2-heptanone). THP-1 cells (ATCC TIB 202) were seeded at a concentration of 2 × 10^6 cells/100-mm dish in 10 ml of growth medium (RPMI 1640 containing 10% (v/v) fetal bovine serum, penicillin G (100 units/ml), streptomycin (100 mg/ml), and 0.1 μM 1,25-dihydroxyvitamin D_3 (Biomol Research Laboratories, Plymouth Meeting, PA)). After 72 h, fresh growth medium (5 ml) was added, and the cells were incubated for another 24 h. For LPS stimulation, an additional 5 ml of growth medium containing LPS at a final concentration of 1 μg/ml was added. Next, the cells were gently removed from the plates using a cell scraper, washed with PBSPV, and lysed in lysis buffer. The lysates were then subjected to solid phase immunoprecipitation and kinase assays as described (18, 26) with minor modifications. Briefly, 96-well U-bottom Microtest III plates (Falcon Labware, Oxnard, CA) were coated with 100 μl of goat anti-mouse or goat anti-rabbit IgG (Sigma) (0.1 mg/ml) at 37 °C for 2 h. The plates were then washed three times with 200 μl of PBS. Next, 100 μl of mouse mAb against CD14, MY4 (20 μg/ml), or rabbit polyclonal antibody against *lyn* (Santa Cruz Biotechnology, Santa Cruz, CA; 10 μg/ml) was added to the wells and incubated overnight at 4 °C. The wells were washed three times with PBS, and blocked with 0.2% gelatin, 1% glycine in PBS for 1 h at 37 °C. The blocking solution was removed, and 100-μl aliquots of the lysates (1 mg/ml) were added to the wells. After a 4-h incubation at 4 °C, the lysates were aspirated, the wells were washed three times with lysis buffer, and 50 μl of kinase assay buffer (25 mM Hepes, pH 7.2, 3 mM $MnCl_2$, 20 mM $MgCl_2$, 0.1% Nonidet P-40, and 5 μCi of [γ-^{32}P]ATP (3,000 Ci/mM; Amersham Corp.)) was added to the wells. After 5 min at room temperature, the proteins were eluted with SDS sample buffer and subjected to SDS-polyacrylamide gel electrophoresis and autoradiography.

RESULTS

LPS Induces NF-κB Translocation in CHO/CD14 Cells—Previous studies have shown that wild-type CHO-K1 cells do not release [^{3}H]20:4 in response to treatment with LPS (9). We have also reported previously the construction of a CHO cell line, CHO/CD14, which expresses moderate levels of surface human CD14 (9). Stimulation of CHO/CD14 cells with as little as 1 ng of LPS/ml resulted in the release of [^{3}H]20:4 into the culture supernatant. This CD14-mediated response closely resembled [^{3}H]20:4 release from the murine macrophage-like cell line, RAW 264.7, in response to LPS stimulation. An additional macrophage response to LPS is the translocation of the transcription factor, NF-κB (19). We therefore chose the EMSA to study LPS-induced NF-κB activation in CHO/CD14 cells in response to LPS to determine whether this response is similar to LPS-induced NF-κB activation in macrophages.

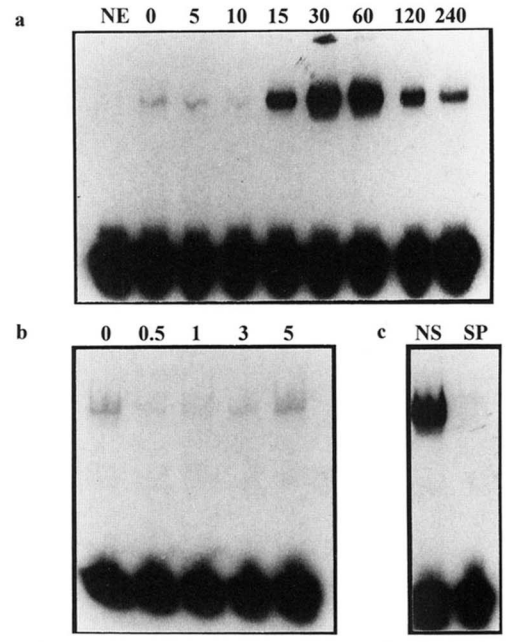

FIG. 1. **LPS-induced NF-κB translocation in CD14-transfected CHO cells.** CHO/CD14 (*panel a*) and CHO/NEO (*panel b*) monolayers containing 2–3 × 10^6 cells were stimulated, in 0.8 ml of Ham's F-12 containing 2% heat-inactivated human serum, with 1 μg of LPS/ml for the times (in min) indicated in the figure. Nuclear extracts were analyzed by EMSA combined with autoradiography as described under "Materials and Methods" using ^{32}P-labeled probe DNA containing the immunoglobulin κ gene enhancer consensus sequence. *Panel c*, competition analysis was performed by including a 25-fold molar excess of unlabeled specific (*SP*) or nonspecific (*NS*) oligonucleotide in the DNA binding reactions assembled with nuclear extracts from CHO/CD14 cells treated with LPS (1 μg/ml) for 1 h.

CHO/CD14 and CHO/NEO cells were treated with LPS (1 μg/ml) for increasing periods of time. Nuclear extracts were prepared as described under "Materials and Methods" and were subjected to EMSA using a DNA probe containing the NF-κB-binding element from the murine immunoglobulin κ light chain gene enhancer. Nuclear localization of NF-κB was evident in CHO/CD14 cells within 15 min and maximal within 30 min following exposure to LPS (Fig. 1*a*). Peak activation of the CHO/CD14 cells resulted in a 10-fold increase in nuclear NF-κB levels as determined by scanning densitometry. Nuclear levels of NF-κB returned to basal levels within 2 h following exposure to LPS. In contrast, treatment of CHO/NEO cells with LPS did not result in NF-κB activation (Fig. 1*b*). Thus, CHO cells can acquire responsiveness to LPS as a result of CD14 expression. These data extend our earlier findings that CHO/CD14 cells release arachidonic acid metabolites in response to LPS, by demonstrating that NF-κB translocation is also induced by LPS in these cells.

Competition analysis was performed on nuclear extracts prepared from CHO/CD14 cells stimulated for 1 h with 1 μg of LPS/ml to demonstrate specificity of the retarded complexes observed in the EMSA. Inclusion of a 25-fold molar excess of unlabeled double-stranded NF-κB oligonucleotide in the DNA binding reaction containing nuclear extracts from stimulated CHO/CD14 cells effectively blocked LPS-induced formation of DNA/protein complexes (Fig. 1*c*). Inclusion of a 25-fold molar

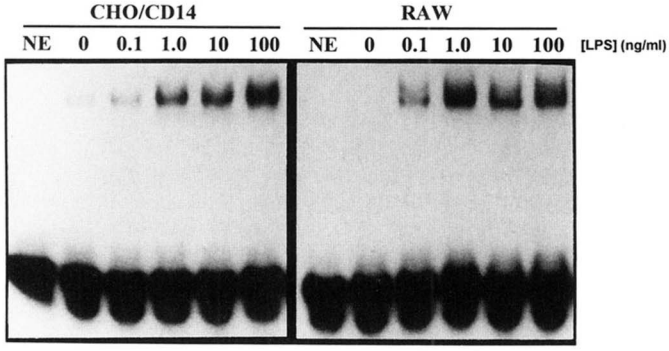

FIG. 2. **LPS dose response in CHO/CD14 cells.** Monolayers of CHO/CD14 and RAW cells (2×10^6/well of a six-well dish) were treated for 1 h with increasing concentrations of LPS (0–100 ng/ml) suspended in Ham's F-12 medium containing 2% human serum. The stimulation was stopped by placing the tissue culture dishes on ice. Nuclear extracts were prepared and subjected to EMSA and autoradiography as described under "Materials and Methods."

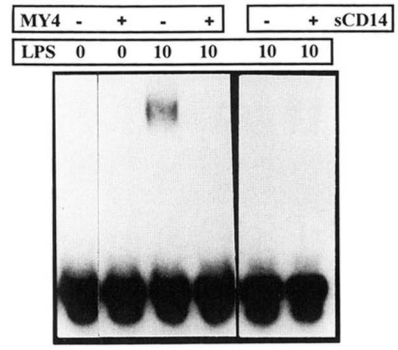

FIG. 3. **Effects of anti-CD14 mAb in CHO/CD14 cells and effect of rsCD14 in CHO-K1 cells.** CHO/CD14 cells were treated for 1 h with 10 ng of LPS/ml in the absence (–) or presence (+) of 200 μg MY4 (anti-CD14 monoclonal antibody)/ml in Ham's F-12 medium containing 2% heat-inactivated human serum; the LPS and the monoclonal antibody were premixed and added simultaneously. As part of the same experiment, monolayers of wild-type CHO-K1 cells were washed free of serum three times with 1 ml of Ham's F-12 and were stimulated with 100 ng of LPS/ml for 1 h in the absence or presence of rsCD14 (10 μg/ml) in macrophage serum-free medium (which contains 0.5% albumin). Nuclear extracts were analyzed by EMSA and autoradiography as described under "Materials and Methods."

excess of a nonspecific double-stranded DNA probe in the DNA binding reaction did not result in competition for protein binding to the radiolabeled DNA probe. These results indicate that the observed DNA-protein complex arises through sequence-specific interactions with the NF-κB consensus sequence. Furthermore, inclusion of anti-human mAbs, specific for NF-κB-related proteins in the DNA binding reactions, demonstrated that NF-κB p65 is a component of the DNA-protein complex observed in CHO/CD14 cells following stimulation with endotoxin (data not shown).

LPS Induces NF-κB Translocation in CHO/CD14 Cells in a Manner Similar to the Murine Macrophage-like Cell Line, RAW 264.7—LPS dose-response experiments were performed to compare NF-κB translocation in CHO/CD14 cells *versus* RAW 264.7 cells. Cells were treated with increasing concentrations of LPS and incubated for 1 h at 37 °C. Nuclear extracts were prepared and subjected to EMSA. As little as 0.1 ng of LPS/ml was sufficient to activate NF-κB translocation in CHO/CD14 cells (Fig. 2). Similar results were observed with LPS dose-response experiments performed on RAW 264.7 cells (Fig. 2). Response was maximal in both cell lines between 10 and 100 ng of LPS/ml. These results were consistent with CD14-mediated signaling in both cell lines through similar signal transduction pathways resulting in NF-κB activation.

mAbs to CD14 Inhibit NF-κB Activation in CHO/CD14 Cells—Many macrophage responses to LPS, including [^{3}H]20:4 release, increased TK activity, and cytokine release, can be inhibited by treating cells with the anti-CD14 mAb MY4 (27). CHO/CD14 cells were treated with LPS in the presence or absence of 200 μg of MY4 mAb/ml. After incubation at 37 °C for 1 h, nuclear extracts were prepared and analyzed by EMSA. Treatment of CHO/CD14 cells with 10 ng of LPS/ml resulted in NF-κB activation. This response was blocked by adding MY4 mAb and LPS simultaneously (Fig. 3). Control experiments using control IgG_{2a} antibodies did not result in loss of NF-κB activation (data not shown). These results demonstrate that LPS-induced NF-κB activation in CHO/CD14 cells is initiated through LPS binding to transfected human CD14.

Soluble CD14 Fails to Enable Responses to LPS in CHO-K1 Cells—Several recent reports have demonstrated that soluble CD14 can confer LPS sensitivity to endothelial cells and other cell lines that do not express detectable levels of CD14 (8, 28–32), even in the absence of LBP. We have reported previously that serum, which contains both LBP and soluble CD14, failed to enable LPS-induced [^{3}H]20:4 release in CHO cells that have not been transfected with CD14 (9). As demonstrated above, serum also failed to substitute for membrane-bound CD14 when LPS-induced translocation of NF-κB was examined. We examined this issue in greater depth using rsCD14. The bioactivity of rsCD14 was confirmed by its ability to enable LPS induction of tissue factor production in endothelial cells.[2] Wild-type CHO-K1 cells were treated with 10 ng of LPS/ml, under serum-free conditions, in the absence or presence of 10 μg of rsCD14/ml. Nuclear extracts were prepared after 1 h of stimulation and analyzed by EMSA. CHO-K1 cells were not responsive to LPS in either the absence or the presence of rsCD14 (Fig. 3). These results suggest that under serum-free conditions, membrane-bound CD14 is required for LPS-induced NF-κB activation in CHO cells.

NF-κB Activation in CHO/CD14 and RAW 264.7 cells Is an Early Event in LPS Signal Transduction and Does Not Require Concomitant mRNA or Protein Synthesis—CHO/CD14 and RAW 264.7 cells were pretreated with either 1.0 μM actinomycin D or cycloheximide for 30 min prior to stimulation with LPS (10 ng/ml). As expected, these conditions inhibited the release of [^{3}H]20:4 metabolites following stimulation of CHO/CD14 cells with endotoxin (data not shown). EMSA of nuclear extracts prepared from these cells demonstrated that, unlike arachidonate release, CD14-dependent LPS-induced NF-κB activation did not require ongoing transcription or translation (Fig. 4).

Treatment of macrophages with anti-inflammatory glucocorticoid hormones can effectively block many of their responses to LPS including the release of cytokines. CHO/CD14 and RAW 264.7 cells were pretreated with dexamethasone for 30 min followed by the addition of LPS to a final concentration of 10 ng/ml. Nuclear localization of NF-κB in response to LPS stimulation was quantitated by EMSA of nuclear extracts (Fig. 4). NF-κB activation in both cell lines was not effected by pretreatment of cells with dexamethasone.

TK Activity Is Not Apparent in CHO/CD14 Cells Stimulated with LPS—Recently, several reports have presented data suggesting that TK activity is involved in CD14-dependent LPS-induced signaling in LPS-responsive monocytes or macrophages (13–15, 18) and a CD14-transfected 70Z/3 pre-B lymphocytic cell line (16). We sought to determine if endotoxin activated a CD14-associated TK in CHO/CD14 cells. CHO/CD14, CHO/NEO, and RAW 264.7 cells were treated with 100

[2] D. T. Golenbock, H. Lichenstein, R. Bach, and C. F. Moldow, manuscript in preparation.

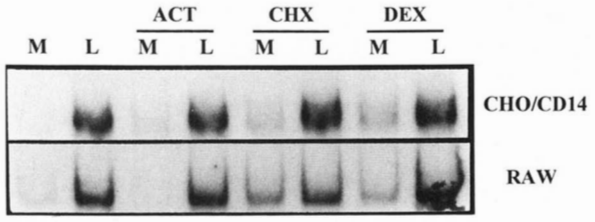

FIG. 4. **Inhibitors of transcription and translation did not block LPS activation of NF-κB.** CHO/CD14 or RAW cells were pretreated for 1 h with a 1 μM concentration of either actinomycin D (*ACT*), cycloheximide (*CHX*), or dexamethasone (*DEX*). This treatment was shown to inhibit LPS-induced release of arachidonate from both CHO/CD14 and RAW 264.7 cells (data not shown). Subsequently, either 800 μl of Ham's F-12 supplemented with 2% heat-inactivated human serum (*M*) or an equal volume of LPS suspended in complete medium at 10 ng/ml (*L*) was added. After 60 min of stimulation, nuclear extracts were analyzed by EMSA and autoradiography as described under "Materials and Methods."

ng/ml LPS for 1 h, lysed with Triton X-100, and analyzed by immunoblotting using the anti-phosphotyrosine mAb 4G10 (Fig. 5*a*). As expected, CHO/NEO did not demonstrate TK activity in response to LPS treatment. CHO/CD14 cells also failed to demonstrate LPS-inducible TK activity. However, treatment of CHO/CD14 cells with phorbol 12-myristate 13-acetate resulted in the induction of phosphotyrosine residues in a low molecular mass protein (~40 kDa), demonstrating that the anti-phosphotyrosine mAb recognized phosphotyrosine residues from CHO cells. Even using short periods of treatment (30 s) we failed to observe inducible tyrosine phosphorylation in the CHO/CD14 cells stimulated by LPS. In contrast to CHO/CD14 cells, we reproducibly observed in RAW 264.7 cells the presence of three proteins (~40–47 kDa) that contain induced tyrosine phosphorylation upon LPS stimulation.

An alternative method of examining LPS-stimulated CHO/CD14 transfectants for inducible TK activity is the solid phase immunoprecipitation and kinase assay (18, 26). This technique has been employed recently by Stefanova and colleagues to describe CD14-associated tyrosine kinases in LPS-stimulated human monocytes (18). These authors reported that LPS activated the nonreceptor tyrosine kinases $p56^{lyn}$, $p58^{hck}$, and $p59^{c\text{-}fgr}$. Furthermore, $p56^{lyn}$ was apparently associated with CD14: immunoprecipitation of stimulated monocytes with anti-CD14 mAb resulted in the coimmunoprecipitation of activated $p56^{lyn}$. When CHO/NEO and CHO/CD14 cells were stimulated with LPS for 15 min and subsequently subjected to immunoprecipitation with anti-CD14 mAb, no CD14-associated LPS-inducible phosphotyrosine residues were observed (Fig. 5*b*, *first four lanes*, from *left*). Similar results were obtained with the CHO transfectants at 5, 30, and 60 min of LPS stimulation (data not shown). In contrast, when vitamin D_3-differentiated THP-1 cells (a human monocytic cell line) were subjected to immunoprecipitation with anti-CD14 mAb (MY4), CD14-associated kinase activity was observed at 75–80, 53/56, 30, and 25 kDa (Fig. 5*b*, *fifth* and *sixth lanes*). When isotype-matched control antibodies were used, no kinase activity was observed from any of the cell lines (data not shown). Simultaneous immunoprecipitations with anti-*lyn* antibodies (*α-lyn*) presumptively identified the 53/56-kDa residues as $p56^{lyn}$ (Fig. 5*b*, *seventh* and *eighth lanes*), consistent with the observations of Stefanova *et al.* (18). Thus, although we confirmed that LPS-inducible activation of a CD14-associated TK occurred in monocytes, this activity was not necessary for NF-κB translocation. LPS stimulation of CD14-transfected CHO cells was not associated with activation of any CD14-associated TK under conditions that result in NF-κB translocation.

To assess further the requirement for TK activity in LPS-induced CD14-dependent signaling, we assayed TK and NF-κB

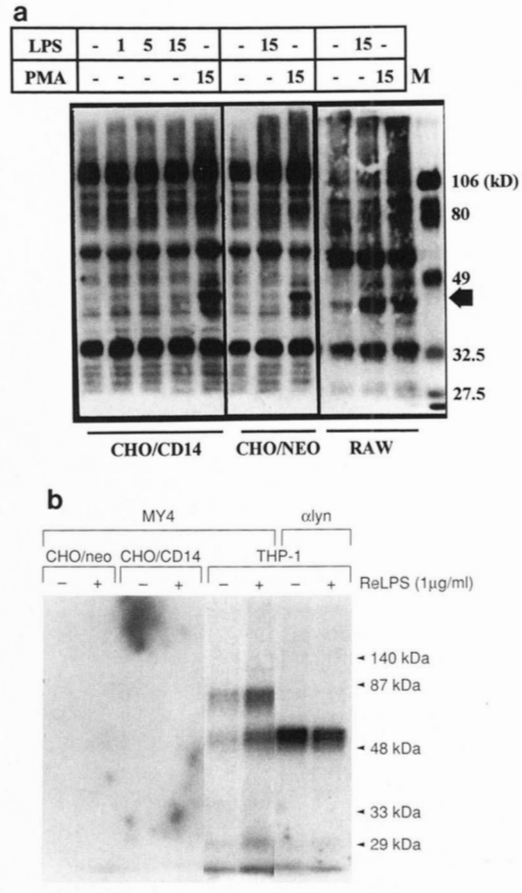

FIG. 5. **LPS failed to induce tyrosine phosphorylation in CHO/CD14 cells.** *Panel a*, monolayers containing 2–3 × 10^6 cells were left untreated (–) or treated with either 100 ng LPS/ml or 30 ng of phorbol 12-myristate 13-acetate/ml in Ham's F-12 medium supplemented with 2% human serum for the times indicated (in min) in the figure. Extracts were prepared using detergent in the presence of protease and phosphatase inhibitors, size fractionated by SDS-polyacrylamide electrophoresis on a 6% gel, then blotted to nitrocellulose. Immunoblot analysis was performed using 4G10 anti-phosphotyrosine mAb and a horseradish peroxidase-based chemiluminescence system as described under "Materials and Methods." The *lane* marked *M* contained prestained molecular mass markers (Bio-Rad). The *bracket* indicates the region of the gel where proteins that have been modified by LPS-inducible tyrosine phosphorylation migrate. *Panel b*, solid phase immunoprecipitation of CD14-associated protein kinases: CHO/NEO, CHO/CD14, or vitamin D_3-differentiated THP-1 cells were incubated for 15 min in the presence (+) or absence (–) of 1 μg of LPS/ml, and lysates were subjected to the solid phase immunoprecipitation and kinase assay as described under "Materials and Methods," using antibody against CD14 (MY4) or *lyn* (*α-lyn*) as indicated. The proteins were separated on 10% SDS-polyacrylamide gels followed by autoradiography. The positions of the molecular mass standards (in kDa) are indicated on the *right*.

activation in cells pretreated with the TK antagonists, herbimycin A (5 μg/ml) and genistein (100 μM). CHO/CD14 and RAW 264.7 cells were pretreated with TK inhibitors for 4 h prior to stimulation with LPS. Immunoblots were performed with cellular extracts prepared from RAW 264.7 cells pretreated with TK antagonists prior to stimulation with LPS, to assess the ability of these agents to block tyrosine phosphorylation. Both herbimycin A and genistein were effective at inhibiting LPS-inducible TK activity in RAW 264.7 macrophage-like cells as demonstrated by the absence of inducible phosphotyrosine residues in cellular proteins following stimulation with LPS, demonstrating the effectiveness of the TK antagonists (data not shown). Herbimycin A and genistein had little effect on the ability of LPS to induce NF-κB translocation in either CHO/CD14 or RAW 264.7 cells (Fig. 6). Thus, NF-κB activation ap-

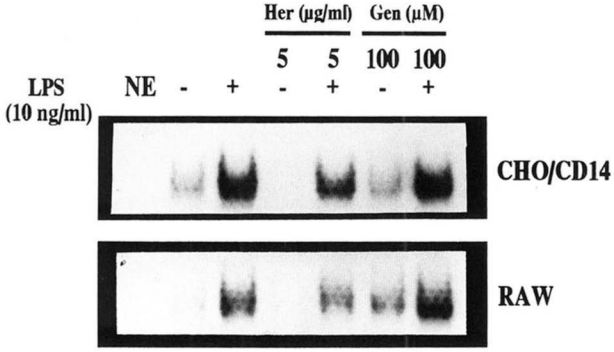

FIG. 6. **Effect of TK inhibitors on LPS activation of NF-κB.** Cells were pretreated with the TK antagonists, herbimycin A (*Her*, 5 μg/ml) or genistein (*Gen*, 100 μM) for 4 h and then incubated for an additional hour either in the absence (–) or presence (+) of 10 ng of LPS/ml in Ham's F-12 supplemented with 2% human serum. Nuclear extracts were prepared and analyzed by EMSA as described under "Materials and Methods." Scanning densitometry of the autoradiographs showed that the ratio of nuclear NF-κB in stimulated/unstimulated cells was CHO/CD14: 3.6, 13, and 4.8; RAW: 8.1, 10.9, and 2.2 (no inhibitor, plus herbimycin A, and plus genistein, respectively).

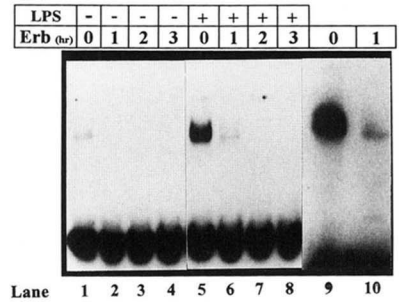

FIG. 7. **Erbstatin analog eliminated NF-κB cytosolic pools from RAW 264.7 cells.** Cell monolayers were treated with 100 μM erbstatin analog in complete medium for increasing periods of time and incubated an additional hour in the absence (–) or presence (+) of 10 ng of LPS/ml. The time with which cells were treated with erbstatin analog prior to LPS stimulation is indicated in hours above the corresponding lane. Nuclear extracts (*lanes 1–8*) were prepared as described under "Materials and Methods," except that the cells were lysed in 100 μl of buffer I prior to analysis by EMSA. Cytoplasmic fractions from unstimulated RAW 264.7 cells (*lanes 9* and *10*) were reserved from the same cells used to prepare the nuclear extracts shown in *lanes 1* and *2*, respectively, and dialyzed against 10,000 volumes of buffer II (see "Materials and Methods") for 12 h. The extracts were adjusted to 0.1% deoxycholate (v/v), incubated on ice for 10 min, then supplemented with Nonidet P-40 to 0.1% (v/v), and incubated an additional 15 min on ice before subjecting to EMSA. Lanes containing nuclear extracts (*lanes 1–8*) were exposed to film overnight with an intensifying screen at –80 °C; lanes containing cytoplasmic extracts (*lanes 9* and *10*) were exposed to film for 5 days with an intensifying screen at –80 °C. Virtually identical results were seen when cells were pretreated 10 μM erbstatin analog (data not shown).

peared to proceed in the absence of TK activity in both CHO/CD14 and RAW 264.7 cells.

Effect of Erbstatin Analog, a TK Antagonist, on Basal NF-κB Levels—Pretreatment of either CHO/CD14 or RAW 264.7 cells with erbstatin analog resulted in a time-dependent decrease in NF-κB DNA binding activity present in nuclear extracts prepared from resting and LPS-stimulated cells (Fig. 7). The observation that basal levels of NF-κB also were diminished in cells treated with erbstatin analog suggested that the drug might cause a decrease in the cellular pools of NF-κB. Thus, its ability to block LPS-induced activation of NF-κB was unrelated to transmembrane signaling. Alternatively, erbstatin analog might actually inhibit the LPS signal transduction machinery required for activation of cellular NF-κB.

To distinguish between these two possibilities, we prepared cytosolic extracts from either untreated RAW 264.7 cells or cells that had been treated with erbstatin analog for 1 h. Cytoplasmic extracts were treated sequentially with deoxycholate and Nonidet P-40 and then analyzed by EMSA. Treating cytoplasmic extracts with deoxycholate followed by Nonidet P-40 effectively dissociates heterodimeric complexes of NF-κB from their inhibitory subunits (*e.g.* IκB), allowing NF-κB to bind specifically to DNA probes *in vitro* (33, 34). Deoxycholate/Nonidet P-40 treatment of cytosolic extracts from cells that had not been treated with erbstatin analog revealed the presence of significant quantities of NF-κB DNA binding activity (Fig. 7). Cytoplasmic extracts prepared from RAW 264.7 cells treated with erbstatin analog for 1 h contained dramatically reduced levels of NF-κB as evidenced by the deoxycholate/Nonidet P-40 treatment (Fig. 7, *lanes 9* and *10*). Similar results were also observed using CHO/CD14 cells (data not shown). Thus, the apparent ability of erbstatin analog to inhibit LPS-induced activation of NF-κB was not the result of inhibition of LPS-induced signal transduction. Rather, pretreatment of cells with erbstatin analog caused the elimination of functional NF-κB from cellular pools.

DISCUSSION

The discovery that LBP-complexed LPS can activate cells by interacting directly with CD14 was a fundamental breakthrough in understanding the mechanism by which endotoxin activates monocytes, macrophages, and neutrophils. The results of CD14 transfection experiments (9, 12, 16, 35) imply that proteins required for LPS signal transduction, subsequent to ligand binding by CD14, are widespread and not limited to myeloid cells. The transcription factor NF-κB has been shown to be rapidly translocated in macrophages following stimulation with endotoxin. Here, we demonstrate that treatment of CHO/CD14 cells with low concentrations of LPS induced rapid nuclear translocation of NF-κB. This response is an early signaling event and does not appear to require transcription or *de novo* protein synthesis. Furthermore, TK activity apparently was not required to observe NF-κB translocation in transfected cells as evidenced by immunoblotting lysates with anti-phosphotyrosine antibodies (Fig. 5) or pharmacologic analysis of both CHO/CD14 and RAW 264.7 cells (Fig. 6). Surprisingly, LPS in conjunction with soluble CD14 did not induce NF-κB activation in wild-type CHO-K1 cells (Fig. 3).

Ciliary neurotrophic factor (CNTF)-induced signal transduction may provide the best model for the study of cell signaling by glycosylphosphatidylinositol-linked proteins. In this signaling system, CNTF binds the glycosylphosphatidylinositol-anchored α component (CNTFR-α) of the CNTF receptor causing CNTFR-α to bind sequentially to the β component of the leukemia inhibitory factor receptor and gp130. Similarly, binding of interleukin-6 to its soluble receptor is followed by the association of this complex to a dimer of gp130 and results in signal transduction. Another variant of this signaling paradigm is signal transduction in response to leukemia inhibitory factor, which binds to leukemia inhibitory factor receptor-β, an integral protein on responsive cells, inducing heterodimerization with gp130, tyrosine phosphorylation of the two subunits, and signal transduction (36). In each signal transduction system described above, tyrosine phosphorylation of the transmembrane subunits occurs concomitantly with dimerization or trimerization of the receptor complexes. Signal transduction across the membrane is observed as evidenced by the subsequent tyrosine phosphorylation of low molecular mass cellular proteins (36). Therefore, specificity of the gp130 signal-transducing molecule is conferred by accessory proteins, which do not necessarily signal directly, but are required for proper receptor function.

Recently, reports have suggested that like the CNTF receptor, CD14 does not signal directly, but signals through an as yet unidentified signal transducing protein. We have even considered that gp130 is the CD14-associated signal transducer, but experiments in our laboratory with human mononuclear cells purified from whole blood have failed to demonstrate that anti-gp130 mAb (gift from Drs. T. Taga and T. Kishimoto, Japan) inhibit LPS-induced tumor necrosis factor-α release.[3] In light of the CNTF model, the rapid appearance of TK activity observed following exposure to endotoxin in RAW 264.7 cells (13, 14), human monocytes (15, 18), and CD14-transfected 70Z/3 lymphocytes (16) suggested that this putative signal transducing protein may function by activating a TK cascade. We were unable to demonstrate LPS-inducible TK activity by immunoblotting protein extracts from LPS-treated CHO/CD14 cells whereas, phorbol 12-myristate 13-acetate induced a distinct pattern of tyrosine phosphorylation (Fig. 5*a*). This suggested that a TK event was not required for LPS-induced activation of NF-κB in CHO/CD14 cells. We have considered the possibility that the immunoblot assay lacked the sensitivity required to detect LPS-induced TK activation in CHO/CD14 cells and that the putative CD14-related signal-transducing element nevertheless might be a TK. However, our observations that pretreatment of CHO/CD14 cells or murine macrophages with genistein and herbimycin A did not inhibit LPS-induced activation of NF-κB (Fig. 6) coupled with the immunoblot data further suggest that this signal transduction pathway does not require TK activity for NF-κB activation in CHO/CD14 and RAW 264.7 cells. Although it might appear that the TK inhibitors had some inhibitory effect on nuclear NF-κB levels in LPS-stimulated cells, basal levels of nuclear NF-κB present in CHO/CD14 and RAW 264.7 cells pretreated with either herbimycin A or genistein were decreased relative to untreated control cells (Fig. 6). Our experimental results, as well as those presented by others (*e.g.* see Fig. 6*b* in Ref. 16) may reflect the ability of some TK inhibitors to decrease overall levels of NF-κB through their effects on NF-κB synthesis or stability. The apparent inhibitory effects of TK inhibitors on nuclear NF-κB levels do not appear to reflect a requirement for TK activity for signal transduction to occur.

We present data here that CHO/CD14 cells do not respond to LPS stimulation by activating TK using three established approaches. First, inducible phosphotyrosine residues are not observed by Western blot. Second, *in vitro* kinase assays fail to show receptor-associated TK activity. Finally, TK inhibitors fail to block an LPS-inducible response. Thus, the CNTF receptor paradigm may not apply directly to CD14-mediated signaling. However, it is likely that signaling pathways other than those that culminate in NF-κB translocation, such as those that result in the production of certain inflammatory mediators, may indeed require TK activity. Macrophages possess a large repertoire of LPS-inducible responses, and these pathways may require the activity of nonreceptor protein tyrosine kinases activated in cells following engagement of CD14 with LPS. The absence of TK activity in CHO/CD14 could explain the small number of macrophage-like responses acquired by CHO-K1 cells following transfection with CD14. Because many of the genes for select tyrosine kinases have been cloned and can be transfected in CHO cells, this hypothesis can now be tested.

Several recent publications have demonstrated that soluble CD14 can bind LPS and activate endothelial and epithelial cells in the absence of LBP (8, 28–32). It was proposed that rsCD14·LPS complexes can interact with specific receptors on the surface of these cell types and effect signal transduction. A soluble form of the CNTF receptor α component was shown to activate normally nonresponsive cells in the presence of CNTF (37). Thus, it was surprising that rsCD14 did not compensate for membrane bound CD14 in CHO-K1 cells. This result was probably not due to the absence of a glycosylphosphatidylinositol anchor because an integral membrane form of CD14 functions in a manner identical with the glycolipid-anchored form of CD14 when transfected into 70Z/3 cells (35). Membrane attachment of CD14 therefore might be a unique requirement for activation of the CHO-K1 signal transduction pathway. Alternatively, expression of high levels of CD14 in transfectants would result in a sufficient density of CD14 molecules on the plasma membrane compared with LPS-activated rsCD14, which might be necessary for interacting with additional signaling molecules in CHO-K1 cells. Finally, the association of LPS with rsCD14 is time-dependent and can be facilitated catalytically by LBP (38). In the experiments presented here, rsCD14 and LPS were added to CHO-K1 cells in the absence of LBP without preincubation, and thus rsCD14 and LPS may not have had time to form active complexes. We have not rigorously assessed this kinetic issue and cannot rule out the possibility that rsCD14 might enable LPS responses in CHO-K1 cells under different stimulation conditions. Further examination of the mechanism of soluble CD14 is beyond the scope of this paper.

The observation that CHO/CD14 cells respond to LPS by nuclear translocation of NF-κB and metabolism of arachidonic acid is strong evidence that signaling molecules similar to those in phagocytes are present in CHO/CD14 cells. The fact that CHO cells are easily manipulated and efficiently transfected makes the CHO/CD14 cell line a valuable tool in which to employ molecular genetic analysis of the LPS signaling pathway culminating in activation of NF-κB and arachidonic acid metabolism. Ultimately, a combination of genetic analysis and pharmacologic approaches should identify the family of LPS signaling proteins which can subsequently be targeted for the development of therapeutic agents for sepsis.

[3] R. I. Ingalls and D. T. Golenbock, unpublished data.

REFERENCES

1. Haziot, A., Chen, S., Ferrero, E., Low, M. G., Silber, R., and Goyert, S. M. (1988) *J. Immunol.* **141,** 547–552
2. Wright, S. D., Tobias, P. S., Ulevitch, R. J., and Ramos, R. A. (1989) *J. Exp. Med.* **170,** 1231–1241
3. Tobias, P. S., Soldau, K., and Ulevitch, R. J. (1986) *J. Exp. Med.* **164,** 777–793
4. Wright, S. D., Ramos, R. A., Patel, M., and Miller, D. S. (1992) *J. Exp. Med.* **176,** 719–727
5. Wright, S. D., Ramos, R. A., Tobias, P. S., Ulevitch, R. J., and Mathison, J. C. (1990) *Science* **249,** 1431–1433
6. Schumann, R. R., Leong, S. R., Flaggs, G. W., Gray, P. W., Wright, S. D., Mathison, J. C., Tobias, P. S., and Ulevitch, R. J. (1990) *Science* **249,** 1429–1431
7. Lynn, W. A., Raetz, C. R. H., Qureshi, N., and Golenbock, D. T. (1991) *J. Immunol.* **147,** 3072–3079
8. Frey, E. A., Miller, D. S., Jahr, T. G., Sundan, A., Bazil, V., Espevik, T., Finlay, B. B., and Wright, S. D. (1992) *J. Exp. Med.* **176,** 1665–1671
9. Golenbock, D., Liu, Y., Millham, F., Freeman, M., and Zoeller, R. (1993) *J. Biol. Chem.* **268,** 22055–22059
10. Heumann, D., Gallay, P., Barras, C., Zaech, P., Ulevitch, R. J., Tobias, P. S., Glauser, M. P., and Baumgartner, J. D. (1992) *J. Immunol.* **148,** 3505–3512
11. Kitchens, R. L., Ulevitch, R. J., and Munford, R. S. (1992) *J. Exp. Med.* **176,** 485–494
12. Lee, J.-D., Kato, K., Tobias, P. S., Kirkland, T. N., and Ulevitch, R. J. (1992) *J. Exp. Med.* **175,** 1697–1705
13. Weinstein, S. L., Gold, M. R., and DeFranco, A. L. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 4148–4152
14. Weinstein, S. L., Sanghera, J. S., Lemke, K., DeFranco, A. L., and Pelech, S. L. (1992) *J. Biol. Chem.* **267,** 14955–14962
15. Weinstein, S. L., June, C. H., and DeFranco, A. L. (1993) *J. Immunol.* **151,** 3829–3838
16. Han, J., Lee, J. D., Tobias, P. S., and Ulevitch, R. J. (1993) *J. Biol. Chem.* **268,** 25009–25014
17. Manthey, C. L., Qureshi, N., Stutz, P. L., and Vogel, S. N. (1993) *J. Exp. Med.* **178,** 695–702
18. Stefanova, I., Corcoran, M. L., Horak, E. M., Wahl, L. M., Bolen, J. B., and Horak, I. D. (1993) *J. Biol. Chem.* **268,** 20725–20728
19. Muller, J. M., Ziegler-Heitbrock, H. W., and Baeuerle, P. A. (1993) *Immunobiology* **187,** 233–256
20. Libermann, T. A., and Baltimore, D. (1990) *Mol. Cell. Biol.* **10,** 2327–2334
21. Shakhov, A. N., Collart, M. A., Vassalli, P., Nedospasov, S. A., and Jongeneel, C.

V. (1990) *J. Exp. Med.* **171,** 35–47
22. Shimizu, H., Mitomo, K., Watanabe, T., Okamoto, S., and Yamamoto, K. (1990) *Mol. Cell. Biol.* **10,** 561–568
23. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) *Nucleic Acids Res.* **11,** 1475–1489
24. Schreiber, E., Matthias, P., Muller, M. M., and Schaffner, W. (1989) *Nucleic Acids Res.* **17,** 6419
25. Hunninghake, G. W., Monks, B. G., Geist, L. J., Monick, M. M., Monroy, M. A., Stinski, M. F., Webb, A. C., Dayer, J. M., Auron, P. E., and Fenton, M. J. (1992) *Mol. Cell. Biol.* **12,** 3439–3448
26. Tamura, G. S., Dailey, M. O., Gallatin, W. M., McGrath, M. S., Weissman, I. L., and Pillemer, E. A. (1984) *Anal. Biochem.* **136,** 458–464
27. Lynn, W. A., Liu, Y., and Golenbock, D. T. (1993) *Infect. Immun.* **61,** 4452–4461
28. Haziot, A., Rong, G. W., Silver, J., and Goyert, S. M. (1993) *J. Immunol.* **151,** 1500–1507
29. Arditi, M., Zhou, J., Dorio, R., Rong, G. W., Goyert, S. M., and Kim, K. S. (1993) *Infect. Immun.* **61,** 3149–3156
30. Read, M. A., Cordle, S. R., Veach, R. A., Carlisle, C. D., and Hawiger, J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 9887–9891
31. Pugin, J., Schurer-Maly, C. C., Leturcq, D., Moriarty, A., Ulevitch, R. J., and Tobias, P. S. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 2744–2748
32. Goldblum, S. E., Brann, T. W., Ding, X., Pugin, J., and Tobias, P. S. (1994) *J. Clin. Invest.* **93,** 692–702
33. Baeuerle, P. A., and Baltimore, D. (1988) *Cell* **53,** 211–217
34. Baeuerle, P. A., and Baltimore, D. (1988) *Science* **242,** 540–546
35. Lee, J. D., Kravchenko, V., Kirkland, T. N., Han, J., Mackman, N., Moriarty, A., Leturcq, D., Tobias, P. S., and Ulevitch, R. J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 9930–9934
36. Davis, S., Aldrich, T. H., Stahl, N., Pan, L., Taga, T., Kishimoto, T., Ip, N. Y., and Yancopoulos, G. D. (1993) *Science* **260,** 1805–1808
37. Davis, S., Aldrich, T. H., Ip, N. Y., Stahl, N., Scherer, S., Farruggella, T., DiStefano, P. S., Curtis, R., Panayotatos, N., Gascan, H., Chevalier, S., and Yancopoulos, G. D. (1993) *Science* **259,** 1736–1739
38. Hailman, E., Lichenstein, H. S., Wurfel, M. M., Miller, D. S., Johnson, D. A., Kelley, M., Busse, L. A., Zukowski, M. M., and Wright, S. D. (1994) *J. Exp. Med.* **179,** 269–277