

# Lipopolysaccharide (LPS) Partial Structures Inhibit Responses to LPS in a Human Macrophage Cell Line without Inhibiting LPS Uptake by a CD14-mediated Pathway

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## Summary

Lipopolysaccharides (LPS) that lack acyloxyacyl groups can antagonize responses to LPS in human cells. Although the site and mechanism of inhibition are not known, it has been proposed that these inhibitory molecules compete with LPS for a common cellular target such as a cell-surface binding receptor. In the present study, we used an in vitro model system to test this hypothesis and to evaluate the role of CD14 in cellular responses to LPS. Cells of the THP-1 human monocyte-macrophage cell line were exposed to 1,25 dihydroxyvitamin D<sub>3</sub> to induce adherence to plastic and expression of CD14, a binding receptor for LPS complexed with LPS-binding protein (LBP). The uptake of picograms of [<sup>3</sup>H]LPS (agonist) and enzymatically deacylated LPS [<sup>3</sup>H]dLPS (antagonist) was measured by exposing the cells to the radiolabeled ligands for short incubation periods. The amounts of cell-associated LPS and dLPS were then correlated with cellular responses by measuring the induction of nuclear NF- $\kappa$ B binding activity and the production of cell-associated interleukin (IL)-1 $\beta$ . We found that similar amounts of [<sup>3</sup>H]LPS or [<sup>3</sup>H]dLPS were taken up by the cells. The rate of cellular accumulation of the ligands was greatly enhanced by LBP and blocked by a monoclonal antibody to CD14 (mAb 60b), yet no cellular responses were induced by dLPS or dLPS-LBP complexes. In contrast, LPS stimulated marked increases of NF- $\kappa$ B binding activity and IL-1 $\beta$ . These responses were enhanced by LBP and inhibited by mAb 60b. dLPS and its synthetic lipid A counterpart, LA-14-PP (also known as lipid Ia, lipid IVa, or compound 406) strongly inhibited LPS-induced NF- $\kappa$ B and IL-1 $\beta$ , yet neither antagonist inhibited the uptake of LPS via CD14. dLPS did not inhibit NF- $\kappa$ B responses to tumor necrosis factor (TNF) $\alpha$  or phorbol ester. Our results indicate that (a) both stimulatory and nonstimulatory ligands can bind to CD14 in the presence of LBP; (b) the mechanism of inhibition by dLPS is LPS-specific, yet does not involve blockade of LPS binding to CD14; and (c) in keeping with previous results of others, large concentrations of LPS can stimulate the cells in the absence of detectable binding to CD14. The findings indicate that the site of dLPS inhibition is distal to CD14 binding in the LPS signal pathway in THP-1 cells, and suggest that molecules other than CD14 are important in LPS signaling.

Animals have sensitive mechanisms for recognizing and responding to gram-negative bacterial LPS (also called endotoxin). Several cellular mechanisms for binding LPS have been described (1-8) and recent evidence suggests that the uptake of LPS by cells of monocytic origin (macrophages, monocytes) or neutrophils is linked to LPS recognition and cellular responses. However, the relationships between LPS-binding proteins in the cell membrane and signaling pathways are not understood.

CD14, a glycosylphosphatidylinositol-anchored protein ex-

pressed on the surface of monocytes, macrophages, and activated neutrophils (9-11), has been shown to be a receptor for complexes of LPS and LPS-binding protein (LBP)<sup>1</sup> (8, 12). Laboratory studies with isolated monocytes, neutrophils,

<sup>1</sup> Abbreviations used in this paper: AOAH, acyloxyacyl hydrolase; dLPS, enzymatically deacylated LPS lacking secondary fatty acyl chains; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; LBP, LPS-binding protein; LPS-LBP and dLPS-LBP, complexes of LPS and dLPS, respectively, with LBP.

freshly drawn whole blood *ex vivo* (8, 12), and CD14-transfected 70Z/3 cells (13) support the contention that the LBP/CD14-dependent pathway is closely linked to initiation of cellular responses. Two models have been proposed to explain how this pathway might function in LPS signaling (1, 2). In the first model, LPS binding to CD14 directly stimulates the cell, whereas in the second, the CD14-LPS interaction facilitates the subsequent interaction of LPS with another signaling molecule. A direct signaling role for CD14 is suggested by reports that certain anti-CD14 mAbs can mimic some of the effects of LPS in human monocytes (14, 15). The second model is supported by the observation that increasing concentrations of LPS can overcome the ability of blockade or depletion of CD14 to inhibit TNF $\alpha$  production (8).

The study of lipid A analogs that can function as LPS antagonists should provide useful information about the LPS signal pathway. One such analog is produced by the leukocyte enzyme, acyloxyacyl hydrolase (AOAH), which selectively removes secondary acyl chains from the lipid A region of LPS (16, 17). Several laboratories have reported that enzymatically deacylated LPS (dLPS) (18–21) and its lipid A counterpart (known variously as synthetic analogs LA-14-PP or 406, or biosynthetic precursors Ia or IVa) (21–26) can inhibit the ability of LPS or lipid A to stimulate human cells. Although most investigators have assumed that these inhibitors compete with LPS for a common cellular target molecule, the site and mechanism of inhibition have not been determined.

In this paper we describe the uptake of picograms of biosynthetically radiolabeled *Escherichia coli* LPS and dLPS by the human monocyte-macrophage cell line, THP-1. (The term "uptake" is used here to refer to the association of LPS with the cell and is a measure of both membrane-bound and internalized LPS.) The uptake of both of these ligands was enhanced in the presence of LBP and blocked by a mAb to CD14. LBP also enhanced the ability of LPS to induce NF- $\kappa$ B and IL-1 $\beta$  responses by these cells, but neither dLPS nor dLPS-LBP triggered these responses. Remarkably, dLPS-LBP and LA-14-PP-LBP inhibited responses to LPS-LBP without diminishing LPS uptake by the cells. These observations indicate that CD14 can participate in the cellular uptake of both stimulatory (LPS) and nonstimulatory (dLPS) ligands. Moreover, the mechanism of inhibition by dLPS does not involve inhibition of LPS binding to CD14. Our data suggest that dLPS may block the binding of LPS to a low-abundance signaling molecule, or that the uptake of dLPS may initiate a negative signal that counteracts or blocks the LPS signal to the cell.

## Materials and Methods

**Cell Culture.** THP-1 cells were obtained from Dr. Dario C. Altieri (The Scripps Research Institute, La Jolla, CA) and were grown in RPMI-1640 with 7% fetal bovine serum (heat-inactivated at 56°C for 30 min) (Hyclone Laboratories Inc., Logan, UT), 2 mM L-glutamine, 50 U/ml penicillin G, and 50  $\mu$ g/ml streptomycin in a 5% CO<sub>2</sub> atmosphere at 37°C. To minimize cell variability, the cells were revived from frozen stock every 2–3 mo. To induce ad-

herence to plastic and expression of CD14, cultures were grown in 6-well plates (Costar, Cambridge, MA) in the presence of 0.1  $\mu$ M 1,25-dihydroxy vitamin D<sub>3</sub> (Biomol Res. Laboratories, Inc., Plymouth Meeting, PA). After 72 h, cultures were fed by adding 0.5 vol of medium containing vitamin D<sub>3</sub> and incubated for another 24 h.

**Antibodies and Reagents.** Anti-CD14 mAbs, 60b (IgG1) (27) and 26ic (IgG2b) (28), were kindly provided by Robert F. Todd (University of Michigan, Ann Arbor). Anti-CD18 mAb, 60.3 (IgG2a) (29), was provided by John M. Harlan (University of Washington, Seattle). Antibodies in ascites fluid were titrated either by FACS<sup>®</sup> analysis (Becton Dickinson & Co., Mountain View, CA) of binding to THP-1 cells, or by their ability to block LPS uptake (mAb 60b). They were used at 1:500 dilution in the experiments reported here. Nonimmune control antibody used for FACS<sup>®</sup> analysis (Becton Dickinson & Co.) was a mixture of 2  $\mu$ g/ml each of murine IgG1, 2a, 2b, and 3 isotypes (Coulter Immunology, Hialeah, FL), and FITC-labeled F(ab')<sub>2</sub> fragments of goat anti-mouse IgG (H + L) were from Tago, Inc. (Burlingame, CA). LBP, purified from rabbit acute phase serum, was generously provided by Peter S. Tobias (Scripps Clinic and Research Foundation, La Jolla, CA). Optimal amounts of LBP were determined empirically by measuring LPS uptake using each lot of LBP. Tissue culture-tested, endotoxin-tested (0.005 ng endotoxin/mg) BSA was obtained from Sigma Chemical Co. (St. Louis, MO). Double-stranded poly(dI-dC) was obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Strict pyrogen-free conditions were maintained by using sterile disposable plasticware and pipette tips, and pyrogen-free distilled water (Baxter Healthcare Corp., Deerfield, IL).

**LPS and Lipid A Preparations.** *E. coli* LCD25, a K12 derivative with Ra or Rb LPS core structure, was biosynthetically radiolabeled with [<sup>3</sup>H]acetate as described (30). Its sp act was 4,200 <sup>3</sup>H dpm/ng, and essentially all of the radioactivity was in the fatty acyl chains. The radiolabeled LPS was deacylated using AOAH (19). Sp act of the dLPS preparations used were 2,885 and 3,020 dpm/ng. Both deacylated and mock-treated LPS were suspended (100  $\mu$ g/ml) in 0.9% NaCl with 5 mg/ml BSA and stored at -70°C. Nonradioactive dLPS was produced from *E. coli* J5 LPS by using trace amounts of [<sup>3</sup>H]LPS to follow the extent of deacylation. Samples containing deacylation reaction components without LPS ("mock dLPS") were used as controls. Synthetic lipid LA-14-PP from ICN Biomedicals, Inc. (Cleveland, OH), was suspended in PBS, sonicated, and stored (0.5 mg/ml) at -70°C. Before use, an aliquot of each LPS or LA-14-PP preparation was diluted to 2  $\mu$ g/ml in RPMI-1640 containing 0.5 mg/ml BSA and sonicated for 1 s on low power with a sonifier with a small steel probe (model 450; Branson Ultrasonics Corp., Danbury, CT), and the radioactivity in the sample was counted. LPS-LBP complexes were prepared by mixing aliquots of sonicated LPS with LBP immediately before use (typically a 10-fold excess of LBP by weight was used for LPS and dLPS, and a 30-fold excess was used for LA-14-PP), incubated 10 min at 37°C, diluted with RPMI/BSA to the working concentration, and kept at room temperature until used. Tritium counting was performed in a liquid scintillation counter (Tricarb 4000 Minaxi; Packard, Downers Grove, IL) with external standardization and quench correction.

**Cell Stimulation.** 96-h cultures of vitamin D<sub>3</sub>-treated THP-1 cells were prepared in 6-well plates as described above. Nonadherent cells were removed by washing with RPMI, and incubations were performed in the absence of serum using 1 ml of RPMI containing penicillin, streptomycin, glutamine, and 0.5 mg/ml BSA. Additions of LPS and other reagents were made in 10–100  $\mu$ l volumes. After incubation, cells were washed at room temperature once with

2 ml of RPMI/BSA medium followed by two washes with 2 ml of PBS. Cells were then placed on ice until addition of buffer A (see below). Assays for uptake, NF- $\kappa$ B, IL-1 $\beta$ , and protein were performed in each experimental sample as described below. Each data point represents the mean value of duplicate wells, and error bars are shown in the figures when the range of the duplicates exceeded the size of the markers. Each observation was repeated at least three times with similar results.

**Nuclear Extracts and Cell Lysates.** The nuclear extract for NF- $\kappa$ B assay and the cell lysate for assay of cell-associated IL-1 $\beta$  and uptake of radiolabeled LPS were prepared from the same experimental sample by a micro-extraction protocol modified from previous methods (31). Adherent THP-1 cells (typically  $10^6$  cells/well) were lysed by adding 1 ml/well of cold buffer A (10 mM Hepes pH 7.9, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM dithiothreitol [DTT], 0.5 mM PMSF, and 0.1% (wt/vol) NP-40; PMSF and DTT were added freshly before use). The cell lysate was mixed in an oscillating ice bath for 5–10 min and transferred to a microcentrifuge tube. Nuclei were pelleted by centrifugation at 750 *g* for 5 min at 4°C, and an aliquot (typically 0.5 ml) of supernatant was removed for scintillation counting (LPS uptake). Another aliquot was stored at -70°C for IL-1 $\beta$  assay and the residual supernatant was aspirated. The nuclear pellet was vortexed in 15  $\mu$ l of buffer C and allowed to stand in ice for 30 min, vortexing at 10-min intervals. Buffer C consisted of 20 mM Hepes, pH 7.9, 25% glycerol (vol/vol), 420 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.5 mM DTT, and 0.5 mM PMSF. Nuclear extracts were centrifuged at 12,000 *g* for 10 min. Each supernatant was removed, mixed with 15  $\mu$ l of buffer D (10 mM Tris (Cl) pH 7.5, 0.2 mM EDTA, 1% NP-40, 0.5 mM DTT, and 0.5 mM PMSF), and stored at -70°C.

**NF- $\kappa$ B Assay.** Nuclear NF- $\kappa$ B was measured by electrophoretic mobility shift assay (EMSA) by modification of a previous method (32). 10  $\mu$ l nuclear extract (typically 4–5  $\mu$ g protein) were mixed with 5  $\mu$ l of a reaction mixture that contained, in 15  $\mu$ l final volume, 10 mM Tris(Cl), pH 7.5, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol, 1 mg/ml BSA, and 0.1 mg/ml double-stranded poly(dI-dC). After standing on ice 15 min, 5  $\mu$ l of <sup>32</sup>P-labeled oligonucleotide (typically 0.2 ng and 50,000 Cerenkov cpm) was added and allowed to stand at room temperature for 20 min. 2  $\mu$ l of 0.1% bromphenol blue was added, and 10  $\mu$ l of the mixture was loaded onto a 4% polyacrylamide mini-gel (7 cm  $\times$  8 cm  $\times$  1 mm) that had been prerun for 30 min. Electrophoresis was for 1 h at 72 V in running buffer (6.7 mM Tris(Cl) pH 7.5, 3.3 mM sodium acetate and 1.0 mM EDTA). The gel was dried onto paper under vacuum at 80°C for 1 h and visualized by exposure to Kodak X-OMAT AR film (Eastman Kodak Co., Rochester, NY). Retarded bands were cut from the dried gel and Cerenkov cpm were measured by counting in distilled water on an open tritium channel. "Relative NF- $\kappa$ B" refers to the increase in cpm/ $\mu$ g nuclear extract protein above the unstimulated control (e.g., relative NF- $\kappa$ B = 2 indicates a value that is twofold higher than the control). The NF- $\kappa$ B oligonucleotide,

5' GTTCGACAGAGGGGACTTTCCGAGAGG 3'  
3' TGTCCTCCCTGAAAAGGCTCTCCGTTG 5'

was prepared from synthetic oligonucleotides as a double-stranded 31 mer with 26 bp corresponding to the wild-type murine  $\kappa$  L chain enhancer sequence (33). <sup>32</sup>P labeling was performed with  $\alpha$ -[<sup>32</sup>P]dCTP or dATP using Klenow fragment. The labeled oligonucleotide was purified by successive passes over two Sephadex G-50 spin columns (Pharmacia Fine Chemicals).

**IL-1 $\beta$  Assay.** Cell-associated IL-1 $\beta$  was measured by ELISA using the high-sensitivity IL-1 $\beta$  kit from Cistron Biotechnology (Pine

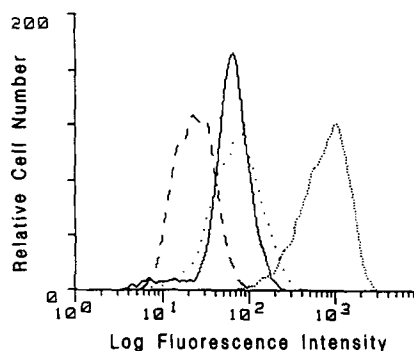
Brook, NJ). 100  $\mu$ l of postnuclear supernatant was used undiluted or diluted in buffer A. The lower limit of detection of IL-1 $\beta$  was 15–20 pg/ml.

**Protein Assay.** Cell number per well was routinely determined by protein quantitation using Coomassie Protein Assay Reagent (Pierce Chemical Co., Rockford, IL) in the manufacturer's microtiter protocol. BSA diluted in the appropriate NP-40-containing diluent was used as a standard. Correlation of protein content with cell number was done in separate experiments by resuspending adherent cells in cold PBS, counting in a hemocytometer, and assaying for protein in the presence of NP-40 as described above. One million cells contained  $\sim$ 275  $\mu$ g of protein.

**FACS<sup>®</sup> Analysis.** Cell-surface expression of CD14 was determined by suspension staining with appropriately diluted anti-CD14 mAb, nonimmune ascites or control IgG. Antibody solutions contained 1% normal goat serum for blocking. Bound mAb was detected with FITC-labeled F(ab')<sub>2</sub> fragments of goat anti-mouse IgG (H+L). Flow cytometry was performed by a FACScan<sup>®</sup> (Becton Dickinson & Co., San Jose, CA).

## Results

**Vitamin D<sub>3</sub> Induces CD14 Expression and Adherence to Plastic.** THP-1, a human leukemia cell line of the monocyte-macrophage lineage (34), was used as a model system for the present study. Although untreated THP-1 cells are not adherent and express little or no cell-surface CD14, exposure to 1,25-dihydroxyvitamin D<sub>3</sub>-induced adherence to plastic and expression of high levels of cell-surface CD14 (35) (Fig. 1). CD14 expression increased over time, reaching a level of  $\sim$ 1–2  $\times$  10<sup>6</sup> molecules/cell by 72 h of culture in vitamin D<sub>3</sub> (Peter S. Tobias, personal communication). Greater than 95% of adherent cells expressed CD14 as detected by mAbs 60b and 26ic. Stimulation with LPS for <1 h induced a



**Figure 1.** Surface expression of CD14 on THP-1 cells: effect of vitamin D<sub>3</sub>. THP-1 cells were incubated for 96 h in complete medium with 0.1  $\mu$ M vitamin D<sub>3</sub>. Nonadherent cells were removed and the adherent cells were resuspended in cold PBS and stained in suspension using mAb 60b, control antibody, or nonimmune mouse ascites. An equivalent number of cells not treated with vitamin D<sub>3</sub> was stained as above with 60b. Histograms from the same experiment were overlaid. (.....) Vitamin D<sub>3</sub>-treated cells stained with 60b; (- - -) control antibody; or (---) nonimmune ascites. (—) Untreated cells stained with 60b.

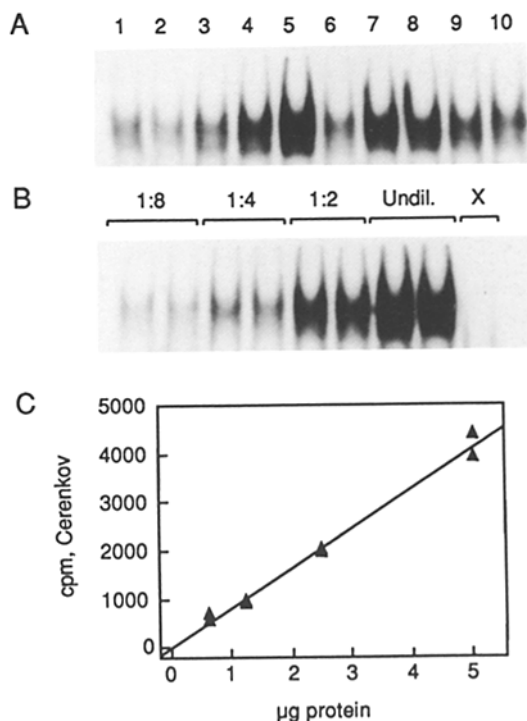
macrophage-like morphologic appearance (i.e., spreading) in many adherent cells.

**LPS Uptake.** The availability of [<sup>3</sup>H]LPS labeled to a high specific activity ( $4.2 \times 10^6$  dpm/ $\mu$ g) provided a unique opportunity to measure the uptake of LPS under conditions that were near the dose threshold for cellular responses. Twice the counting background (63 dpm) is equivalent to 15 pg [<sup>3</sup>H]LPS. Assuming an average of  $10^6$  cells/well and an average molecular mass of 4,000 kD for the LPS monomer, this represents 2,250 molecules of LPS per cell. The term "uptake" refers to cell-associated radioactivity derived from [<sup>3</sup>H]LPS or [<sup>3</sup>H]dLPS after incubation at 37°C. The location of the cell-associated ligands on or in the cell is not known. In studies not shown here, we found that <15% of the fatty acyl chains are removed from the LPS by cellular deacylation during the short incubation periods used here, suggesting that most of the cell-associated radioactivity is in intact LPS. Ligand-binding studies were not done at 4°C since significant

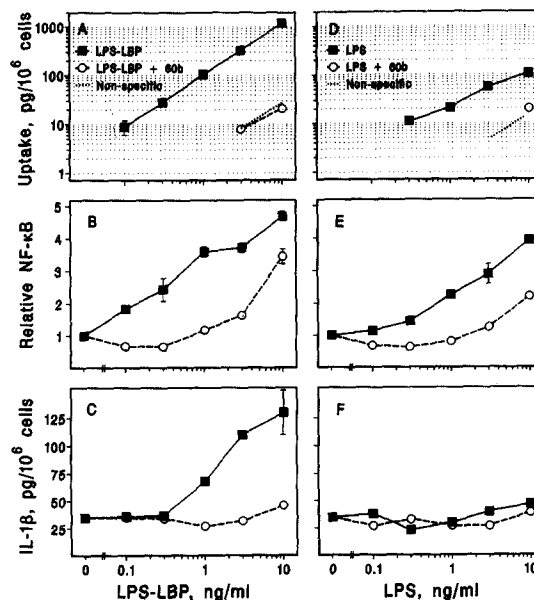
LPS binding could not be demonstrated at this temperature (data not shown).

**NF- $\kappa$ B and IL-1 $\beta$  Responses.** To correlate the uptake of [<sup>3</sup>H]LPS with a quantifiable early activation marker, we used a micro-assay for nuclear NF- $\kappa$ B binding activity. The NF- $\kappa$ B response under various conditions is shown in Fig. 2A. Unstimulated THP-1 cells expressed low levels of NF- $\kappa$ B (lane 1), and a 1-h incubation with LPS-LBP stimulated a marked increase in NF- $\kappa$ B above control levels (lanes 4 and 5). By contrast, dLPS-LBP did not stimulate NF- $\kappa$ B (lanes 2 and 3). As anticipated, dLPS was also an effective inhibitor of the NF- $\kappa$ B response to LPS (lane 6). This antagonistic effect appeared to be specific for LPS since dLPS did not inhibit the NF- $\kappa$ B response to TNF $\alpha$  (lanes 7 and 8) or to phorbol ester (lanes 9 and 10). Serial dilutions of a nuclear extract from LPS-stimulated cells showed good proportionality with Cerenkov cpm, and the ability of unlabeled oligonucleotide to block binding of the labeled oligonucleotide showed specificity (Fig. 2, B and C).

To obtain an independent assessment of cell responses, we measured the accumulation of IL-1 $\beta$  in the same experimental samples used for LPS uptake and NF- $\kappa$ B assay. The 1-h time points used in this study were too early to measure IL-1 release into the culture medium, but an early response could



**Figure 2.** EMSA for nuclear NF- $\kappa$ B in vitamin D<sub>3</sub>-treated THP-1 cells. (A) THP-1 cells ( $0.8 \times 10^6$  cells/well) were stimulated for 1 h at 37°C, as indicated, and NF- $\kappa$ B was assayed using 4.5  $\mu$ g nuclear extract protein. (1) No addition; (2) 3 ng/ml dLPS-LBP; (3) 30 ng/ml dLPS-LBP; (4) 0.1 ng/ml LPS-LBP; (5) 3 ng/ml LPS-LBP; (6) 3 ng/ml LPS-LBP + 3 ng/ml dLPS-LBP; (7) 10 pM TNF $\alpha$ ; (8) 10 pM TNF $\alpha$  + 3 ng/ml dLPS-LBP; (9) 50 nM PMA; and (10) 50 nM PMA + 3 ng/ml dLPS-LBP. An autoradiogram of the dried gel is shown. (B) Cells were incubated for 1 h at 37°C with 3 ng/ml LPS-LBP, and pooled nuclear extracts were prepared in duplicate twofold serial dilutions (10  $\mu$ l undiluted extract = 5  $\mu$ g protein). The far right lane (X) represents undiluted extract containing a 100-fold excess (25 ng) of unlabeled competitor oligonucleotide. (C) NF- $\kappa$ B bands were cut from lanes in B, <sup>32</sup>P was measured by Cerenkov counting, and the cpm were plotted against the amount of nuclear extract used.



**Figure 3.** [<sup>3</sup>H]LPS dose-response: effect of LBP and anti-CD14 mAb 60b on LPS uptake, nuclear NF- $\kappa$ B, and cell-associated IL-1 $\beta$ . Vitamin D<sub>3</sub>-treated THP-1 cells ( $10^6$  cells/well) were preincubated at 10–15°C with or without mAb 60b for 15 min. Cells were warmed to room temperature and varying amounts of [<sup>3</sup>H]LPS or [<sup>3</sup>H]dLPS (in 10–100  $\mu$ l vol) were added and incubated for 1 h at 37°C. (A) Uptake of LPS-LBP. (■-■) LPS-LBP; (○-○) LPS-LBP + 60b; and (---) Nonspecific binding of LPS-LBP to plastic. (B) NF- $\kappa$ B response. (■-■) LPS-LBP; and (○-○) LPS-LBP + 60b. (C) IL-1 $\beta$  response. (■-■) LPS-LBP; and (○-○) LPS-LBP + 60b. (D) Uptake of LPS without LBP. (■-■) LPS; (○-○) LPS + 60b; and (---) Nonspecific binding to plastic in wells with cells removed. (E) NF- $\kappa$ B response. (■-■) LPS; and (○-○) LPS + 60b. (F) IL-1 $\beta$  response. (■-■) LPS; and (○-○) LPS + 60b.

be measured in cell lysates. Although at 1 h the IL-1 $\beta$  levels induced by LPS were low (100–500 pg/10<sup>6</sup> cells compared with 25–35 pg/10<sup>6</sup> cells in unstimulated controls) and varied in separate experiments, the measurements were internally consistent within each experiment.

**LBP Augments the Uptake of LPS and Increases LPS-induced NF- $\kappa$ B and IL-1 $\beta$  Responses.** As shown in Fig. 3, A and D, LBP increased the uptake of LPS by the cells. NF- $\kappa$ B binding activity was increased by LBP throughout the dose range from 0.1 to 10 ng/ml LPS (Fig. 3, B and E), and an IL-1 $\beta$  response was observed only in the presence of LBP (Fig. 3, C and F).

**Anti-CD14 mAb 60b Blocks Uptake of LPS but Does Not Completely Prevent Cell Responses.** Also shown in Fig. 3, A and D is the impact of mAb 60b on LPS uptake. 60b blocked all detectable uptake of LPS, whether or not it was complexed with LBP. By contrast, Table 1 shows that uptake was not inhibited by mAb 26ic, previously reported to be a nonblocking

antibody to CD14 (8), or by anti-CD18 mAb 60.3. Virtually all of the residual uptake observed after blocking with 60b could be accounted for by nonspecific adherence of LPS to plastic (Table 1 and Fig. 3, A and D). In control experiments, 6-well plates were either incubated with complete medium without cells for 96 h, or adherent cells were removed from the wells by vigorous washing. [<sup>3</sup>H]LPS-LBP was then added to the wells, the plates were incubated for 1 h at 37°C, and residual <sup>3</sup>H was measured after washing the wells and adding detergent lysis buffer as described in Materials and Methods. In wells that had been incubated without cells, the average binding was 0.13%  $\pm$  0.05% SD ( $n$  = 3) of the added [<sup>3</sup>H]LPS. [<sup>3</sup>H]LPS binding was only slightly higher (0.28%  $\pm$  0.06% SD,  $n$  = 6) when cells had been removed from the plates by washing. For comparison, the uptake of LBP-bound [<sup>3</sup>H]LPS by adherent cells averaged 11%  $\pm$  4% SD ( $n$  = 26) of the added [<sup>3</sup>H]LPS.

**Table 1.** Effect of mAbs on Uptake and Response

	Uptake (pg/10 <sup>6</sup> cells)	NF- $\kappa$ B (Relative)	IL-1 $\beta$ (pg/10 <sup>6</sup> cells)
<b>LBP alone</b>			
No Ab	—	1.0 $\pm$ 0.05	26 $\pm$ 2.5
60b ( $\alpha$ CD-14)	—	0.6 $\pm$ 0.03	29 $\pm$ 0.5
26ic ( $\alpha$ CD-14)	—	0.8 $\pm$ 0.02	24 $\pm$ 0
60.3 ( $\alpha$ CD-18)	—	1.0 $\pm$ 0.20	27 $\pm$ 0
<b>[<sup>3</sup>H]LPS:LBP</b>			
No Ab	393 $\pm$ 3	5.3 $\pm$ 0.15	273 $\pm$ 45
60b ( $\alpha$ CD-14)	7 $\pm$ 2	1.7 $\pm$ 0.09	20 $\pm$ 1
26ic ( $\alpha$ CD-14)	350 $\pm$ 16	5.3 $\pm$ 0.33	282 $\pm$ 14
60.3 ( $\alpha$ CD-18)	344 $\pm$ 26	5.8 $\pm$ 0.10	246 $\pm$ 16
Nonspecific*	8	—	—
<b>[<sup>3</sup>H]dLPS:LBP</b>			
No Ab	366 $\pm$ 5	1.1 $\pm$ 0.03	23 $\pm$ 2
60b ( $\alpha$ CD-14)	19 $\pm$ 2	0.7 $\pm$ 0.04	30 $\pm$ 0.5
26ic ( $\alpha$ CD-14)	306 $\pm$ 11	0.9 $\pm$ 0.15	27 $\pm$ 4
60.3 ( $\alpha$ CD-18)	303 $\pm$ 5	1.1 $\pm$ 0.08	26 $\pm$ 0.5
Nonspecific*	14	—	—
<b>[<sup>3</sup>H]dLPS:LBP + [<sup>3</sup>H]LPS:LBP</b>			
No Ab	797 $\pm$ 3 <sup>†</sup>	1.7 $\pm$ 0.10	56 $\pm$ 1
60b ( $\alpha$ CD-14)	27 $\pm$ 0.5 <sup>†</sup>	2.0 $\pm$ 0.06	12 $\pm$ 12

Vitamin D<sub>3</sub>-treated THP-1 cells (1.1  $\times$  10<sup>6</sup> cells/well) were preincubated at 10–15°C with or without mAb for 15 min. Cells were warmed to room temperature and 3 ng/ml (50  $\mu$ l) of [<sup>3</sup>H]LPS:LBP or [<sup>3</sup>H]dLPS:LBP, or 3 ng/ml each of [<sup>3</sup>H]LPS:LBP + [<sup>3</sup>H]dLPS:LBP were added and incubated for 1 h at 37°C.

\* Nonspecific binding to plastic: cells were removed from wells by washing before incubation with labeled compounds as described above.

† Uptake in dpm from both labeled compounds was  $\sim$  equal to the sum of the uptake of the individual compounds. Conversion to pg/10<sup>6</sup> cells is based on the average specific radioactivity of the two compounds.

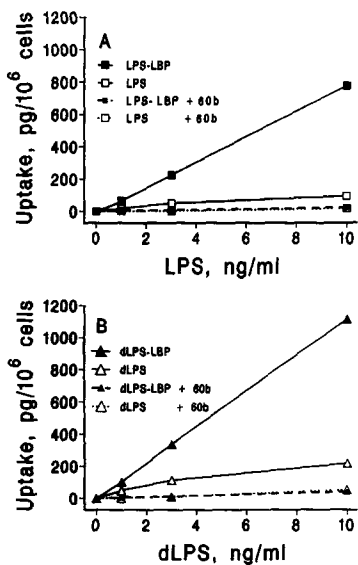
This was reduced to  $0.35\% \pm 0.14\%$  ( $n = 8$ ) after blocking with mAb 60b.

As shown in Fig. 3, B and E, mAb 60b greatly reduced cellular responses to LPS. The NF- $\kappa$ B response was suppressed by 60b at low LPS concentrations (0.1–1 ng/ml), but this inhibition was partially overcome at 3 and 10 ng/ml. mAb 60b also suppressed the IL-1 $\beta$  response (Fig. 3 C).

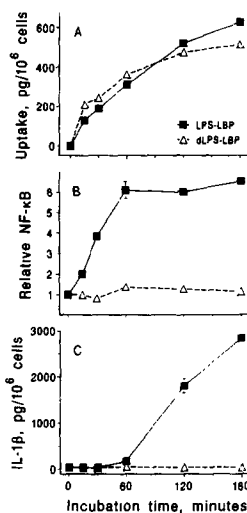
*dLPS Uptake Is Also Enhanced by LBP and Blocked by mAb 60b* Fig. 4 shows dose-response curves for [ $^3$ H]dLPS and [ $^3$ H]LPS uptake, in the presence and absence of a 10-fold excess of LBP. LBP greatly enhanced the uptake of both compounds. Optimal amounts of each batch of purified LBP were determined empirically. It was typical for a 5- to 10-fold excess (by weight) of LBP to produce maximal uptake when preincubated in concentrated solution with LPS or dLPS (see Materials and Methods). Using LPS-LBP or dLPS-LBP complexes preformed at a ratio of 1:10, LBP increased LPS uptake 5.3 fold ( $\pm 2.6$  SD,  $n = 22$ ) and dLPS uptake 4.2 fold ( $\pm 1.2$  SD,  $n = 6$ ). Fig. 4 also shows that, as for LPS (A), the uptake of dLPS (B) was inhibited by 60b, whether or not the dLPS was complexed with LBP.

The time course for uptake of [ $^3$ H]dLPS-LBP was nearly identical to that of [ $^3$ H]LPS-LBP (Fig. 5 A).

*LPS Induces Nuclear NF- $\kappa$ B Binding Activity and Cell-associated IL-1 $\beta$ . dLPS Does Not.* LPS was a potent stimulus to NF- $\kappa$ B binding activity and IL-1 $\beta$  synthesis, but dLPS did not elicit either of these responses (Figs. 2 and 3, and Fig. 5, B and C). Since the uptake of dLPS was enhanced by LBP and blocked



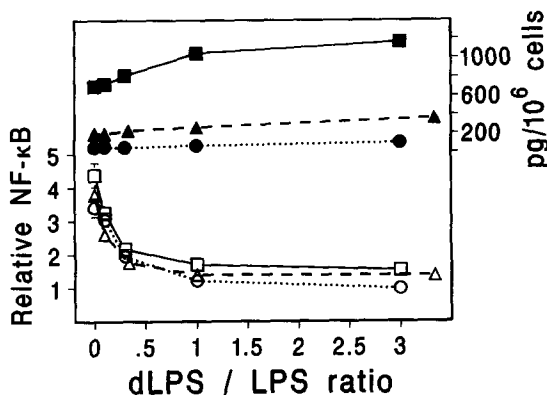
**Figure 4.** Dose-response: effect of LBP and anti-CD14 mAb 60b on the uptake of [ $^3$ H]LPS and [ $^3$ H]dLPS. Vitamin D<sub>3</sub>-treated THP-1 cells ( $10^6$  cells/well) were preincubated at 10–15°C with or without 60b for 15 min. Cells were warmed to room temperature and varying amounts of radiolabeled ligand (10–100  $\mu$ l) were added and incubated for 1 h at 37°C. LPS-LBP and dLPS-LBP denote preformed complexes made by preincubation of a 2  $\mu$ g/ml solution of [ $^3$ H]LPS or [ $^3$ H]dLPS with 20  $\mu$ g/ml LBP for 10 min at 37°C.



**Figure 5.** Time course for [ $^3$ H]LPS and [ $^3$ H]dLPS uptake and responses. Vitamin D<sub>3</sub>-treated THP-1 cells ( $0.7 \times 10^6$  cells per well) were incubated with 3 ng/ml [ $^3$ H]LPS-LBP or [ $^3$ H]dLPS-LBP for 15, 30, 60, 120, and 180 min at 37°C.

by mAb 60b, consistent with uptake via CD14, this observation indicates that engaging CD14 with an LBP-complexed ligand does not necessarily trigger these cell responses.

*dLPS and LA-14-PP Inhibit Responses to LPS without Inhibiting LPS Uptake.* To characterize the antagonistic effects of LPS in terms of both [ $^3$ H]LPS uptake and cellular response, we looked at the interaction of unlabeled dLPS and [ $^3$ H]LPS ligands that were each prebound to optimal amounts of LBP before they were added to the cells. dLPS-LBP was added in varying amounts to three concentrations of [ $^3$ H]LPS-LBP. As shown in Fig. 6, dLPS inhibited NF- $\kappa$ B responses without inhibiting LPS uptake. In experiments not shown, when dLPS-LBP and LPS-LBP were added to cells in equal concentrations, NF- $\kappa$ B levels were inhibited by  $66 \pm 6\%$  SD ( $n = 9$ ) compared to control levels obtained with LPS-LBP alone, yet LPS uptake was not inhibited. The uptake of [ $^3$ H]LPS



**Figure 6.** Inhibition of LPS response by dLPS without inhibition of LPS intake. Vitamin D<sub>3</sub>-treated THP-1 cells ( $0.7 \times 10^6$  cells/well) were incubated with 1, 3, or 10 ng/ml [ $^3$ H]LPS-LBP with varying amounts of unlabeled dLPS-LBP for 1 h at 37°C. (Top, lines with filled markers) LPS uptake. (Bottom, lines with open markers) Nuclear NF- $\kappa$ B response. (Circles with dotted line) 1 ng/ml LPS; (Triangles with dashed line) 3 ng/ml LPS; and (Squares with solid line) 10 ng/ml LPS.

that occurred in the presence of dLPS was also inhibitable by mAb 60b (Table 1, and data not shown). In experiments not shown, when [<sup>3</sup>H]LPS and dLPS were not prebound to LBP but were added directly to cells in incubation medium without LBP or with limiting concentrations of LBP, LPS uptake was inhibited to a variable extent (<50% inhibition). This inhibition was largely overcome as the LBP concentration was increased. NF- $\kappa$ B and IL-1 $\beta$  responses were dramatically inhibited by dLPS in the presence or absence of LBP.

To eliminate the possibility that inhibition by dLPS is due to a biological contaminant in the dLPS preparation, similar experiments were performed using LA-14-PP. LA-14-PP is a chemically defined, synthetic analog of lipid A which is structurally equivalent to the lipid A moiety of dLPS. As shown in Fig. 7, LA-14-PP-LBP also inhibited NF- $\kappa$ B and IL-1 $\beta$  responses without inhibiting [<sup>3</sup>H]LPS-LBP uptake.

Similar experiments in which [<sup>3</sup>H]dLPS-LBP was added to cells with [<sup>3</sup>H]LPS-LBP (Table 1) were done to exclude the possibility that any characteristics of dLPS inhibition are due to unanticipated structural differences between the unlabeled *E. coli* J5 dLPS and the *E. coli* K12 [<sup>3</sup>H]LPS. As expected, the uptake of each of the labeled ligands was additive, cellular responses were inhibited, and mAb 60b blocked the uptake of both ligands. Control experiments (not shown), in which dLPS was substituted with "mock dLPS" (see Materials and Methods), confirmed that the presence of residual AOA in the dLPS preparations did not inhibit the ability of LPS to stimulate a cellular response.

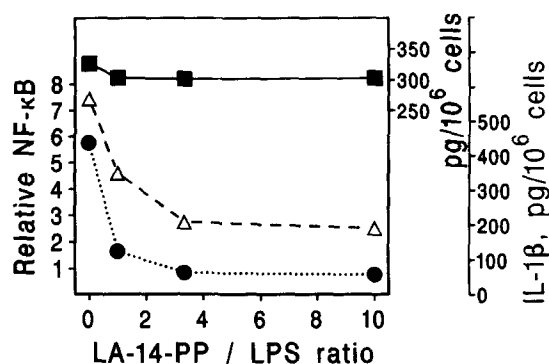
**Inhibition by dLPS Is Not a Result of LPS Sequestration.** Since LPS can form aggregates in solution (36, 37), we addressed the possibility that dLPS might sequester LPS binding site(s) that are important for inducing cell responses. We incubated THP-1 cells with varying amounts of dLPS-LBP (0–1 ng/ml) for 1 h, washed the cells, and added 3 ng/ml of [<sup>3</sup>H]LPS-LBP. As shown in Fig. 8, preincubation with as little as 0.3 ng/ml dLPS for 1 h inhibited the NF- $\kappa$ B response to 3 ng/ml LPS by 50%, yet LPS uptake was not inhibited. In experi-

ments not shown, NF- $\kappa$ B responses to submaximal stimulatory amounts of TNF $\alpha$  and phorbol ester were not inhibited by dLPS preincubation.

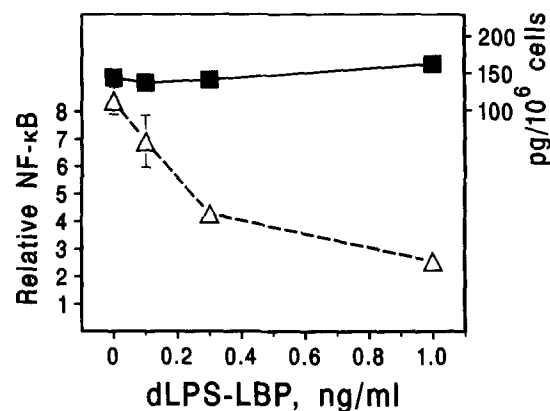
## Discussion

Two general models have been put forward to account for the role of CD14 in cellular responses to LPS (1, 2). The first model proposes that when LPS or LPS-LBP binds CD14, the LPS signal is directly transduced to the cell. In keeping with this model, mAbs to CD14 can trigger a number of responses in monocytes and neutrophils, including ligand internalization, transient oxidative burst, IL-1 production (14, 38), homotypic adhesion (15), and calcium fluxes (38). CD14 itself need not be the signal transducer, however, since the signaling molecule might associate with CD14 after initial ligand binding (like the interaction of gp130 with IL-6R) (39), or may be part of a molecular complex with CD14. Close association of CD14 with a protein kinase (40), for example, might account for the finding that protein tyrosine phosphorylation is induced as an early response to LPS in murine macrophages (41). In the second model, CD14 is not so directly involved in signaling. Rather, it binds LPS on the cell membrane, where the LPS can interact (possibly by transfer within the membrane or cell) with the actual signaling molecule(s) (1). This model was proposed to account for the observation that high LPS concentrations overcome the inhibitory effect of blockade or depletion of CD14 on LPS-stimulated TNF $\alpha$  production (2, 8).

We found that the binding of both LPS and dLPS to THP-1 cells was augmented by LBP and inhibited by anti-CD14 mAb 60b, indicating that both ligands were interacting with LBP and CD14, yet only LPS induced NF- $\kappa$ B and IL-1 $\beta$  responses. The ability of LPS and dLPS to associate with the cells in the absence of LBP was also inhibited by mAb 60b, suggesting



**Figure 7.** Inhibition of LPS response by LA-14-PP without inhibition of LPS uptake. Vitamin D<sub>3</sub>-treated THP-1 cells ( $1.1 \times 10^6$  cells/well) were incubated with 3 ng/ml [<sup>3</sup>H]LPS-LBP with varying amounts of unlabeled LA-14-PP-LBP for 1 h at 37°C. (Filled squares with solid line) LPS uptake; (Open triangles with dashed line) NF- $\kappa$ B; and (Filled circles with dotted line) cell-associated IL-1 $\beta$ .



**Figure 8.** Inhibition of LPS response by preincubation with dLPS. Vitamin D<sub>3</sub>-treated THP-1 cells ( $0.4 \times 10^6$  cells/well) were preincubated with varying amounts of unlabeled dLPS-LBP for 1 h at 37°C. Cells were washed with RPMI-1640 and incubated with 3 ng/ml [<sup>3</sup>H]LPS-LBP for 1 h at 37°C. (Filled squares with solid line) LPS uptake; and (Open triangles with dashed line) nuclear NF- $\kappa$ B response.

that both ligands may bind directly to CD14, but only LPS elicited cell responses. CD14 may therefore bind both stimulatory (LPS) and nonstimulatory (dLPS) ligands in THP-1 cells, suggesting that engagement of CD14 is insufficient to produce signals resulting in NF- $\kappa$ B binding activity and IL-1 $\beta$  production.

Another remarkable finding, that dLPS and LA-14-PP blocked responses to LPS without inhibiting LPS uptake via CD14, seems most consistent with the second model. The site of apparent competition between LPS and dLPS occurs after the interaction of LPS with CD14 and might therefore involve binding to a low abundance molecule, the putative signal transducer. A different mechanism for dLPS inhibition of responses to LPS cannot be excluded, however. Since dLPS-LBP is able to inhibit responses to 10-fold or greater amounts of LPS-LBP, yet does not block the same responses to TNF $\alpha$  or phorbol ester, dLPS may be able to induce a LPS-specific inhibitory pathway. Thus, binding dLPS to CD14 or another molecule might initiate a negative signal that counteracts the positive signal from LPS.

In keeping with the results of Wright et al. (8), we also observed that high concentrations (10 ng/ml) of LPS partially overcame the inhibition of NF- $\kappa$ B binding activity which resulted from blocking LPS binding to CD14 by mAb 60b. Since the uptake of LPS by the 60b-treated cells was not above background (nonspecific) binding using our radiolabeled probe, small amounts of LPS may have been taken up by the cells via non-CD14 mechanisms and then interacted with another molecule or molecules in the signal pathway.

It seems likely that dLPS and LA-14-PP did not inhibit LPS uptake in our studies because the number of molecules of cell-surface CD14 greatly exceeded the number of molecules of ligand bound to the cells. By using LPS that was biosynthetically radiolabeled to high specific activity (30), we were able to measure much smaller amounts of LPS and dLPS than had previously been possible in studies of LPS-cell interactions. The picogram amounts of cell-associated LPS that were measured in these experiments were near the dose

threshold for cellular responses in this *in vitro* system. The LPS uptake was equivalent to 2,250–165,000 molecules per cell, well below the number of CD14 molecules on the cells ( $10^6$  or more). The great excess of cell-surface CD14 thus provides a likely explanation for the lack of inhibition of LPS-LBP uptake by dLPS-LBP. Recently, Couturier et al. (42) reported binding of derivatized [ $^3$ H]LPS to human monocytes in the presence of FCS at room temperature. Binding appeared to be dose-dependent, saturable, displaceable, and inhibitable by antibodies to CD14. Saturating amounts of LPS ( $\sim 1$   $\mu$ g added per million cells) were much higher than the concentrations used in the present study (0.1–10 ng LPS per million cells), again suggesting that saturation of CD14 occurs at LPS concentrations far greater than the low concentrations used here.

The present results seem most consistent with a scenario in which CD14 binds LPS and LPS-LBP, accumulating LPS on the plasma membrane where the fully acylated lipid A moiety can then interact with other key molecules in the response cascade. dLPS may also interact with these molecules but, lacking the necessary structural information (acyloxyacyl groups), dLPS is unable to initiate the signal. The ability of dLPS to inhibit responses to much larger amounts of LPS, despite the presence of a large excess of CD14 on the cells, suggests that the putative signaling molecules are much less abundant than CD14.

The ability of preincubation with dLPS to prevent cellular responses to much larger amounts of LPS reinforces the suggestion (18–20) that dLPS, if generated *in vivo*, might blunt responses to LPS and provide an LPS-specific mechanism for controlling the inflammatory response. Our findings also suggest that dLPS, generated intracellularly, might play a role in the induction of tolerance to LPS. Finally, it is possible, as suggested by Kovach et al. (23), that part-structures of lipid A or LPS that lack acyloxyacyl groups could be useful therapeutics for gram-negative bacterial sepsis. Our results provide support for all of these speculations and encourage further efforts to evaluate them experimentally.

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