Monocyte Adherence Induced by Lipopolysaccharide Involves CD14, LFA-1, and Cytohesin-1

REGULATION BY Rho AND PHOSPHATIDYLINOSITOL 3-KINASE

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Mechanisms regulating lipopolysaccharide (LPS)-induced adherence to intercellular adhesion molecule (ICAM)-1 were examined using THP-1-cell transfected with CD14-cDNA (THP-1wt). THP-1wt adherence to ICAM-1 was LPS dose-related, time-dependent, and inhibited by antibodies to either CD14 or leukocyte function associated antigen-1 (LFA)-1, but was independent of any change in the number of surface expressed LFA-1 molecules. A potential role for phosphatidylinositol (PI) 3-kinase (PI 3-kinase) in LPS-induced adherence was examined using the PI 3-kinase inhibitors LY294002 and Wortmannin. Both inhibitors selectively attenuated LPS-induced adherence, but not phorbol 12-myristate 13-acetate-induced adherence. Inhibition by these agents was unrelated to any changes in either LPS binding to or LFA-1 expression by THP-1wt cells. LPS-induced adherence was also abrogated in U937 cells transfected with a dominant negative mutant of PI 3-kinase. Toxin B from Clostridium difficile, an inhibitor of the Rho family of GTP-binding proteins, abrogated both PI 3-kinase activation and adherence induced by LPS. Cytohesin-1, a phosphatidylinositol 3,4,5-triphosphate-regulated adapter molecule for LFA-1 activation, was found to be expressed in THP-1wt cells. In addition, treatment of THP-1wt with cytohesin-1 antisense attenuated LPS-induced adherence. These findings suggest a model in which LPS induces adherence through a process of “inside-out” signaling involving CD14, Rho, and PI 3-kinase. This converts low avidity LFA-1 into an active form capable of increased binding to ICAM-1. This change in LFA-1 appears to be cytohesin-1-dependent.

Adherence of monocytes to endothelial cells is an essential requirement for the localization of these cells to sites of tissue inflammation (1–3). Several reports have shown that this process is dependent upon the monocyte surface molecule lymphocyte function-associated antigen-1 (LFA-1)α (CD11a/CD18; αβ2) (Refs. 3–5 and reviewed in Refs. 6 and 7). Intercellular adhesion molecule-1 (ICAM-1) (CD54) has been identified as a high affinity counter-receptor for LFA-1 (8). Interactions of ICAM-1 with LFA-1 mediate several important functions in the immune system in addition to adherence (6). The basal affinity of LFA-1 for ICAM-1 or its other ligands is low and LFA-1 must be activated to mediate stable adherence (4, 5). Indeed, in its activated form, the affinity of LFA-1 for ICAM-1 increases 200-fold in comparison with its affinity in the resting state (9). This is consistent with a process of “inside-out” signaling that converts LFA-1 into an activated form capable of mediating increased adhesion. It is important to note that conditions which give rise to increased adherence do not necessarily lead to increased cell surface expression of LFA-1 (5, 10).

The signaling events that link cell stimulation to the activation of LFA-1 are incompletely understood. Recently, a regulatory protein that interacts with the cytoplasmic tail of CD18 has been cloned (11). This protein, cytohesin-1, contains a pleckstrin homology domain that binds the phosphatidylinositol 3-kinase (PI 3-kinase) metabolite, phosphatidylinositol 3,4,5-trisphosphate (PtdIns3,4,5-P3), leading to changes in properties of the protein (12). These findings suggest a potential role for PI 3-kinase in regulating the activity of LFA-1.

Bacterial lipopolysaccharide (LPS) is known to enhance the accumulation of leukocytes at inflammatory foci (13) and the adherence of leukocytes to endothelial cells in vitro (14, 15). Although it has also been shown that LPS-induced adherence is mediated at least in part by LFA-1 (16), the pathway linking LPS to LFA-1 has not been characterized. A dominant LPS signaling pathway involves the membrane receptor CD14 (17, 18). Binding of LPS to CD14 results in the activation of multiple Src family protein tyrosine kinases, and this appears to involve the physical association of p53/p56Fyn with the receptor (19). It has also been shown that LPS induces the CD14-dependent association of an activated form of PI 3-kinase with p53/p56Fyn (20). Furthermore, activation of monocyte PI 3-kinase by LPS results in the generation of PtdIns3,4,5-P3 (21), which as discussed above is known to regulate various effector functions including cell adhesion (22, 23). Taken together, THP-1 cells transfected with CD14-cDNA; THP-1wt, THP-1wt cells transfected with vector alone; PI 3-kinase, phosphatidylinositol 3-kinase; PMA, phorbol 12-myristate 13-acetate; PtdIns3,4,5-P3, phosphatidylinositol 3,4,5-trisphosphate; HBSS, Hanks’ balanced salt solution; Wpsa, wild-type bovine PI 3-kinase subunit p85; Δp85a, α-chain, mutant bovine p85α; MFI, mean fluorescence intensity; FCS, fetal calf serum; S-abc, phosphorothioate modified oligonucleotide; PTTC, fluorescence isothiocyanate; α-Mb, monoclonal antibody; BSA, bovine serum albumin; RT-PCR, reverse transcription-polymerase chain reaction; FACS, fluorescence-activated cell sorter.
these findings suggest the possibility that LPS-induced adhesion may be mediated through a pathway involving PI 3-kinase leading to changes in LFA-1 activity. The results of the present study show that LPS binding to CD14 induces monocyte adherence dependent upon LFA-1, ICAM-1, and cytohesin-1, via a PI 3-kinase-dependent pathway regulated by the small GTP-binding protein Rheo.

**EXPERIMENTAL PROCEDURES**

**Reagents and Chemicals**—RPMI 1640, Hanks’ balanced salt solution (HBSS), and penicillin/streptomycin were from Stem Cell Technologies (Vancouver, British Columbia). Human AB serum was purchased from The Canadian Red Cross (Vancouver, British Columbia). LPS from *Escherichia coli* O127:B8 was from Difco. LPS was labeled with FITC as described (24, 25). Purified soluble, recombinant ICAM-1 (sICAM-1) was a generous gift from Dr. J. R. Woska, Jr. (Boehringer Ingelheim, Ridgefield, CT). Phorbol 12-myristate 13-acetate (PMA), wortmannin, p85-α-phosphatidyl (α,ω-serine), p-α-phosphatidylidylinositol (PtdIns), and p-α-phosphatidylinositol 4,5-diphosphate were purchased from Sigma. LY294002 was from Calbiochem. Toxin B purified from *Clostridium difficile* was generously provided by Dr. G. Armstrong (University of Alberta, Edmonton, Alberta). Protein A-agarose, and electrophoresis reagents were purchased from Bio-Rad. [γ-32P]ATP was from Amer sham (Oakville, Ontario, Canada).

**Monoclonal Antibodies** (mAbs)—The following mAbs were used: 3C10 (IgG2b, anti-CD14 mAb, a gift from Dr. W. C. Van Voorhis, University of Washington, Seattle, WA), W6/32 (IgG2a, anti-HLA-class I, American Type Culture Collection, Manassas, VA), TS1/18 (IgG1, anti-CD18), and TS1/22 (IgG1, anti-CD11a) were from the Hybridgetm Bank of the University of Iowa, Iowa city, IA. Anti-PI 3-kinase mAb was from Upstate Biotechnology (Lake Placid, NY).

**Cell Lines**—The monocytic cell lines THP-1wt (THP-1 cells stably expressing glycosylphosphatidylinositol-anchored CD14) and THP-1-rsv (THP-1 cells transfected with vector alone) were kindly provided by Dr. R. Ulevitch (The Scripps Research Institute, La Jolla, CA). Transfection of the promonocytic cell line U937 with cDNA encoding the entire coding region of either wild-type bovine PI 3-kinase subunit p85α (Wp85α) or mutant bovine p85α (Ap85α) was as described (21). The mutant has a deletion of 35 amino acids from residues 479–513 of bovine p85α and the insertion of two other amino acids (Ser-Arg) in the deleted position. This alteration prevents the association of mutant p85α with the p110 catalytic subunit. Mutant p85α competes with native p85 for binding to essential signaling proteins, thereby acting as a dominant negative mutant (21, 26). These transfected cell lines are referred to as Wp85-U937 and Ap85-U937. All cell lines were cultured in RPMI 1640 supplemented with 10% FCS (HyClone, Logan, UT), 2 mm L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml).

**Adhesion to Immobilized sICAM-1**—Flat-bottomed, polystyrene, cell culture-treated, microtiter plates (Becton Dickinson, Franklin Lakes, NJ) were loaded with purified sICAM or BSA (coating control) by carefully washing three times with 200 μl of warm (37 °C) incubation buffer and three times with 10 mM Tris-HCl (pH 7.4). PI 3-kinase activity was measured as described (20). Briefly, immunoprecipitates were incubated for 10 min at 4 °C with 10 μg of sonicated (3 × 20 s in a ultrasonic cell disruptor, Branson Sonic Power Co., Danbury, CT) PtdIns in 10 μl of 50 mM Hepes to which was added 60 μl of kinase buffer and three times with 10 mM Tris-HCl (pH 7.4). PI 3-kinase activity was monitored by measuring incorporation of [γ-32P]ATP into PI 3-phosphatidyl-L-serine, L- phosphatidyl-serine, and phosphatidylinositol 4,5-diphosphate (27). PI 3-kinase activity was expressed as dpm incorporated as described (20).

**RESULTS**

**THP-1 Phenotype Analysis**—Surface expression of CD14 and LFA-1 molecules was analyzed by immunofluorescence and FACS analysis. The representative record shown in Fig. 1 demonstrates that cells transfected with CD14 (THP-1wt) and control cells transfected with vector alone (THP-1-rsv) expressed similar levels of both LFA-1α and β chains (CD11a and CD18, respectively). However, THP-1wt cells expressed about 90-fold more CD14 than did THP-1-rsv cells.

**Adherence of THP-1 Cells to Immobilized sICAM-1-coated Plates**—Initial experiments were carried out to standardize the model system of LPS-induced adherence of THP-1 cells to sICAM-1. CD14-transfected THP-1wt cells and control THP-1-rsv cells were incubated in microtiter wells coated with sICAM-1 at concentrations ranging from 20 to 40 μg/ml and treated with LPS in the presence of 0.5% normal AB serum. THP-1wt cells adhered to sICAM-1 in a dose-dependent manner with a maximum of 48 ± 8.7% cells binding to wells coated with 20 μg/ml of sICAM-1 (Fig. 2A). In contrast, control THP-1-rsv cells adhered at maximum rate of 8.3 ± 2%. In the absence of sICAM-1, only 4–5% of the THP-1wt cell adhered nonspecifically to the plate (data not shown). Binding specificity for ICAM-1 was also demonstrated by control experiments in which only 4.8 ± 3.2% of THP-1wt cells adhered to wells coated with BSA at 20 μg/ml (n = 4, data not shown).

THP-1wt and THP-1-rsv cells were also examined for adher-
A concentration of 10
countries of anti-CD14 and anti-LFA-1 mAbs were tested,
(TS1/18) or anti-CD18 mAb (TS1/22) (30, 31). When a range
significant inhibition with either neutralizing anti-CD11a mAb
of LFA-1 in this model. The results obtained (Fig. 3
with mAb was also used to examine the extent of involvement
mately 80% inhibition of adherence. Competitive inhibition
cells with mAb 3C10 prior exposure to LPS led to approxi-
absence of serum resulted in markedly reduced adherence
for the second hour. The importance of serum was also ad-
lated adherence was maximal by 60 min and remained stable
course of THP-1 adherence to sICAM-1 (Fig. 2
pendent. In other experiments, cells were incubated with LPS
LPS-induced adherence in this system was largely CD14-de-
resulted in maximal
5000 events. In each panel, histograms displaced to the
pressed as histograms of fluorescence intensity (log scale) derived from
fixed in 2% paraformaldehyde before FACS analysis. Results are ex-
gated F(ab)² sheep anti-mouse IgG. Samples were washed twice and
a
b
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Fig. 1. CD14 and LFA-1 surface expression on THP-1 cell lines.
THP-1rsv and THP-1wt cells were incubated for 30 min at 4 °C with
either anti-CD14 (a), anti-CD18 (LFA-1 β-chain) (b), or anti-CD11a
(LFA-1 α-chain) (c), then washed twice and labeled with FITC-conju-
gated Fab', sheep anti-mouse IgG. Samples were washed twice and
fixed in 2% paraformaldehyde before FACS analysis. Results are ex-
pressed as histograms of fluorescence intensity (log scale) derived from
5000 events. In each panel, histograms displaced to the right represent
cells stained with specific mAbs, and histograms on the left represent
cells stained with irrelevant isotype-matched IgG. Numerical values in
the top right of each frame indicate the MFI index, which corresponds
to the ratio: MFI of cells incubated with specific antibody/MFI of cells
stained with irrelevant isotype-matched IgG. The data shown are rep-
resentative of results obtained in five separate experiments yielding
similar results.
ence in the presence of a range (0.1–10 µg/ml) of concentrations
of LPS (Fig. 2B). Treatment of THP-1wt cells with LPS resulted
in a dose-dependent increase in adherence that was maximal
(52.1 ± 6.1%, mean ± S.E., n = 2) at 1 µg/ml. Maximal adherence
observed with THP-1rsv was only 10.7 ± 1.8% (mean ±
S.E., n = 2), and this was not affected by LPS, indicating that
LPS-induced adherence in this system was largely CD14-de-
pendent. In other experiments, cells were incubated with LPS
(1 µg/ml in 0.5% AB⁺ serum) for up to 2 h to determine the time
course of THP-1 adherence to sICAM-1 (Fig. 2C). LPS-stimu-
lated adherence was maximal by 60 min and remained stable
for the second hour. The importance of serum was also ad-
dressed. The results showed that exposure of cells to LPS in the
absence of serum resulted in markedly reduced adherence
(12.1 ± 0.8%, mean ± S.E., n = 2). Conversely, treatment with
serum alone did not induce cell adherence (data not shown).

Cell Surface Molecules Involved in LPS-induced THP-1 Ad-
herence to sICAM-1—As shown above, only cells transfected
with CD14 displayed enhanced adherence in response to LPS.
The role of CD14 in cell adherence was investigated further by
competitive inhibition with a neutralizing anti-CD14 mAb
3C10 (18, 29). Fig. 3A shows that preincubation of THP-1wt
cells with mAb 3C10 prior exposure to LPS led to approxi-
mately 80% inhibition of adherence. Competitive inhibition
with mAb was also used to examine the extent of involvement
of LFA-1 in this model. The results obtained (Fig. 3A) show
significant inhibition with either neutralizing anti-CD11a mAb
(TS1/18) or anti-CD18 mAb (TS1/22) (30, 31). When a range
of concentrations of anti-CD14 and anti-LFA-1 mAbs were tested,
concentration of 10 µg/ml was observed to result in maximal
inhibition of LPS-induced adherence (data not shown). Furt-
thermore, when compared with blockade of either CD11a or

CD18 alone, inhibition (85%) was enhanced when anti-CD18
and anti-CD11a were used in combination. Specificity of inhi-
bition by anti-CD14 and anti-LFA-1 mAbs was validated using
anti-MHC class I mAb, W6/32. Despite its binding to constitu-
tively expressed cell surface molecules, W6/32 did not affect
LPS-induced adherence.

Certain agonists are known to induce increased adherence
by either up-regulating integrin expression, by increasing in-
tegrin affinity for substrate or by a combination of these effects

Fig. 2. THP-1 cell adherence to immobilized sICAM-1. Flat-
topped, polystyrene cell culture-treated 96-well microtiter plates
were loaded with 10⁵ cells/well in 200 µl of RPMI 1640 (final volume),
and cells were allowed to adhere at 37 °C in a humidified atmosphere
containing 5% CO₂. Unbound cells were washed away, and attached
cells were fixed with 2% paraformaldehyde/HBSS and stained with
0.05% crystal violet. Excess staining solution was rinsed away, and the
absorbance of the dye retained by the adherent cells (eluted by addition
of 100% methanol) was measured in individual wells at 570 nm. Dupli-
cate determinations were made for each data point. Adherence was quan-
titated using a standard curve generated with a range of known
input cell numbers. A, adherence of THP-1wt or THP-1rsv cells in response to 2 µg/ml LPS (in presence of 0.5% AB⁺) assayed for 1 h in
wells precoated with a range of concentration of sICAM-1 (0–40 µg/ml).
B, THP-1wt cells were stimulated with a range of concentration of LPS
(0–10 µg/ml) in presence of 0.5% AB⁺ serum for 1 h in wells coated with
20 µg/ml of sICAM-1 or BSA. C, adherence of THP-1wt and THP-1rsv
cells stimulated with LPS (1 µg/ml in 0.5% AB⁺ serum) for different
times (7 min to 2 h) in wells coated with 20 µg/ml sICAM-1. The values
shown in each panel are the averages of two independent determina-
tions obtained in separate experiments.
not affect the expression of these proteins by THP-1wt cells. In contrast, incubation of cells with fMet-Leu-Phe, an agonist known to up-regulate integrin expression (33), induced significant increases in expression of both CD11a and CD18. Taken together, these findings suggest that LPS does not modify the number of surface expressed LFA-1 molecules, but rather initiates a signaling sequence through CD14 leading to increased avidity of LFA-1 for ICAM-1.

LPS-induced Adherence Is Phosphatidylinositol 3-Kinase-dependent—Recent evidence has suggested a role for PI 3-kinase in signaling pathways activated by LPS (20, 21). To examine the potential involvement of PI 3-kinase in LPS-induced adherence, cells were incubated with various concentrations of the PI 3-kinase inhibitors wortmannin and LY294002 for 20 min prior to the addition of LPS. Preincubation with wortmannin inhibited LPS-induced adherence in a dose-dependent manner (Fig. 4A, maximum inhibition 92.9 ± 6.7%, mean ± S.E., n = 3) with an IC50 of approximately 1 nM. This value is 10 times lower than the IC50 for wortmannin as determined for inhibition of fMet-Leu-Phe-stimulated neutrophil homotypic aggregation (33). LY294002, an inhibitor of PI 3-kinase that acts via a distinct mechanism, when used at concentrations known to be relatively selective for inhibition of PI 3-kinase, also attenuated LPS-induced adherence (Fig. 4B, maximum inhibition 89.5 ± 2.1%, mean ± S.E., IC50 ~0.45 μM, n = 3). In contrast to abrogation of LPS-induced adherence, neither wortmannin nor LY294002 had significant effects on PMA-induced adherence, except at high concentrations. These results show that inhibition of LPS-induced adherence by wortmannin or LY294002 is not due to nonspecific toxicity. Moreover, they suggest that the pathways regulating adherence in response to PMA and LPS are distinct.

PI 3-kinase inhibitors have been shown to affect the expression of some cell surface receptors (34). Experiments were done, therefore, to examine the effects of wortmannin and LY294002 on the expression of CD11a and CD18. The data shown in Fig. 4C indicate that high concentration of inhibitors (100 nM wortmannin and 16 μM LY294002) resulted in only small changes (20–30% reductions) in surface expression of CD18 and CD11a. These modest changes appear insufficient to explain the marked attenuation of LFA-1-mediated adherence to sICAM-1. Binding of LPS to cells was also examined using FITC-LPS and FACS analysis. LPS binding was not altered in THP-1wt cells pretreated with either wortmannin or LY294002 (data not shown).

The requirement for PI 3-kinase in LPS-induced adherence was also examined in cells transfected with a dominant negative mutant of p85 (Δp85). Stable transfection with Δp85 resulted in a significant reduction in LPS-stimulated PI 3-kinase activity (Fig. 5A), and this correlated with marked attenuation of cell adherence to sICAM-1 (Fig. 5B). In contrast, cells transfected with wild-type p85 showed both LPS-stimulated PI 3-kinase activity and adherence. Taken together, these findings suggest that PI 3-kinase activation plays a central role in LPS-induced adherence.

Rho Regulates PI 3-Kinase Activation and LPS-induced Adherence—Small GTP-binding proteins of the Rho family participate in various important signaling pathways, including those regulating cellular adhesion (35–37). C. difficile toxin B, which specifically inhibits Rho proteins (38, 39), was used to investigate the potential role of Rho in the regulation of LPS-induced adherence. Pretreatment for 30 min with 2 nM toxin B resulted in significant and maximal attenuation of LPS-induced adherence of THP-1wt to sICAM-1 (Fig. 6A) (63.5 ± 5.0% inhibition, mean ± S.E., n = 3). In contrast, induction of adherence in response to PMA was toxin-resistant. To analyze further the
inhibitory effect of toxin B, a dose response analysis was performed. Three separate experiments showed that IC_{50} for toxin inhibition of LPS-induced adherence was ~4 nM and that 20 nM produced maximal inhibition (86.0 ± 2.8% inhibition, mean ± S.E., n = 3). In contrast, maximal inhibition of PMA-induced adherence was less than 20%. To address whether Rho regulates activation of PI 3-kinase in response to LPS, cells were incubated with toxin B under the same experimental condition as those used for adherence and then stimulated with LPS for 20 min. The data shown in Fig. 6 indicate that toxin B reduced LPS-induced PI 3-kinase activity in a dose-dependent manner.

LPS-induced Adherence Is Cytohesin-1-dependent—Cytohesin-1 is an adaptor molecule that interacts specifically with the cytoplasmic tail of CD18 to increase cell adhesion to ICAM-1 (11). It is also known that binding of PtdIns-3,4,5-P3 to the pleckstrin homology domain of cytohesin-1 is required for activating cellular adhesion (12). Given that cytohesin-1 is not ubiquitously expressed (11), expression of this adaptor molecule in THP-1 cells was examined. Semiquantitative RT-PCR using primers for cytohesin-1 was carried out using total RNA of THP-1 cells. The results shown in Fig. 7 demonstrate that cytohesin-1 mRNA is expressed in both THP-1wt and THP-1rsv cells. An antisense strategy to inhibit cytohesin-1 expression was used to examine whether cytohesin-1 is involved in LPS-induced adherence. THP-1wt cells were incubated in the presence of antisense S-oligos spanning the cytohesin-1 translation initiation region (including the ATG initiation codon) and then assayed for adherence in response to LPS. As shown in Fig. 8A, treatment of cells with antisense S-oligo to cytohesin-1 mRNA significantly attenuated LPS-induced adherence in a concentration-dependent manner with maximal inhibition (72 ± 8%) at 5 μM. In contrast, at 5 μM of control, sense S-oligo, only a

FIG. 4. Wortmannin and LY294002 attenuate LPS-induced adherence. 1 × 10^5 THP-1wt cells (in 200 μl of RPMI 1640, final volume) were dispensed into 96-well microtiter plates and incubated for 20 min at 37 °C and 5% CO2 with various concentrations of wortmannin (0–100 nM) (A) or LY294002 (0–32 μM) (B). Either LPS (1 μg/ml in 0.5% AB serum) or PMA (20 ng/ml) were then added for 60 min at 37 °C. Adherent cells were stained with crystal violet, and absorbances were measured as described in the legend to Fig. 2. Duplicate determinations were made for each data point. The data are presented as percent of maximal adherence, calculated as described in the legend to Fig. 3. In C, cells were incubated for 20 min in RPMI 1640 alone (A), in RPMI 1640 plus 100 nM wortmannin (B), or in RPMI 1640 plus 16 μM LY294002. Cells were then washed and stained for CD11a and CD18 as described in the legend to Fig. 1. Results are expressed as histograms of fluorescence intensity and MFI indices as described in the legend to Fig. 1. In A and B the values shown are the averages of two independent determinations obtained in separate experiments. C shows results obtained in one of two independent experiments that yielded similar results.

FIG. 5. PI 3-kinase activity and adherence of U937 cells transfected with either wild-type bovine p85α or dominant negative mutant Δp85α. A, cells were stimulated with either 1 μg/ml LPS or medium alone followed by detergent lysis and immunoprecipitation with anti-PI 3-kinase antibody. Phosphatidylinositol kinase activity was assayed as described under “Experimental Procedures.” Radioactivity observed at the origin (ORI) reflects residual, water-soluble ^32P-labeled material in the samples. Spots corresponding to phosphatidylinositol phosphate (PIP) were cut and analyzed by scintillation counting. Activities, expressed as percent of control (untreated) cells transfected with wild-type p85α, are shown at the top of the figure. B, 1 × 10^5 cells of either p85α or Δp85α transfected U937 cells were exposed to LPS (indicated concentration) in the presence of 0.5% AB and allowed to adhere to either BSA or sICAM-1 for 60 min at 37 °C and 5% CO2. Unbound cells were washed away, and adherence was assayed as described in the legend to Fig. 2. The data shown in A are the means ± S.E. of values obtained in three separate experiments. Results in B are from one of two independent experiments that yielded similar results.
minimal effect on adherence was observed. This finding suggested a direct role of cytohesin-1 in the response of THP-1 cells to LPS. FACS analysis of cells exposed to fluorescein modified antisense S-oligo, in the same conditions used for unmodified S-oligos, revealed that THP-1 cells readily incorporated foreign DNA. However, a significant proportion of cells (24%) remained S-oligo-free (Fig. 8B). This finding may explain why antisense S-oligo treatment did not result in complete inhibition of LPS-induced adherence. Increasing the concentration of either S-oligos, LipofectAMINE, or both, to achieve a transfection rate approaching 100% resulted in toxicity thereby reducing the specificity of the antisense S-oligo treatment (data not shown).

**DISCUSSION**

This study examined signaling events required for LPS-induced adherence. The system used involved a quantitative, microtiter adhesion assay, CD14 transfected THP1 cells, and immobilized sICAM-1. Adherence in this system was found to be dependent upon CD14 (Fig. 2). Experiments that examined competitive inhibition of LPS-induced adherence using mAbs to CD14, CD18, and CD11a (Fig. 3A) provided direct evidence that LPS-induced adherence to sICAM-1 involves a CD14 mediated signal leading to activation of cell surface expressed LFA-1. These findings are consistent with previous data showing that antibody cross-linking of cell surface CD14 induces LFA-1 activation (40). LPS effects on LFA-1 did not involve changes in the expression of CD18 or CD11a. This indicates that LPS-induced adhesion was related to increased affinity of LFA-1 for ICAM-1 rather than to increased expression of cell surface LFA-1. Such changes in the properties of LFA-1 are presumably mediated by a specific pathway of inside-out signaling initiated through CD14.

The requirement for PI 3-kinase activity in a variety of leukocyte functions, together with its apparent role in the adhesion of platelets (41), lymphocytes (42), and neutrophils (33), made this enzyme an attractive candidate for mediating signaling through CD14 for monocyte adhesion. This hypothesis was supported further by the finding that LPS induces the CD14-dependent association of an activated form PI 3-kinase with p53/p56{	extsuperscript{lin}} (20). The role of PI 3-kinase in LPS-induced adherence was examined using two different approaches. The first involved the use of two structurally unrelated PI 3-kinase inhibitors wortmannin and LY294002. LPS-induced adherence was attenuated by both of these agents (Fig. 4). The effects of...
wortmannin are considered to be relatively specific for PI 3-kinase by experiments in which a dominant negative mutant of PI 3-kinase (p85) expressed in U937 cells completely abrogated inhibition of the formation of PtdIns-3,4,5-P$_3$. Thus, the most likely mechanism for the attenuation of the PMA-induced, monocyte adherence to sICAM-1 (Fig. 6, B) shows that LPS triggers Rho-mediated activation of PI 3-kinase, leading to downstream effects on LFA-1 and monocyte adherence. An important question arising from these observations is how PI 3-kinase activation modulates the properties of LFA-1. Recently, cytohesin-1 has been shown to interact with the cytoplasmic tail of CD18 (11). Cytohesin-1 contains a domain homologous to the yeast Sec7 gene product and a pleckstrin homology domain. The Sec7 domain binds to and regulates PtdIns-3,4,5-P$_3$ (4). LPS binding to CD14 (1) engages the small G protein Rho (2) leading to activation of PI 3-kinase (3). PtdIns-3,4,5-P$_3$ (4) binds to the pleckstrin homology (PH) domain of cytohesin-1, thereby modifying its interaction, through its Sec7 domain, with the cytoplasmic tail of CD18 (5). This leads to altered properties of LFA-1 (6) and to increased affinity for its counter receptor ICAM-1.

It has been reported previously that the small G-protein Rho regulates PI 3-kinase activation in different cell systems (45–47). This finding suggested the possibility that LPS-induced adherence may be Rho-regulated and mediated by PI 3-kinase. In this report, a requirement for Rho in LPS-induced adherence was suggested by studies that used C. difficile toxin B, which specifically inhibits Rho family proteins (38, 48). Pretreatment of THP-1wt cells with toxin B for 30 min. attenuated LPS-induced adherence to sICAM-1 (Fig. 6, A and B). In contrast, PMA-induced adherence appeared to be mediated by a toxin B-insensitive pathway (Fig. 6, A and B). This dichotomy is consistent with reports showing that PMA-induced responses in a variety of cell types may be either resistant to Rho toxins or that inhibition of these responses requires prolonged periods of incubation with toxins (24 h and more) (49, 50). For example 8–24 h of pretreatment with botulinum C3 exoenzyme, another inhibitor of Rho family proteins, was required to attenuate PMA-induced, LFA-1/ICAM-1-dependent aggregation of the lymphoblastoid cell line JY (49). On the other hand, PMA-induced activation of phospholipase D in HEK-98 (human embryonic kidney) cells was resistant to treatment with C. difficile toxin B for as long as 24 h (50). In the present study, THP-1 cells were incubated with toxin B for up to a maximum of 3 h. Under these conditions it is clear that adherence induced by LPS was toxin-sensitive, whereas the response to PMA was markedly resistant.

The findings that both Rho and PI 3-kinase appeared to be essential for LPS-induced adherence raised the question as to whether they act independently or whether they are positioned together in a single signaling pathway. Fig. 6C shows that toxin B prevented activation of PI 3-kinase in LPS-stimulated THP-1wt cells, suggesting that Rho regulates this LPS response in monocytes. This observation is consistent with previous reports showing involvement of Rho in PI 3-kinase activation in other systems (45–47). Although we cannot completely eliminate the possibility of a direct, PI 3-kinase-independent role for Rho in regulating monocyte adherence, the data suggest that LPS triggers Rho-mediated activation of PI 3-kinase, leading to downstream effects on LFA-1 and monocyte adherence.
expressed in THP-1 cells. The results shown in Fig. 7 confirmed that the cytohesin-1 gene is transcribed under basal conditions. To directly address the role of cytohesin-1 in LPS-induced adherence, cytohesin-1-specific antisense oligonucleotides were used. To ensure maximal specificity of the antisense oligonucleotide, the sequence selected was from a region lacking significant homology with other sequenced human genes. The oligonucleotides were also phosphorothioate-modified to limit degradation and purified by high performance liquid chromatography to remove incomplete synthesis products. In addition fluorescein-modified antisense and FACS analysis were used to monitor oligonucleotide incorporation into cells. The finding that antisense treatment of THP-1 cells, but not treatment with sense oligonucleotide, significantly attenuated LPS-induced adherence to ICAM-1 (Fig. 8A) provided compelling evidence to suggest that cytohesin-1 plays an essential role in adherence induced by LPS. Of note, the proportion of cells that incorporated antisense-oligonucleotide (Fig. 8B) correlated closely with the fraction of cells that failed to adhere in response to LPS (Fig. 8A).

Taken together, the results presented are consistent with a model (Fig. 9) in which LPS binding to CD14 switches on the response to LPS (Fig. 8A) in close agreement with the fraction of cells that failed to adhere in response to LPS (Fig. 8A).