INFECTION AND IMMUNITY, Feb. 1995, p. 486–497 Vol. 63, No. 2
0019-9567/95/$04.00+0

CD14 Is Not Involved in *Rhodobacter sphaeroides* Diphosphoryl Lipid A Inhibition of Tumor Necrosis Factor Alpha and Nitric Oxide Induction by Taxol in Murine Macrophages

FUMIKO KIRIKAE,[1] TERUO KIRIKAE,[1]* NILOFER QURESHI,[2,3] KUNI TAKAYAMA,[2,3] DAVID C. MORRISON,[4] AND MASAYASU NAKANO[1]

Department of Microbiology, Jichi Medical School, Minamikawachi-machi, Tochigi-ken 329-04, Japan[1]*; Mycobacteriology Research Laboratory, William S. Middleton Memorial Veterans Hospital,*[2] *and Department of Bacteriology, College of Agricultural and Life Sciences, University of Wisconsin,*[3] *Madison, Wisconsin 53706; and The Cancer Center, University of Kansas Medical Center, Kansas City, Kansas 66160-7832*[4]

Received 30 June 1994/Returned for modification 8 August 1994/Accepted 14 November 1994

Taxol, a microtubule stabilizer with anticancer activity, mimics the actions of lipopolysaccharide (LPS) on murine macrophages in vitro. Recently, it was shown that taxol-induced macrophage activation was inhibited by the LPS antagonist *Rhodobacter sphaeroides* diphosphoryl lipid A (RsDPLA). To investigate the mechanisms of taxol-induced macrophage activation, the present study focused on the interaction of LPS, RsDPLA, and taxol in the activation of and binding to macrophages. Taxol alone induced murine C3H/He macrophages to secrete tumor necrosis factor alpha (TNF) and to produce nitric oxide (NO) with kinetics similar to that of LPS. Macrophages from LPS-hyporesponsive C3H/HeJ mice, in contrast, did not yield any detectable TNF and NO production in response to LPS or taxol. RsDPLA inhibited taxol-induced TNF and NO production from C3H/He macrophages in a dose-dependent manner. The inhibition by RsDPLA was specific for LPS and taxol in that RsDPLA did not inhibit heat-killed *Listeria monocytogenes*- or zymosan-induced TNF production. Polymyxin B blocked the inhibitory effect of RsDPLA on taxol-induced TNF production. The inhibitory activity of RsDPLA appeared to be reversible since macrophages still responded to taxol in inducing TNF production after the RsDPLA was washed out with phosphate-buffered saline prior to the addition of taxol. Taxol-induced TNF production was not inhibited by colchicine, vinblastine, or 10-deacetylbaccatine III. A mutant cell line, J7.DEF3, defective in expression of a CD14 antigen, responded equally well to taxol by producing TNF as did the parent J774.1 cells. This suggested that the activation of macrophages by taxol does not require CD14. Taxol-induced TNF production by the mutant cells was also inhibited by RsDPLA. ^{125}I-labeled LPS and ^{3}H-labeled taxol was reported to bind to J774.1 cells predominantly via CD14 and microtubules, respectively. The binding of ^{125}I-labeled LPS to J7.DEF3 cells was about 30 to 40% of that to J774.1 cells. The binding of ^{125}I-LPS to J774.1 cells was inhibited by unlabeled LPS and RsDPLA but not by taxol. On the other hand, ^{3}H-labeled taxol bound to both J774.1 cells and J7.DEF3 cells in similar time- and dose-dependent manners. The binding of [^{3}H]taxol to these cells was inhibited by taxol but not by LPS or RsDPLA. Although the binding studies failed to examine cross competition for binding to macrophages, a possible explanation of these results is that LPS, RsDPLA, and taxol share the same molecule(s) on murine macrophages for their functional receptor(s), which is neither CD14 nor tubulin.

Bacterial lipopolysaccharide (LPS) activates macrophages to produce and release various pharmacologically active mediators, including tumor necrosis factor alpha (TNF), interleukin-1, and nitric oxide (NO) (28, 35, 37). Many recent studies have sought to elucidate the molecular mechanisms of LPS-triggered macrophage activation to determine how the cells recognize and respond to LPS by the receptor-ligand interaction (27, 55). Consequently, a number of specific LPS-binding proteins have been identified on macrophages, including CD11/18 adhesins (56), a scavenger receptor (11), and specific proteins with molecular masses of 73 (19), 40/45 (13), and 38 (20) kDa. In addition, an important role for CD14 (58) in the recognition of LPS complexed to LPS-binding serum protein has been reported (44). Although the evidence for the functional role of 73-kDa LPS-binding protein (3) and CD14 (58) has been documented, most of the molecular mechanisms of cell activation induced by LPS remain to be fully defined.

An alternative approach to elucidate the functional properties of LPS receptors on macrophages and the mechanisms of cell activation initiated by LPS is to perform binding study and cell activation analysis on LPS antagonists and compounds having LPS-like activity (LPS agonist). The present study was undertaken to investigate the various interactions of an LPS agonist in the activation of macrophages and an LPS antagonist in the inhibition of those interactions by using taxol as an LPS agonist (Fig. 1C) (35) and *Rhodobacter sphaeroides* diphoshoryl lipid A (RsDPLA) as an LPS antagonist (Fig. 1B) (21).

Taxol, a diterpene compound, was originally isolated from the stem bark of *Taxus brevifolia* and shown to have antiproliferative activity against various cultured cells as well as antineoplastic activity in tumor patients (53). These effects of taxol appear to be related to its ability to bind to tubulin, to promote microtubule assembly, and to stabilize microtubules by bundle formation (17, 42, 43). Recently, Ding et al. (5)

* Corresponding author. Mailing address: Department of Microbiology, Jichi Medical School, 3311-1 Yakushiji Minamikawachi-machi, Tochigi-ken 329-04, Japan. Phone: 81-285-44-2111, ext. 3162. Fax: 81-285-44-1175.

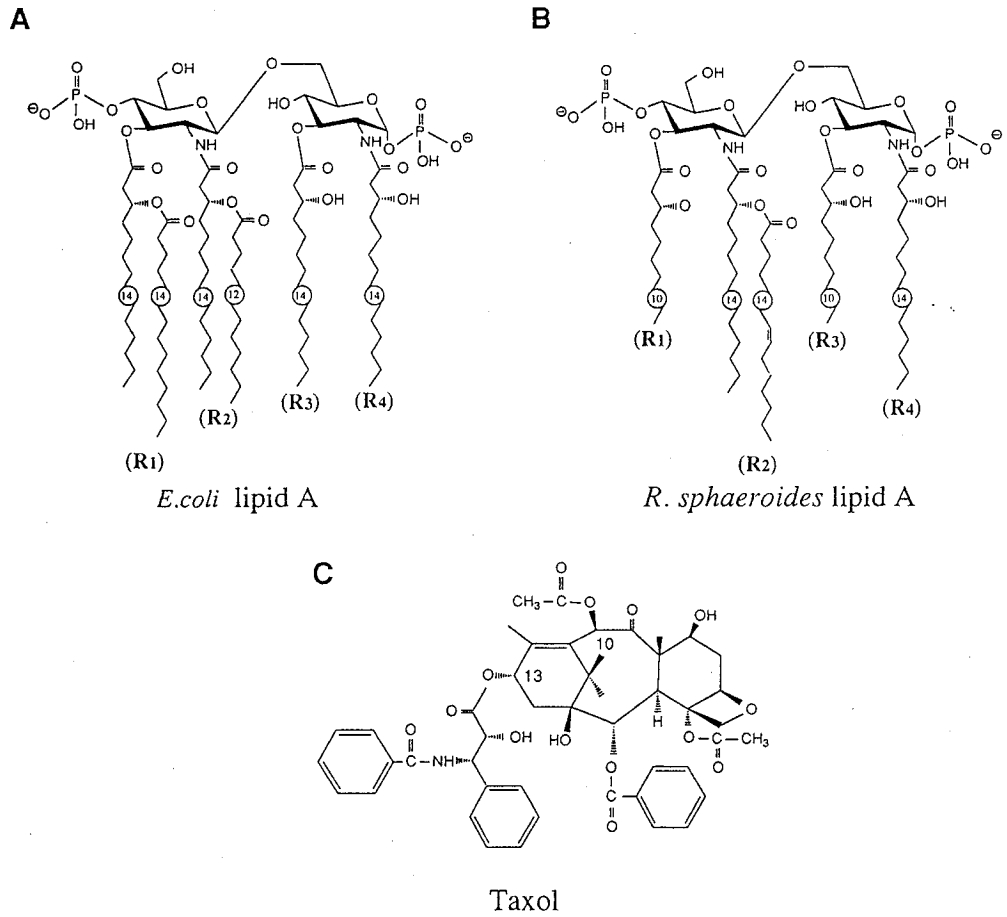

FIG. 1. Chemical structure of *E. coli*-type lipid A (A), RsDPLA (B), and taxol (C) (25, 32, 41, 47). The structure given for RsDPLA is one of two predominant structural forms, the other form lacking the unsaturated fatty acid.

discovered that taxol exhibits cell cycle-independent and LPS-like effects on murine macrophages. Taxol, like LPS, was shown to activate murine macrophages to down-regulate TNF receptors and to initiate synthesis and secretion of TNF protein. Further studies revealed that responsiveness to taxol was genetically linked to responsiveness to LPS. Macrophages from the LPS-hyporesponsive C3H/HeJ mice (48) were found to be hyporesponsive to taxol as a result of a mutation at a single genetic locus (39), and genetic analysis of recombinant inbred mice demonstrated a close linkage between the mutant LPS^d allele and an ability to activate macrophages in response to taxol. These data have provided strong evidence that taxol may share the actions of LPS for macrophage activation, i.e., that taxol is an LPS agonist.

Several synthetic and natural lipid A analogs are known to be biologically inactive but to retain the capacity to block LPS stimulation in various assay systems (21, 37). Pentaacyl RsDPLA is a well-characterized and potent LPS antagonist. RsDPLA has been shown to block LPS-dependent TNF production in cultured macrophages (49) and monocytes (7) and to inhibit LPS-initiated interleukin-1 secretion in macrophages (33) and interleukin-6 secretion in a macrophage cell line (14). RsDPLA is thought to function as a competitive inhibitor of LPS binding to its putative receptor(s), and this is due to its structural similarity to the "toxic" lipid A (Fig. 1A) (25, 32, 41, 47), the active moiety of the LPS molecule. We have recently demonstrated that RsDPLA competes with LPS for the binding of LPS to cultured macrophages, possibly via a CD14-dependent pathway (14).

In the present study, we have investigated further the relationship between the agonistic actions of LPS and taxol and the antagonistic action of RsDPLA on murine macrophages. Our findings suggest that LPS, taxol, and RsDPLA may recognize an identical molecule(s) on macrophages, a putative LPS receptor(s), which is neither CD14 nor tubulin.

(This research was conducted by F. Kirikae in partial fulfillment of the requirements for a Ph.D. from the Department of Microbiology, Jichi Medical School.)

MATERIALS AND METHODS

Mice. C3H/He and C3H/HeJ mice were bred and maintained in the Animal Faculty of the Jichi Medical School under standard care. Female mice were used at 10 to 15 weeks of age. In individual experiments, age-matched mice were used.

Reagents. The Re chemotype LPS (ReLPS) from *Salmonella minnesota* R595 was kindly provided by K. Hisatsune, Josai University, Sakado, Japan. The S-form LPS from *S. minnesota* wild type was purchased from Sigma Chemical Co., St. Louis, Mo. The RsDPLA derived from the LPS of *R. sphaeroides* ATCC 17023 was prepared as described previously (34). Synthetic lipid A (compound 506) (12) was kindly provided by Daiichi Pharmaceutical Co., Tokyo, Japan. The molecular weights of subunits of ReLPS and lipid A were estimated from the known chemical structures to be 2,800, 1,496, and 2,001 for ReLPS, RsDPLA, and synthetic lipid A, respectively. Taxol was purchased from Sigma and stored at −20°C as a 10 mM stock solution in dimethyl sulfoxide (DMSO). Taxol was insoluble at 300 μM in serum-free culture medium or medium containing less than 0.1% fetal bovine serum (FBS), and FBS at >1% was necessary to solubilize taxol in medium. Taxol was insoluble at >600 μM in medium containing 2% FBS

(2% FBS–medium). Before addition to cells, therefore, taxol was dissolved in 2% FBS–medium at 300 μM. At 300 μM taxol in 2% FBS–medium, the LPS concentration was estimated to be <1 pg/ml (<0.003 endotoxin unit per ml), as measured by a chromogenic endotoxin-specific assay using recombined *Limulus* coagulation enzymes (ES test; Seikagaku Kogyo Co., Tokyo, Japan; a reference endotoxin, *Escherichia coli* O111:B4 LPS) (29), in close agreement with earlier studies that also excluded LPS contamination of taxol (1, 23). In addition, taxol does not affect *Limulus* coagulation enzymes in the ES test because addition of taxol to the enzyme mixture did not affect responsiveness of the enzymes to LPS (50). Heat-killed *Listeria monocytogenes* (HKLM) was the generous gift of S. W. Russell, the University of Kansas Medical Center, Kansas City. Zymosan, colchicine, vinblastine, and 10-deacetylbaccatine III were purchased from Sigma and stored at −20°C as a 100 mM stock solution in DMSO for colchicine and 10 mM stock solutions in DMSO for vinblastine and 10-deacetylbaccatine III. These reagents contained LPS at 37 pg/ml in a 10^6/ml concentration of HKLM cells, 3.9 ng/ml in a 1-μg/ml concentration of zymosan, 3 pg/ml in 300 μM colchicine, 2 pg/ml in 300 μM vinblastine, and <1 pg/ml in 300 μM 10-deacetylbaccatine III. [^{3}H]taxol (19 Ci/mmol; 1.0 mCi/ml in ethanol) was purchased from Moravek Biochemicals, Inc., Brea, Calif. Before use, [^{3}H]taxol was evaporated in a centrifugal concentrator (model CC-101; Tomy Co., Tokyo, Japan) and dissolved in DMSO at 10 μM.

Radioiodinated LPS was prepared as reported previously (52). Briefly, *S. minnesota* R595 LPS was incubated at 1 mg/ml with 50 mM *p*-OH-methylbenzimidate (Sigma) in 50 mM borate buffer (pH 8.0) for 18 h at 37°C. LPS coupled to *p*-OH-methylbenzimidate was radioiodinated with sodium ^{125}I (Amersham Co., Tokyo, Japan) by incubation with IODO-BEADS iodination reagent (Pierce, Rockford, Ill.). The specific activity of ^{125}I-labeled LPS was 1.2 μCi/μg, and labeled LPS retained nearly full activity to induce TNF and NO production by J774.1 cells (data not shown).

Cell preparations. Mouse macrophages were isolated by peritoneal lavage 4 days after intraperitoneal injection of 1.5 ml of sterile 2.9% thioglycolate broth (Nissui Pharmaceutical Co., Tokyo, Japan). Cells were washed with serum-free RPMI 1640 (Flow Laboratories, Irvine, Scotland) and resuspended in RPMI 1640 containing 2% heat-inactivated FBS (Flow Laboratories), 4 mM L-glutamine (Kanto Chemical Co., Tokyo, Japan), 100 U of penicillin (Meiji-Seika Co., Tokyo, Japan) per ml, and 100 μg of streptomycin (Meiji-Seika) per ml. Cells were plated at 2×10^5 per well in 96-well plates (Nunc, Roskilde, Denmark) for TNF and NO induction or at 1×10^5 per well in 24-well plates (Sumitomo Bakelite Co., Tokyo, Japan) for the ^{125}I-LPS and [^{3}H]taxol binding assay. Cells were cultured for 2 h at 37°C with 5% CO_2 and then washed three times with serum-free RPMI 1640 to remove nonadherent cells. The remaining adherent cells were greater than 95% macrophages, as determined by morphologic criteria and nonspecific esterase staining.

Mouse macrophage-like J774.1 cells were kindly provided by T. Suzuki, the University of Kansas Medical Center. TNF-sensitive L929 cells were donated by M. J. Parmely, the University of Kansas Medical Center. A mutant J7.DEF3 cell line derived from J774.1 cells which has been characterized previously was also used in some studies (15). These cells were grown at 37°C with 5% CO_2 in RPMI 1640 containing 8% FBS, 4 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml.

Stimulation of TNF and NO production by macrophages. J774.1 and J7.DEF3 cells were prepared for stimulation as described previously (15). When these cells, grown in flasks (Nunc), reached confluency, the culture medium and nonadherent cells were removed and replaced with fresh culture medium. After an additional 24 h of culture, the cells were collected by vigorous pipetting, centrifuged, washed once with the same culture medium by centrifugation, and plated in 96-well culture plates at 2×10^5 cells per well in 200 μl of RPMI 1640 medium containing 2% FBS (2% FBS–RPMI 1640). Peritoneal macrophages were plated and washed as described above and overlaid with 200 μl of 2% FBS–RPMI 1640. Cells were incubated with stimulant, and supernatants were collected at times indicated in individual experiments and stored at −80°C until used for determination of TNF and NO.

TNF assay. TNF activity was determined by a functional cytotoxic assay using the TNF-sensitive cell line, L929, as described previously (40). L929 cells were plated in 96-well culture plates at 5×10^4 cells per well in 100 μl of 5% FBS–RPMI 1640 and incubated for 20 h. The cells were then cultured for an additional 18 h in the presence of serial dilutions of test supernatant and 5 μg of actinomycin D (Sigma) per ml. The viability of cells was determined by a quantitative colorimetric staining assay using a tetrazolium salt (3-[4,5-dimethylthiazol-yl]-2,5-diphenyltetrazolium bromide [MTT], Sigma). TNF activity is expressed in units per milliliter, with 1 U being the amount of TNF causing 50% lysis of L929 cells.

NO assay. NO formation was measured as the stable end product nitrite (NO_2^-) in culture supernatants with the Griess reagent (9). Briefly, 100 μl of culture supernatants was added to each well of 96-well plates and mixed with 100 μl of Griess reagent (1:1 [vol/vol]; 0.1% *N*-[1-naphthyl]ethylenediamine dihydrochloride in H_2O, 1% sulfanilamide in 5% H_2PO_4), and the A_{540} was read with a model Biomek 1000 spectrophotometer (Beckman Instruments, Palo Alto, Calif.).

^{125}I-LPS binding assay. Binding of ^{125}I-LPS to J774.1 cells was performed as described previously (15). Briefly, cells were plated and cultured overnight at 10^5 per well of tissue culture plates in 1 ml of 5% FBS–RPMI 1640. The plates were washed three times with phosphate-buffered saline (PBS), which was replaced with 180 μl of ice-cold Hanks' balanced salt solution (HBSS) containing 5% FBS and 0.1% sodium azide (HBSS binding buffer), and cooled to 0°C. The cells were incubated for 7 h after addition of either 20 μl of ^{125}I-LPS or ^{125}I-LPS plus an indicated competitor in HBSS binding buffer at 0°C. In some experiments, the cells were incubated with ^{125}I-LPS or ^{125}I-LPS plus an indicated competitor in RPMI 1640 containing 5% FBS at 37°C for the indicated times. The incubation was terminated by rapid washing of the cells in each well two times with 1 ml of ice-cold HBSS binding buffer and then by two washes with 1.0 ml of ice-cold PBS. The cells were lysed with 500 μl of 0.1 N NaOH, and the radioactivity in 450 μl of the NaOH extracts was measured with a model ARC-600 gamma counter (Aloka Co., Tokyo, Japan).

[^{3}H]taxol binding assay. Determination of binding of [^{3}H]taxol to J774.1 cells and peritoneal macrophages was carried out as described by Manfredi et al. (22), with some modifications. The cells were plated and cultured for 16 h at 10^5 per well in tissue culture plates containing 1 ml of 8% FBS–RPMI 1640 per well. The plates were washed with PBS twice, and then 200 μl of 8% FBS–RPMI 1640 containing the appropriate competitors and [^{3}H]taxol was added to each well. The final concentration of [^{3}H]taxol was 10 nM unless specified otherwise. The cells were incubated for 2 h in a humidified incubator at 37°C with 5% CO_2. The incubation was terminated by washing the cells in each well two times with $2 \times$ 1 ml of ice-cold HBSS binding buffer and then with 2×1 ml of ice-cold PBS. The cells were lysed in 300 μl of 0.1 N NaOH at room temperature for 10 min, neutralized with an equal volume of glacial acetic acid, and then added to 8 ml of ACS II scintillation fluid (Amersham). The radioactivity was measured with a model LSC-3500 liquid scintillation counter (Aloka). Efficiency of counting averaged 38%.

RESULTS

Taxol induces TNF and NO production in LPS-responsive C3H/He macrophages but not in LPS-hyporesponsive C3H/HeJ macrophages. Ding et al. (5) demonstrated previously that macrophages from LPS-responsive mice respond to taxol by inducing TNF production, whereas macrophages from LPS-hyporesponsive C3H/HeJ mice do not respond to taxol. To confirm and extend these findings, both LPS-responsive and -hyporesponsive macrophages were assessed for their responsiveness to taxol in inducing TNF and NO production. The results of these initial experiments are shown in Fig. 2. In LPS-responsive C3H/He macrophages, taxol induced TNF and NO production in a dose-dependent manner with minimal inducing doses of 1.9 and 3.8 μM and optimal doses of 7.5 and 15 μM, respectively (Fig. 2A and C). The 50% effective doses [i.e., the doses of taxol required to induce half-maximal responses for log (TNF or NO production)] were estimated to be 2.2 and 5.8 μM, respectively. The time course of TNF secretion and NO production by C3H/He macrophages after taxol stimulation closely resembled that of LPS (Fig. 2B and D). In LPS-hyporesponsive C3H/HeJ macrophages, neither taxol nor LPS induced significant detectable levels of TNF and NO at any dose or time (Fig. 2).

RsDPLA inhibits taxol-induced TNF and NO production. RsDPLA is known as an LPS antagonist for many biological activities of macrophages stimulated by LPS (7, 16, 21, 33, 49). Manthey et al. (23) demonstrated recently that RsDPLA inhibits taxol-induced TNF and interleukin-1β production and protein-tyrosine phosphorylation in mouse macrophages. To confirm and extend those observations, we next assessed the inhibitory effects of RsDPLA on taxol-induced TNF and NO production by LPS-responsive C3H/He macrophages. When increasing doses of RsDPLA were added to C3H/He macrophages 2 h prior to the addition of taxol, there were dose-dependent decreases in both TNF secretion and NO formation (Fig. 3A and C). The ID_{50}s [the doses of RsDPLA needed to inhibit by 50% the amount of log (TNF or NO produced in response to taxol alone)] of RsDPLA for macrophage responses to 7.5 and 15 μM taxol were 30 and 120 ng/ml for TNF secretion and 98 and 650 ng/ml for NO production, respectively. Kinetics studies of the inhibition by RsDPLA of taxol-induced TNF secretion and NO production were carried out,

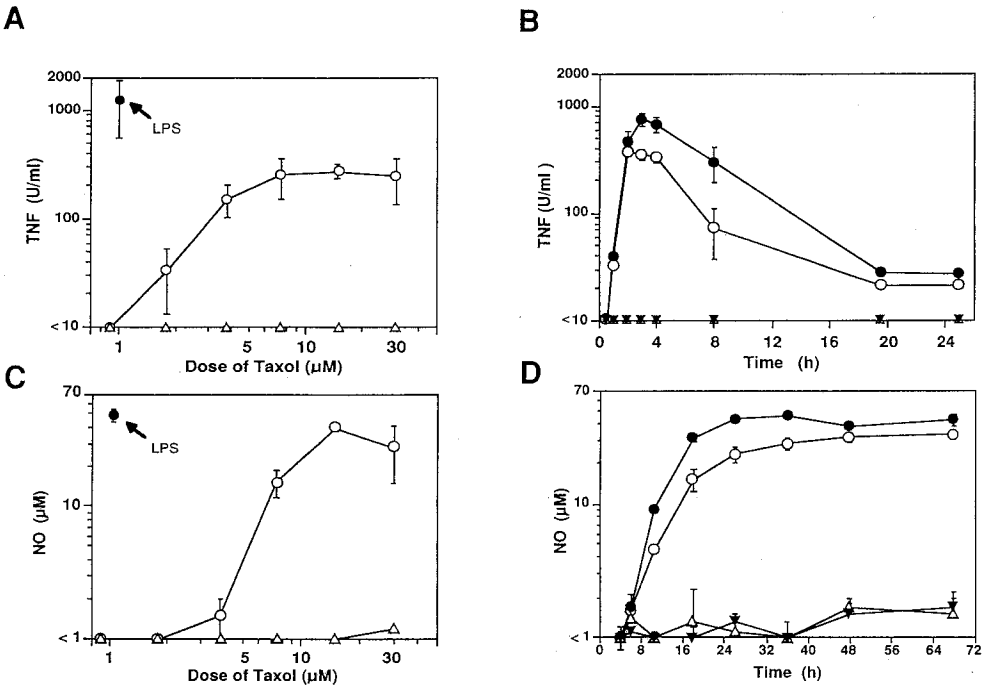

FIG. 2. Induction of TNF and NO production by C3H/He and C3H/HeJ macrophages in response to taxol and LPS. (A) Dose response of taxol-induced TNF secretion. Macrophages from C3H/He mice (○) and C3H/HeJ mice (△) were incubated with taxol at various doses for 4 h for TNF in triplicate wells, and C3H/He macrophages were also incubated with 10 ng of *S. minnesota* R595 LPS per ml (●). (B) Time course of taxol- and LPS-induced TNF secretion. Macrophages from C3H/He (○ and ●) or C3H/HeJ (△ and ▼) were incubated with LPS (10 ng/ml) (● and ▼) or taxol (15 μM) (○ and △) for the indicated time in triplicate wells. (C) Dose response of taxol-induced NO production. Experiments were carried out in the same manner as that described for panel A, except that the cells were incubated for 48 h. (D) Time course of taxol- and LPS-induced NO production. Experiments were carried out in the same manner as that described for panel B. TNF activity in the supernatants was determined by the cytotoxic assay using L929 cells. NO production was measured as nitrite in culture supernatants with Griess reagent. TNF secretion and NO production by unstimulated cells were below detectable levels in all experiments. Each point represents the mean ± standard error of the mean. Panels A and C show the mean of three experiments. The data in panels B and D represent one of three experiments with similar results.

and the data are shown in Fig. 3B and D. The inhibition by RsDPLA was observed to occur in a dose-dependent manner during all time periods tested, with almost complete inhibition at the higher dose of 333 ng/ml.

RsDPLA does not inhibit TNF production induced by HKLM and zymosan. Because HKLM and zymosan are also potent inducers of TNF in macrophages, experiments were carried out to assess whether RsDPLA would also inhibit TNF induction by C3H/He and C3H/HeJ macrophages stimulated with HKLM and zymosan. As shown in Fig. 4, C3H/He macrophages responded to LPS, taxol, HKLM, and zymosan by secretion of TNF, whereas LPS-hyporesponsive C3H/HeJ macrophages did not respond to LPS and taxol but did respond to HKLM and zymosan by inducing TNF release. RsDPLA markedly inhibited both LPS- and taxol-induced TNF release by LPS-responsive macrophages. On the other hand, RsDPLA did not inhibit HKLM-induced TNF secretion by either LPS-responsive or -hyporesponsive macrophages. RsDPLA significantly inhibited zymosan-induced TNF secretion by LPS-responsive macrophages at lower doses of zymosan. This inhibitory effect of RsDPLA appeared to be due to LPS contamination of zymosan because the inhibition by RsDPLA was not observed in LPS-hyporesponsive macrophages stimulated with zymosan. Furthermore, as described in Materials and Methods, the zymosan tested here contained 3.9 ng of LPS per ml by a *Limulus* assay, which corresponds to 0.1 ng of LPS per ml in the lowest concentration of zymosan used in this study (0.025 μg of zymosan per ml); this is more than the minimum concentration of LPS required to induce TNF production by LPS-responsive macrophages (0.01 to 0.1 ng/ml) and relatively higher than the lowest concentration of HKLM (<1 pg of LPS per 5×10^2 HKLM) needed to induce production. Collectively, these data suggest that the inhibitory effects of RsDPLA on macrophage activation are specific for LPS and taxol.

Polymyxin B blocks the inhibitory effect of RsDPLA on taxol-induced TNF production. Although polymyxin B has been documented to neutralize many of the biological activities of LPS by binding to lipid A (26), it is not known whether polymyxin B might also neutralize the inhibitory activity of RsDPLA. To examine whether polymyxin B inhibits activities of taxol itself, taxol (7.5 or 15 μM) was preincubated with polymyxin B (5 or 20 μg/ml) for 30 min and then added to C3H/He macrophages. Polymyxin B did not show any inhibitory activity in inducing TNF secretion by taxol, in confirmation of an earlier study (24) (data not shown). Experiments were then carried out to investigate whether polymyxin B could inhibit the antagonistic activities of RsDPLA against taxol. As shown by the data in Fig. 5, polymyxin B treatment of RsDPLA completely blocked the inhibitory effects of RsDPLA on taxol-induced TNF secretion. These data indicate that the structural components of RsDPLA which are responsible for inhibition by RsDPLA of taxol-induced macrophage activation may be related to the same structural components of lipid A by which polymyxin B inactivates the biological activity of LPS.

Taxol shares a common binding site with RsDPLA in the activation of macrophages. To investigate further the nature of the interaction between taxol and RsDPLA on macrophages, time interval experiments were performed. RsDPLA was added to macrophages before, at the same time, or after stimulation with taxol. As shown by the data in Fig. 6A, RsDPLA

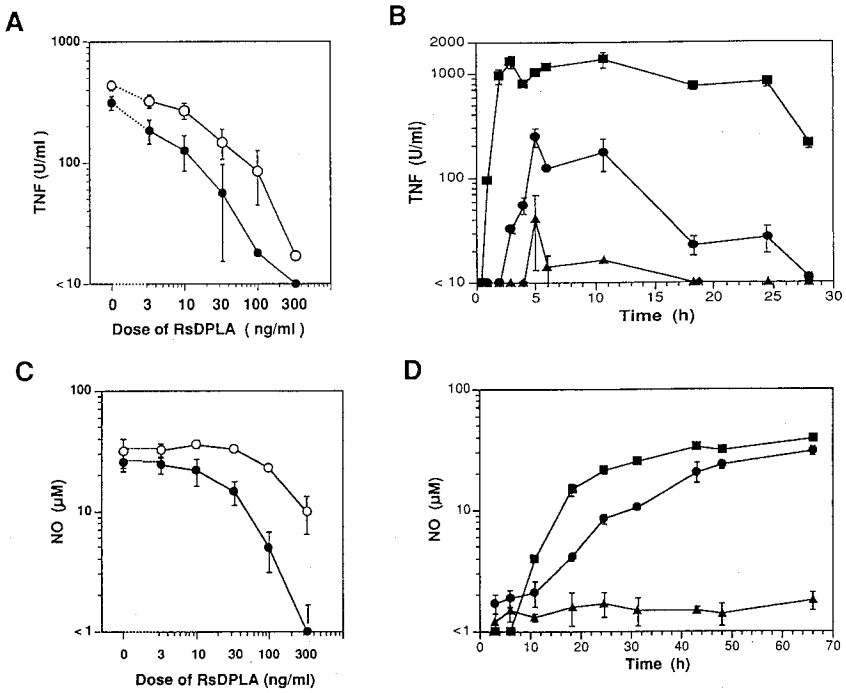

FIG. 3. RsDPLS inhibition of taxol-induced TNF secretion and NO production. (A) Dose-dependent inhibition by RsDPLA of taxol-induced TNF secretion. Macrophages from C3H/He mice were preincubated with various doses of RsDPLA for 1 h prior to incubation with 15 μM taxol (○) or 7.5 μM taxol (●) for 4 h. (B) Time course of taxol-induced TNF secretion by RsDPLA-pretreated macrophages. Macrophages from C3H/He mice were preincubated with RsDPLA at 0 (■), 33 (●), or 333 (▲) ng/ml for 1 h prior to incubation with 15 μM taxol for the indicated time. (C) Dose-dependent inhibition of taxol-induced NO production by RsDPLA. Experiments were carried out in the same manner as that described for panel A, except that the cells were incubated for 48 h. (D) Time course of taxol-induced NO production by RsDPLA-pretreated macrophages. Experiments were carried out in the same manner as that described for panel B. TNF activity in the supernatants was determined by the cytotoxic assay. NO formation was measured as nitrite in culture supernatants. TNF secretion and NO production by unstimulated cells were below detectable levels in all experiments. Each point represents the mean ± standard error of the mean. Panels A and C show the means of three experiments. The data in panels B and D represent one of three experiments with similar results.

almost completely inhibited taxol-induced TNF production when RsDPLA was added to cells 2 h to 5 min before taxol stimulation or simultaneously with taxol. In contrast, when RsDPLA was added to cells following taxol stimulation, RsDPLA only partially inhibited TNF secretion, and the inhibitory effects of RsDPLA decreased in a time-dependent manner.

The data shown in Fig. 6A indicate that RsDPLA must be present prior to the interaction of taxol with the macrophage to manifest its inhibitory properties. This experiment does not, however, distinguish whether prior macrophage exposure to RsDPLA is necessarily sufficient for manifestation of its inhibitory properties. To determine whether RsDPLA is required to be present with taxol to manifest its inhibitory effects on taxol-induced TNF secretion, macrophages from C3H/He mice were preincubated with or without RsDPLA for 1 h, washed with PBS three times, and further incubated in the presence or absence of taxol and/or RsDPLA. When macrophages were preincubated with RsDPLA, washed, and then incubated with RsDPLA and taxol together, the cells did not induce detectable TNF secretion, as would be expected from the data shown in Fig. 6A (Fig. 6C). On the other hand, macrophages preincubated with RsDPLA, washed in the same way, and incubated with taxol alone induced as much TNF secretion as could be induced by taxol alone. These results indicate that RsDPLA must be present with taxol to effectively mediate inhibitory effects. An essentially similar profile of TNF induction was observed in stimulation with LPS (Fig. 6B and D). Although we cannot exclude the possibility that RsDPLA would inhibit taxol by binding it directly, the results shown in Fig. 6 suggest collectively that taxol and RsDPLA share a common binding site which is involved in the activation of macrophages by taxol.

Response of CD14-deficient J7.DEF3 cells to taxol and RsDPLA in induction of TNF secretion. We have recently characterized a mutant cell line (J7.DEF3) that has been selected from J774.1 cells on the basis of a defect of specific ^{125}I-LPS binding in the presence of serum (15). The available data would indicate that, in these mutant cells, the defect is the lack of expression of CD14 (8, 51). To determine whether taxol activates macrophages via a CD14 molecule, the TNF production of J774.1 cells and that of J7.DEF3 cells were compared in response to taxol (Fig. 7A). In the parent J774.1 cells, taxol induced significant TNF release in a dose-dependent manner, with a minimal inducing dose of 1.9 μM, a 50% effective dose of 5 μM, and an optimal dose of 15 μM. The J7.DEF3 mutant cells also responded to taxol by induction of TNF release characterized by a dose-response relationship that is virtually indistinguishable from that of the J774.1 cells (Fig. 7A).

To test whether RsDPLA is equally capable of blocking the taxol-induced TNF secretion observed in both J774.1 and J7.DEF3 cells, cells were preincubated with RsDPLA for 1 h prior to the addition of taxol and TNF secretion was measured at 4 h. The results of this experiment for J774.1 and J7.DEF3 cells are shown in Fig. 7B and C, respectively. In J774.1 cells, RsDPLA inhibited TNF release by stimulation with both 7.5 and 15 μM taxol in a dose-dependent manner, and the ID_{50} values were 50 and 70 ng/ml, respectively. In J7.DEF3 cells, RsDPLA could also inhibit TNF secretion induced by taxol, with profiles of dose-response curves similar to those seen in the parent cells. The ID_{50} values, 60 ng/ml for 7.5 μM taxol and

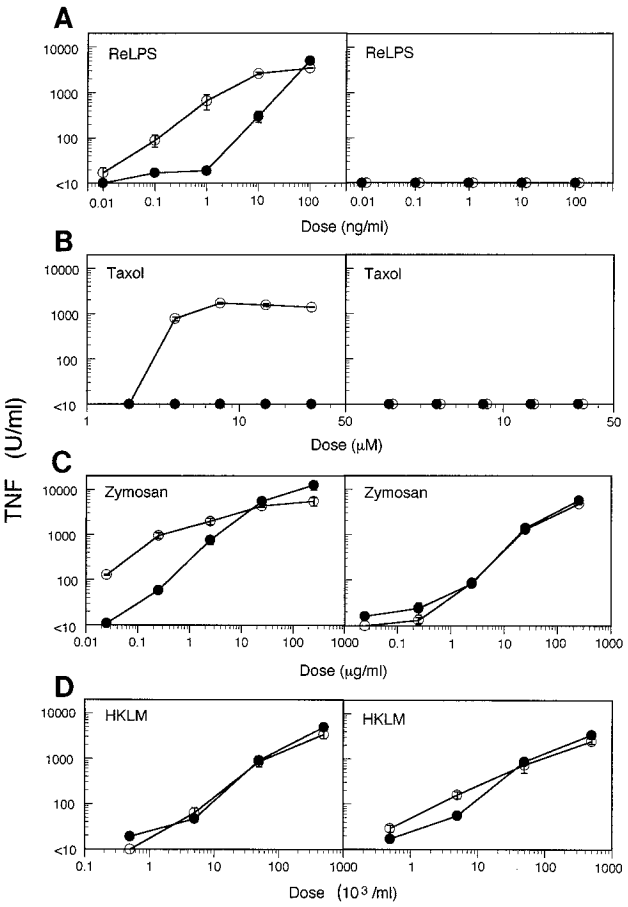

FIG. 4. Effects of RsDPLA on TNF secretion by C3H/He and C3H/HeJ macrophages stimulated with HKLM and zymosan. Macrophages from C3H/He mice (left panels) or C3H/HeJ mice (right panels) were preincubated without RsDPLA (○) or with 333 ng of RsDPLA per ml (●) for 2 h prior to incubation with *S. minnesota* R595 LPS, taxol, zymosan, or HKLM in triplicate wells at the indicated doses for 4 h. TNF activity in the supernatants was determined in the cytotoxic assay. Each point represents the mean ± standard error of the mean. The data are from one of two independent experiments with similar results.

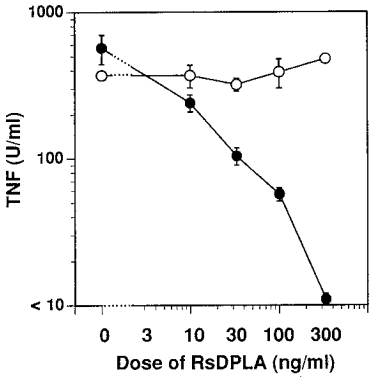

FIG. 5. Effects of polymyxin B on inhibitory actions of RsDPLA in taxol-induced TNF secretion. RsDPLA was incubated previously with or without polymyxin B (20 μg/ml) for 30 min. Polymyxin B-RsDPLA mixture (○) or RsDPLA only (●) was added to C3H/He macrophages, and the cells were incubated with taxol (15 μM) in triplicate wells for 4 h. TNF activity in the supernatants was determined in the cytotoxic assay. Each point represents the mean ± standard error of the mean. The data are from one of two independent experiments with similar results.

100 ng/ml for 15 μM, are essentially equivalent to those determined for the J774.1 cells. These results indicate that there is no significant difference between the parental and mutant cells in their responsiveness to taxol and in the antagonistic effects of RsDPLA on taxol-induced cell activation. These data, therefore, provide support for the concept that taxol induces macrophage activation independently of the CD14 antigen.

Taxol-induced TNF production is not inhibited by colchicine, vinblastine, or 10-deacetylbaccatine III. It is well documented that taxol binds directly to microtubules (22, 30) and inhibits the disassembly of microtubules (17, 42, 43). As a result, cell replication is inhibited (53). One taxol derivative, 10-deacetylbaccatine III, which lacks the side chain at C-13 of taxanes, is incapable of either binding to tubulin or inhibiting cell growth (10). On the other hand, there are other mitotic inhibitors, such as colchicine and vinblastine, which like taxol also bind directly to the β subunit of tubulin (54). To investigate whether the observed LPS agonist-like activities of taxol on macrophages are closely related to binding of taxol to microtubules, the effects of these mitotic inhibitors on macrophage activation were examined. Colchicine, vinblastine, and 10-deacetylbaccatine III essentially did not induce any TNF secretion in C3H/He macrophages or in J774.1 cells over the range of 1.25 to 150 μM for colchicine, 1.25 to 30 μM for vinblastine, and 2 to 125 μM for 10-deacetylbaccatine III (data not shown). We also examined whether these reagents might inhibit taxol-induced TNF production. These results are shown in Fig. 8. Pretreatment with neither colchicine, vinblastine, nor 10-deacetylbaccatine III for 2 h essentially affected taxol-induced TNF secretion by C3H/He macrophages, suggesting that the binding sites of colchicine and vinblastine on the β subunit of tubulin may be irrelevant in TNF induction by taxol.

Taxol does not inhibit specific binding of ^{125}I-LPS to macrophages. We reported previously that ^{125}I-labeled LPS binds to intact J774.1 cells in a specific and saturable manner (15) and that J7.DEF3 cells manifest a significant decrease in specific ^{125}I-LPS binding (16). The time kinetics of ^{125}I-LPS binding to J774.1 cells and J7.DEF3 cells are shown in Fig. 9. At 0°C and in the presence of sodium azide, which is known to inhibit membrane fluidity, total and specific binding of ^{125}I-LPS to J774.1 cells increased in a time-dependent manner and reached a steady state by 6 to 8 h (Fig. 9A). Under the same conditions, both total and specific binding of ^{125}I-LPS to J7.DEF3 cells was about 30 to 40% (Fig. 9B) of that to J774.1 cells.

At 37°C and in the absence of sodium azide, the total binding to J774.1 cells increased rapidly over the first 1 h and then increased gradually until 6 h (Fig. 9C). The nonspecific binding to J774.1 cells increased gradually, and the increase continued until the end of the experiments. As a result, the specific binding, defined as the difference between the total and nonspecific binding, did not reach a steady state. ^{125}I-LPS bound to J7.DEF3 cells with a profile essentially identical to that of ^{125}I-LPS binding to J774.1 cells at 37°C (Fig. 9D).

In these binding assays, we observed predominantly CD14-dependent uptake of LPS by J774.1 cells at 0°C, since J7.DEF3 cells, which do not express the CD14 antigen on the cell surface (8, 51), showed lower levels of specific ^{125}I-LPS binding at 0°C than J774.1 cells (Fig. 9B) (15); on the other hand, the observed ^{125}I-LPS binding at 37°C was CD14-independent uptake of LPS, which may be related to LPS-induced activation of cells, i.e., enhanced endocytosis and pinocytosis. Competitive binding studies were performed mainly at 0°C. As shown in Fig. 9E, unlabeled ReLPS inhibited the binding of ^{125}I-LPS

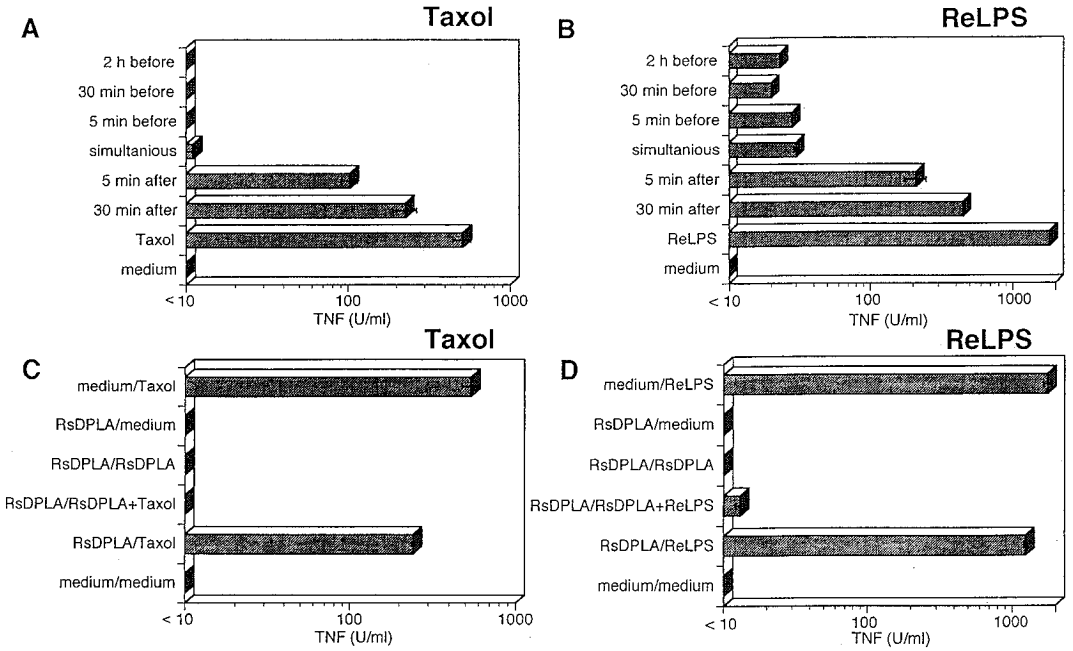

FIG. 6. Nature of the interaction on macrophages between taxol and RsDPLA in taxol-induced TNF secretion. (A and B) RsDPLA (333 ng/ml) was added to C3H/He macrophages at the indicated times before or after stimulation with taxol (15 μM) (A) or with ReLPS (10 ng/ml) (B), and the macrophages were then incubated for 4 h. (C and D) C3H/He macrophages were preincubated with or without 333 ng of RsDPLA per ml for 1 h, washed with PBS three times, and further incubated for 4 h in the presence or absence of taxol and/or RsDPLA (C) or in the presence or absence of ReLPS and/or RsDPLA (D). The supernatants were collected, and TNF activity in the supernatants in triplicate was determined by the cytotoxic assay. Each point represents the mean ± standard error of the mean. The data are from one of two or three experiments with similar results.

binding to J774.1 cells in a dose-dependent manner. Half-maximal binding inhibition (ID_{50}) occurred at 2.2 μM. A methylbenzimidate derivative of ReLPS (M-ReLPS; ID_{50}, 2 μM), S-form LPS (ID_{50}, 700 nM), lipid A (compound 506; ID_{50}, 3 μM), and RsDPLA (ID_{50}, 350 nM) also inhibited the binding of ^{125}I-LPS to J774.1 cells to extents similar to that of ReLPS. On the other hand, taxol did not inhibit the binding of ^{125}I-LPS to J774.1 cells compared with the DMSO control. Colchicine, vinblastine, and 10-deacetylbaccatine III also did not inhibit the binding of ^{125}I-LPS at the concentrations tested. These results suggest that if the majority of the observed binding of LPS is, in fact, to CD14, then CD14 is most likely not a taxol-binding site on the cell surface.

To examine whether taxol blocks CD14-independent LPS binding or uptake to cells, experiments of competitive binding to J7.DEF3 cells at 0 and 37°C or to J774.1 cells at 37°C were performed. Under the experimental conditions tested, we failed to detect that taxol blocked ^{125}I-LPS binding to these cells. As shown by the data (Fig. 9F), binding of ^{125}I-LPS to J7.DEF3 cells at 0°C was also inhibited by ReLPS (ID_{50}, 2 μM), M-ReLPS (ID_{50}, 2 μM), S-form LPS (ID_{50}, 3 μM), lipid A (ID_{50}, 7 μM), and RsDPLA (ID_{50}, 800 nM), but not by colchicine, vinblastine, or 10-deacetylbaccatine III (Fig. 9F). RsDPLA (6.7 μM; 10 μg/ml) also inhibited the binding of ^{125}I-LPS to both J774.1 cells and J7.DEF3 cells for 1 h at 37°C to 12 and 9% of control values, whereas neither taxol (30 and 300 μM) nor colchicine (3 mM) significantly inhibited the binding of ^{125}I-LPS to both cells (data not shown).

LPS and RsDPLA do not inhibit binding of [^{3}H]taxol. Taxol is known to bind directly to microtubules in a specific manner (22). Thus, to investigate whether LPS binds to cellular microtubules or to other taxol-binding sites in cultured J774.1 and J7.DEF3 cells, binding studies using [^{3}H]taxol were performed, and the results of these studies are shown in Fig. 10. Binding of

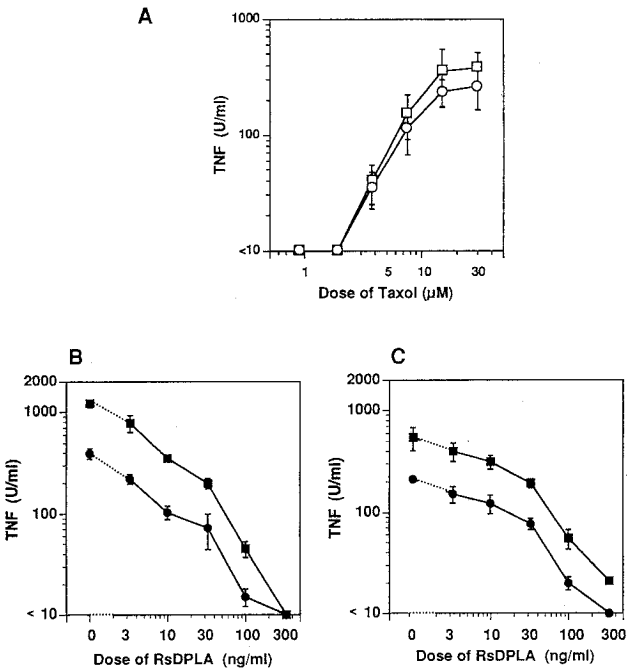

FIG. 7. Response of J774.1 cells and J7.DEF3 cells to taxol and RsDPLA. (A) J774.1 cells (□) and J7.DEF3 cells (○) were incubated with 15 μM taxol for 4 h. (B and C) J774.1 cells (B) and J7.DEF3 cells (C) were preincubated wth RsDPLA at the indicated doses for 1 h prior to incubation with 15 (■) or 7.5 (●) μM taxol for 4 h. TNF activity in the supernatants in triplicate was determined by the cytotoxic assay. Each point represents the mean ± standard error of the mean of five experiments in panel A and one of two experiments with similar results in panels B and C.

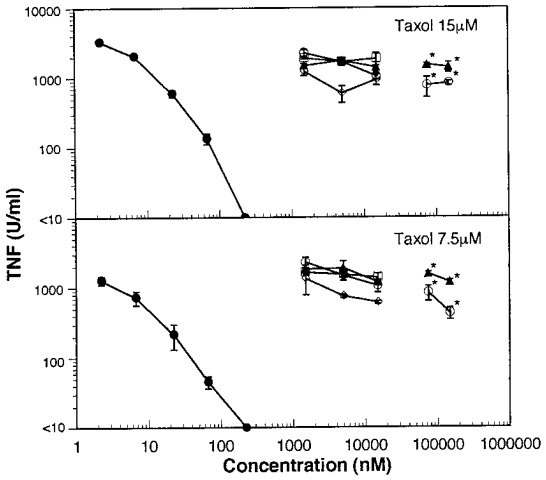

FIG. 8. Effects of pretreatment with mitotic inhibitors on TNF secretion by C3H/He macrophages stimulated with taxol. Cells were incubated with RsDPLA (●), colchicine (○), vinblastine (◇), 10-deacetylbaccatine III (□), or DMSO (▲ and ▲* [equivalent to concentrations of DMSO contained in these reagent solutions]) for 2 h and then stimulated with taxol (15 [top] or 7.5 [bottom] μM) for an additional 4 h. These competitors were prepared from a 10 mM stock solution in DMSO. Colchicine at higher doses was from a 100 mM stock (○*). Concentrations of RsDPLA are expressed in molarity (M), i.e., 100 ng of RsDPLA per ml is equal to 67 nM. TNF activity in the supernatants was determined by the cytotoxic assay. Each point represents the mean ± standard error of the mean. The data represent one of two experiments with similar results.

10 nM [^{3}H]taxol to J774.1 cells reached saturation within 180 min at 37°C. The binding of higher concentrations of taxol (a mixture of [^{3}H]taxol and unlabeled taxol) reached saturation more rapidly, within 60 min for 50 nM taxol and 15 min for 5 μM taxol (Fig. 10A). The kinetics of [^{3}H]taxol binding to J7.DEF3 cells was similar to that to J774.1 cells (Fig. 10B).

Binding of 10 nM [^{3}H]taxol to J774.1 cells was inhibited with increasing concentrations of unlabeled taxol (Fig. 10C), indicating that competition occurred for a limited number of binding sites. Half-maximal binding inhibition occurred at 100 nM. Scatchard analysis of the competitive binding data indicated two classes of binding sites: a K_d of 8.4×10^{-8} M and 9.1×10^6 binding sites per cell, and lower-affinity binding sites, with a K_d of 1.7×10^{-5} M and 3.0×10^8 binding sites per cell (Fig. 10C, insert). Binding of [^{3}H]taxol to J7.DEF3 cells was inhibited by unlabeled taxol, and its profile of biphasic Scatchard curves was similar to that for binding to J774.1 cells (Fig. 10D). For J7.DEF3 cells, there were K_ds of 7.7×10^{-8} M and 7.8×10^6 binding sites per cell for the higher-affinity binding sites and K_ds of 1.4×10^{-5} M and 2.0×10^8 binding sites per cell for the lower-affinity binding sites. There is no significant difference in these values for J774.1 cells and J7.DEF3 cells.

Competitive binding studies were performed to determine whether mitotic inhibitors and LPS preparations inhibit the binding of [^{3}H]taxol to both cells. In these studies, to examine properties of two classes of taxol-binding sites, [^{3}H]taxol was tested at a lower dose of 10 nM and at a higher dose of 5 μM (as a mixture of [^{3}H]taxol and unlabeled taxol), for higher and lower affinities, respectively. These data are shown in Fig. 10E to H. Either colchicine or vinblastine also markedly inhibited binding of [^{3}H]taxol to J774.1 cells (Fig. 10E). Half-maximal binding inhibition occurred at 200 and 10 nM, respectively. Since colchicine and vinblastine are known to bind to tubulin, these results suggest that the observed binding inhibition of [^{3}H]taxol at a lower concentration by colchicine and vinblastine is microtubule specific. 10-Deacetylbaccatine III, which is known not to bind to tubulin from mammalian cells (10), did not inhibit the binding of [^{3}H]taxol to the cells. On the other hand, neither ReLPS, RsDPLA, the S form of LPS, nor lipid A (compound 506) significantly inhibited the binding of [^{3}H]taxol to J774.1 cells. These data suggest that LPS does not bind to the major taxol-binding sites on microtubules. Profiles of [^{3}H]taxol-binding inhibition in J7.DEF3 cells were essentially identical (Fig. 10F).

As described above, both J774.1 cells and J7.DEF3 cells had other [^{3}H]taxol-binding sites with lower affinity. To examine whether ReLPS and RsDPLA inhibit the binding of [^{3}H]taxol to these binding sites, competitive binding studies were performed at a high concentration of 5 μM [^{3}H]taxol. This concentration of taxol is sufficient for inducing TNF secretion by J774.1 and J7.DEF3 cells. These results are shown in Fig. 10G and H. Colchicine and vinblastine at the concentrations tested manifested only slight inhibition of [^{3}H]taxol binding in both cells, indicating that the observed binding is not to microtubules. Under these conditions, neither ReLPS nor RsDPLA inhibits the binding of [^{3}H]taxol to J774.1 and J7.DEF3 cells.

DISCUSSION

In this report, we have examined the mechanism(s) by which LPS and taxol activate murine macrophages to induce TNF secretion and NO production. Taxol induced responses in LPS-responsive C3H/He but not LPS-hyporesponsive C3H/HeJ macrophages. RsDPLA, a lipid A antagonist, blocked taxol-induced TNF secretion and NO production. The inhibition of TNF secretion by RsDPLA appears to be specific for LPS and taxol stimulation because RsDPLA did not inhibit TNF responses induced by other activators, such as HKLM and zymosan. Mutant macrophage-like J7.DEF3 cells, which are defective in expression of CD14 antigen, responded to taxol by TNF secretion to an extent equivalent to that of the parental CD14-positive J774.1 cells, and RsDPLA inhibited taxol-induced TNF production equally in both J7.DEF3 and J774.1 cells. The binding of ^{125}I-LPS to J774.1 cells was significantly inhibited by both unlabeled LPS and RsDPLA but not by taxol. The binding of [^{3}H]taxol to the cells, in contrast, was significantly inhibited by unlabeled taxol but not by LPS or RsDPLA. Collectively, these results are consistent with the concepts that (i) both LPS and taxol interact with a functional LPS receptor on the cell surface, (ii) that RsDPLA blocks both LPS- and taxol-induced macrophage activation by occupancy of that functional LPS receptor, and (iii) that neither CD14 nor microtubules, which are the dominant LPS- and taxol-binding proteins, respectively, associated with the cell, serve as the functional receptors for LPS or taxol.

Binding studies with [^{3}H]taxol showed that taxol binds specifically and reversibly to polymerized tubulin in cell-free systems (30) as well as to microtubules in cultured cells (22). More recently, direct photoaffinity labeling with [^{3}H]taxol has demonstrated that taxol preferentially binds to the β subunit of tubulin (36). It is possible that a tubulin protein, perhaps β tubulin, would be a target molecule for LPS. Ding et al. (4) have recently demonstrated by the use of the photoactivable, radioiodinated LPS probe, ^{125}I-LPS-2-(*p*-azidosalicylamido)-1,3-dithiopropionamide, that LPS can bind specifically to β tubulin. Our results reported here, nevertheless, would not support the concept that microtubules would serve as functional LPS receptors, since (i) LPS did not inhibit the binding of [^{3}H]taxol in macrophages, (ii) taxol did not inhibit the binding of ^{125}I-LPS in macrophages, (iii) RsDPLA most likely blocks the taxol-induced macrophage activation at the cell surface, presumably by blocking the specific binding of taxol to the

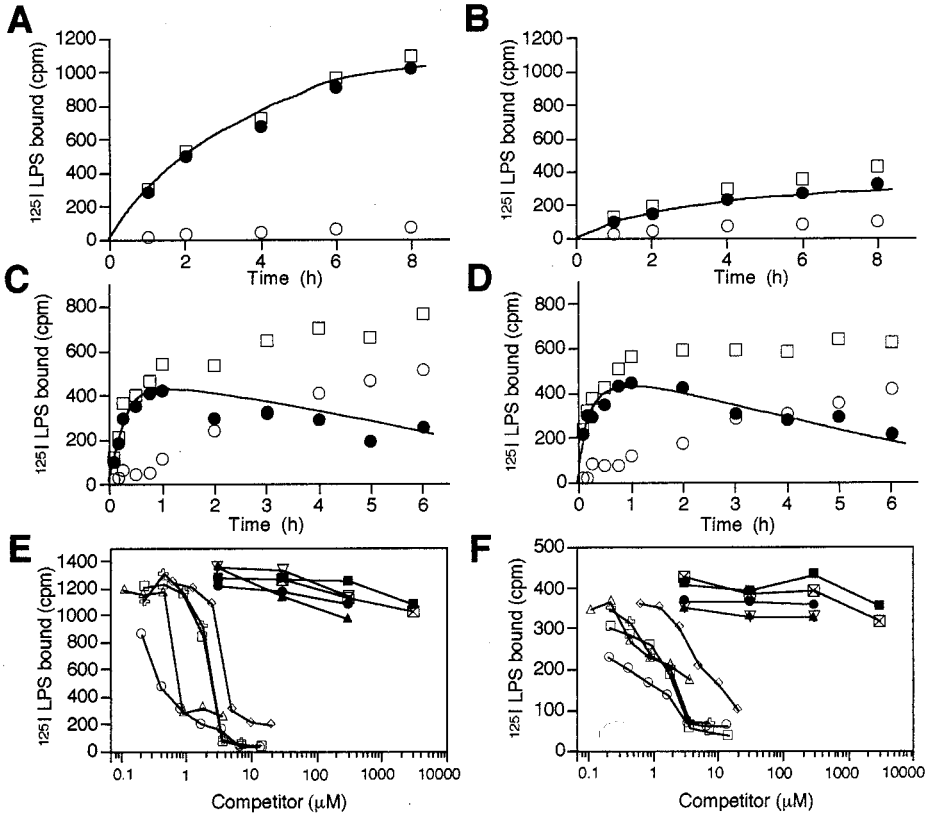

FIG. 9. Kinetics of ^{125}I-LPS binding to J774.1 and J7.DEF3 cells and competitive inhibition of the binding by LPS preparations and mitotic inhibitors. (A to D) Kinetics of ^{125}I-LPS binding. J774.1 (A and C) and J7.DEF3 (B and D) cells were incubated for various time intervals at 0°C (A and B) or 37°C (C and D) with ^{125}I-LPS (100 ng/ml) alone (defined as total binding [□]) or in the presence of unlabeled LPS (20 μg/ml; defined as nonspecific binding [○]). Specific binding (●) was defined as the difference between the total binding and nonspecific binding. Cells were washed, and the radioactivity bound to the cells was determined. The amount of radioactivity added to each well was 3,380 ± 380 cpm. Data represent mean values for two determinations from one of two or three similar experiments. (E and F) Competitive inhibition of ^{125}I-LPS binding to J774.1 (E) and J7.DEF3 (F) cells by LPS preparations and mitotic inhibitors. The cells were incubated at 0°C for 6 h with ^{125}I-LPS (100 ng/ml) in the presence or absence of unlabeled competitors at the indicated doses. Tested competitors were taxol (●), colchicine (■), vinblastine (▲), 10-deacetylbaccatine III (▽), ReLPS (□), RsDPLA (○), S-form LPS (△), synthetic lipid A (◇), M-ReLPS (✣), and DMSO (☒ [equivalent to concentrations of DMSO contained in these reagent solutions]). The amounts of radioactivity bound to control cells were 1,220 (E) and 340 (F) cpm. Each point represents a value for one determination from one of two representative experiments.

functional LPS receptor, and (iv) other microtubule-binding agents, colchicine and vinblastine, did not activate macrophages to induce TNF secretion nor did these agents inhibit taxol-induced macrophage activation. In addition to these findings, macrophages from LPS-hyporesponsive C3H/HeJ mice possess normal microtubule morphology, and taxol induced the formation of microtubule bundles in C3H/HeJ macrophages similar to that observed in other cell types (23). Microtubules derived from C3H/HeJ and LPS-responsive C3H/HeN mice are indistinguishable in their ability to bind LPS (4). Collectively, these results strongly support the conclusion that microtubules do not function as a specific LPS receptor in generating transmembrane signals leading to macrophage activation. The putative LPS receptor might be a microtubule-associated protein like MAP-2, as suggested by Ding et al. (4).

CD14, a differentiation antigen of monocytes and macrophages and a glycosylphosphatidylinositol-anchored protein, serves as a specific binding site for the complex of LPS and LPS-binding protein (58). This CD14-dependent LPS recognition by the cells is thought to contribute to enhanced opsonization and phagocytic uptake of LPS-coated particles and gram-negative bacteria (59). It also enhances the binding of LPS (15, 44, 58) and responds to LPS in inducing the secretion of inflammatory cytokines (44, 57, 58). A soluble form of CD14 lacking the glycosylphosphatidylinositol anchor also functions as a coligand for LPS in potentiating responses to LPS in CD14-deficient cells (6, 31). These observations suggest that CD14, regardless of whether it is in the membrane-bound or soluble form, complexes with the LPS, which then potentiates its interaction with the functional LPS receptor. The CD14 may function to facilitate the ability of macrophages to recognize and respond to LPS.

We have recently examined the responsiveness to various chemotypes of LPS (15) of mutant J7.DEF3 cells, which are defective in CD14 expression (8), and have suggested a functional role for the CD14-dependent LPS recognition system. At the LPS-active site, the lipid A moiety of LPS is normally masked by the polysaccharide portion of LPS, making it difficult to bind directly to lipid A-binding sites (LPS receptors) on the cells. By forming a complex with LPS, CD14 would expose the lipid A portion of the LPS molecule and correctly present it to the functional LPS receptor. The present studies have shown that the mutant J7.DEF3 cells readily respond to taxol in inducing TNF secretion and that taxol-induced TNF production is blocked by RsDPLA to the same extent as occurs with CD14-positive parent cells. These results indicate that taxol activates macrophages in a CD14-independent manner and that CD14 is not the target molecule for taxol. It is well

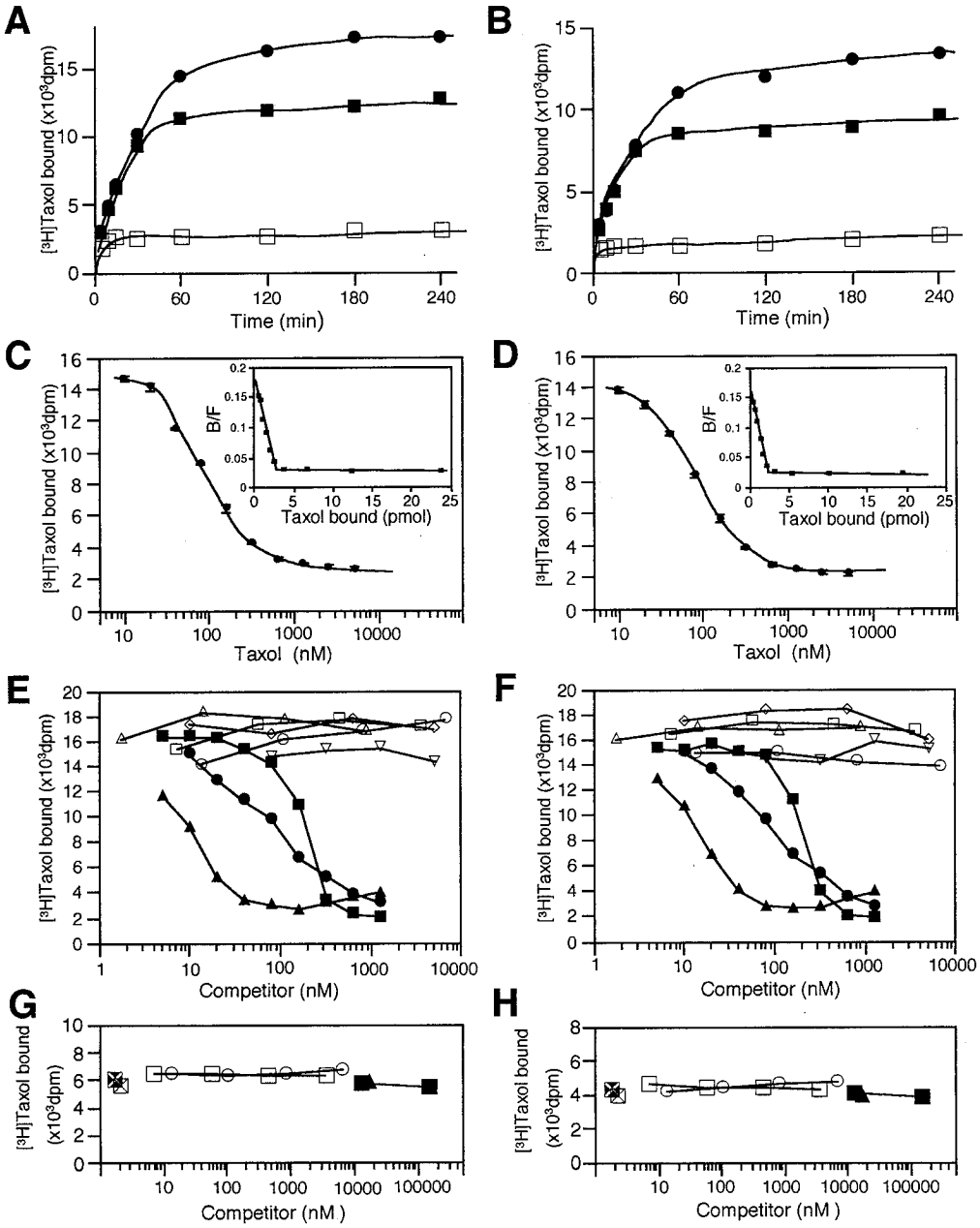

FIG. 10. Binding of [^{3}H]taxol to J774.1 cells and J7.DEF3 cells and competitive inhibition of the binding by LPS preparations and mitotic inhibitors. (A and B) Kinetics of [^{3}H]taxol binding. The J774.1 (A) and J7.DEF3 (B) cells were incubated at 37°C for the indicated times with [^{3}H]taxol (10 nM) alone (●) or in the presence of unlabeled taxol (40 nM [■] or 5 μM [□]). The cells were washed, and [^{3}H]taxol binding was then measured. (C and D) Competitive inhibition of [^{3}H]taxol binding to J774.1 (C) and J7.DEF3 (D) cells by unlabeled taxol. The cells were incubated at 37°C for 3 h with [^{3}H]taxol (10 nM) in the presence of unlabeled taxol at the indicated doses. The amounts of radioactivity bound to control cells were 14,580 (C) and 13,050 (F) dpm. Scatchard plots (inserts) of these data were used to determine apparent K_d values and valences (binding sites per cell). B/F, [^{3}H]taxol bound to cells per free [^{3}H]taxol. Units on the abscissa are picomoles of [^{3}H]taxol per 2×10^5 cells. (E and F) Competitive inhibition of [^{3}H]taxol binding with higher-affinity binding sites to J774.1 (E) and J7.DEF3 (F) cells by LPS preparations and mitotic inhibitors. The cells were incubated at 37°C for 3 h with [^{3}H]taxol (10 nM) in the presence or absence of unlabeled competitors at the indicated doses. Tested competitors were taxol (●), colchicine (■), vinblastine (▲), 10-deacetylbaccatine III (▽), ReLPS (□), RsDPLA (○), S-form LPS (△), and synthetic lipid A (◇). The amounts of radioactivity bound to control cells and 0.015% DMSO control cells were 16,900 and 16,600 dpm (E) and 16,200 and 16,300 dpm (F), respectively. (G and H) Competitive inhibition of [^{3}H]taxol binding with lower-affinity binding sites to J774.1 (E) and J7.DEF3 (F) cells by LPS preparations and mitotic inhibitors. The cells were incubated at 37°C for 3 h with a mixture of [^{3}H]taxol (20 nM) and unlabeled taxol (10 μM) in the presence of unlabeled ReLPS (□), RsDPLA (○), colchicine (■), or vinblastine (▲) at the indicated doses. Values for control cells (⊠) and DMSO-control cells (⊠) are shown. Data represent the means ± standard errors of the means of triplicate wells (C and D) or the means of duplicate wells. The amount of radioactivity added to each well was 93,000 dpm for 10 nM [^{3}H]taxol. The data are from one of two or three representative experiments.

documented that serum compounds, such as LPS-binding protein (44) and septin (57), are important for the CD14-dependent response to LPS in macrophages. We have not been able to determine the effects of serum on taxol-induced cell activation since taxol was not soluble in serum-free medium. Additional studies using LPS-responsive cells which do not express absolutely the surface CD14 would be needed to exclude completely the contribution of a CD14-dependent pathway in taxol-induced macrophage activation.

The present studies have provided further evidence that there exists a functional LPS receptor on the cell surface which recognizes LPS, RsDPLA, and taxol and which generates a transmembrane signal resulting in macrophage activation. Since the chemical structure of taxol is quite different from that of LPS or RsDPLA, it may be difficult to imagine that a single receptor recognizes these structures. The possibility that the receptor for taxol differs from that for LPS and RsDPLA cannot be ruled out. If this is so, then these receptors must be located in close proximity to each other to allow mutual interference. Presently, there are several good candidates for the putative LPS receptor protein. These include the 73-kDa (18) and 40-kDa (13, 18, 19) proteins which were identified by photo-cross-linking and ligand-blotting assays. The p73 protein is expressed on mouse B cells, T cells, and macrophages and localized on the cell membrane (19). There exist similar p73 proteins on peripheral blood mononuclear cells of a variety of endotoxin-sensitive mammalian species, including humans (38). The binding of LPS to the p73 protein was inhibitable by lipid A (18). A hamster monoclonal antibody, 5D3, raised against p73 (2) could activate macrophages for tumor cell killing (3). These data provide support for the concept that the p73 protein serves as a functional receptor for LPS in triggering lymphoreticular cell responses. Our data summarized above would predict, therefore, that the p73 protein must be recognized by all LPS, RsDPLA, and taxol. Indeed, Lei et al. (20) have recently demonstrated that RsDPLA inhibits the binding of LPS to p73, indicating that p73 protein can recognize both LPS and RsDPLA. We are currently carrying out experiments to determine whether taxol inhibits the binding of LPS to the p73 protein.

Using coimmunoprecipitation and kinase assays, Števanová et al. (45) have previously demonstrated that a number of glycosylphosphatidylinositol-anchored cell surface molecules are associated with protein tyrosine kinases related to Src. Their recent studies have shown that CD14 is coupled to protein tyrosine kinase p53/56^{lyn} in human monocytes and that LPS activates CD14-associated p53/56^{lyn} (46). However, it is still unclear exactly how a glycosylphosphatidylinositol-anchored CD14 molecule without a cytoplasmic domain is associated with an intracellular protein tyrosine kinase, Src-related p53/56^{lyn}. It is reasonable to hypothesize that the functional LPS receptor, possibly p73 protein, can be linked to both CD14 and p53/56^{lyn} and that this receptor complex can be associated with microtubules. Our current studies are designed to confirm or refute this hypothesis.

ACKNOWLEDGMENTS

We would like to thank K. Hisatsune for providing *S. minnesota* R595 LPS, M. J. Parmely for providing the L929 cell line, S. W. Russell for providing HKLM, T. Suzuki for providing the J774.1 cell line, and H. Tamura and Y. Arimori for comments and personal communications on the ES test. We also thank Daiichi Pharmaceutical Co. for providing synthetic lipid A (compound 506).

This work was supported by grants from the Ministry of Education, Science and Culture (06670302 to T.K. and 05454194 to M.N.), the National Institutes of Health (R37 AI23447 and PO1 CA54474 to D.C.M), and the Research Service of the Department of Veterans Affairs and National Institutes of Health Grant (GM36054 to K.T.).

REFERENCES

1. **Bogdan, C., and A. Ding.** 1992. Taxol, a microtubule-stabilizing antineoplastic agent, induces expression of tumor necrosis factor α and interleukin-1 in macrophages. J. Leukocyte Biol. **52:**119–121.
2. **Bright, S. W., T. Chen, L. M. Flebbe, M. Lei, and D. C. Morrison.** 1990. Generation and characterization of hamster-mouse hybridomas secreting monoclonal antibodies with specificity for lipopolysaccharide receptor. J. Immunol. **145:**1–7.
3. **Chen, T., S. W. Bright, J. L. Pace, S. W. Russell, and D. C. Morrison.** 1990. Induction of macrophage-mediated tumor cytotoxicity by a hamster monoclonal antibody with specificity for lipopolysaccharide receptor. J. Immunol. **145:**8–12.
4. **Ding, A., E. Sanchez, M. Tancinco, and C. Nathan.** 1992. Interactions of bacterial lipopolysaccharide with microtubule proteins. J. Immunol. **148:** 2853–2858.
5. **Ding, A. H., F. Porteu, E. Sanchez, and C. F. Nathan.** 1990. Shared actions of endotoxin and taxol on TNF receptors and TNF release. Science **248:**370–372.
6. **Frey, E. A., D. S. Miller, T. G. Jahr, A. Sudan, B. V., T. Espevik, B. B. Finlay, and S. D. Wright.** 1992. Soluble CD14 participates in the response of cells to lipopolysaccharide. J. Exp. Med. **176:**1665–1671.
7. **Golenbock, D. T., R. Y. Hampton, N. Qureshi, K. Takayama, and C. R. H. Raetz.** 1991. Lipid A-like molecules that antagonize the effects of endotoxins on human monocytes. J. Biol. Chem. **266:**19490–19498.
8. **Goyert, S. M., T. Kirikae, F. Kirikae, and D. C. Morrison.** 1992. Identification of a defective molecule in LPS-binding mutant J7.DEF3 cells isolated from macrophage-like J774.1 cells, abstr. 159. *In* program and abstracts of the 2nd Conference of the International Endotoxin Society, Vienna.
9. **Green, L. C., D. A. Wagner, J. Glogowski, P. L. Skipper, J. S. Wishnok, and S. R. Tannenbaum.** 1982. Analysis of nitrate, nitrite, and [^{15}N]nitrate in biological fluids. Anal. Biochem. **126:**131–138.
10. **Gueritte-Voegelein, F., D. Guenard, F. Lavelle, M. le Goff, L. Mangatal, and P. Potier.** 1991. Relationships between the structure of taxol analogues and their antimitotic activity. J. Med. Chem. **34:**992–998.
11. **Hampton, R. Y., D. T. Golenbock, P. Marsha, M. Krieger, and C. R. H. Raetz.** 1991. Recognition and plasma clearance of endotoxin by scavenger receptors. Nature (London) **352:**342–344.
12. **Imoto, M., N. Yoshimura, S. Kusumoto, and T. Shiba.** 1984. Total synthesis lipid A, active principle of bacterial endotoxin. Proc. Jpn. Acad. Ser. B **60:**285–288.
13. **Kirikae, T., F. Kirikae, F. U. Schade, M. Yoshida, S. Kondo, K. Hisatsune, S. Nishikawa, and E. T. Rietschel.** 1991. Detection of lipopolysaccharide-binding proteins on membranes of murine lymphocyte and macrophage-like cell lines. FEMS Microbiol. Immunol. **76:**327–336.
14. **Kirikae, T., F. U. Schade, F. Kirikae, N. Qureshi, K. Takayama, and E. T. Rietschel.** 1994. Diphosphoryl lipid A derived from the lipopolysaccharide (LPS) of *Rhodobacter sphaeroides* ATCC 17023 is a potent competitive LPS inhibitor in murine macrophage-like J774.1 cells. FEMS Immunol. Med. Microbiol. **9:**237–244.
15. **Kirikae, T., F. U. Schade, F. Kirikae, E. T. Rietschel, and D. C. Morrison.** 1993. Isolation of a macrophage-like cell line defective in binding of lipopolysaccharide. Influence of serum and lipopolysaccharide chain length on macrophage activation. J. Immunol. **151:**2742–2752.
16. **Kirikae, T., F. U. Schade, U. Zahringer, F. Kirikae, H. Brade, S. Kusumoto, T. Kusama, and E. T. Rietschel.** 1994. The significance of the hydrophilic backbone and the hydrophobic fatty acid regions of lipid A for macrophage binding and cytokine induction. FEMS Immunol. Med. Microbiol. **8:**13–26.
17. **Kumar, N.** 1981. Taxol-induced polymerization of purified tubulin. J. Biol. Chem. **256:**10435–10441.
18. **Lei, M., and D. C. Morrison.** 1988. Specific endotoxic lipopolysaccharide-binding proteins on murine splenocytes. I. Detection of lipopolysaccharide-binding sites on splenocytes and splenocyte subpopulations. J. Immunol. **141:**996–1005.
19. **Lei, M., and D. C. Morrison.** 1988. Specific endotoxic lipopolysaccharide-binding proteins on murine splenocytes. II. Membrane localization and binding characteristics. J. Immunol. **141:**1006–1011.
20. **Lei, M., N. Qureshi, and D. C. Morrison.** 1993. Lipopolysaccharide (LPS) binding to 73-kDa and 38-kDa surface proteins on lymphoreticular cells: preferential inhibition of LPS binding to the former by *Rhodopseudomonas sphaeroides* lipid A. Immunol. Lett. **36:**245–250.
21. **Lynn, W. A., and D. T. Golenbock.** 1992. Lipopolysaccharide antagonists. Immunol. Today **13:**271–276.
22. **Manfredi, J. J., J. Parness, and S. B. Horwitz.** 1982. Taxol binds to cellular microtubules. J. Cell Biol. **94:**688–696.
23. **Manthey, C. L., M. E. Brandes, P. Y. Perera, and S. N. Vogel.** 1992. Taxol increases steady-state levels of lipopolysaccharide-inducible genes and protein-tyrosine phosphorylation in murine macrophages. J. Immunol. **149:**2459–2465.
24. **Manthey, C. L., N. Qureshi, P. L. Stutz, and S. N. Vogel.** 1993. Lipopoly-

saccharide antagonists block taxol-induced signaling in murine macrophages. J. Exp. Med. **178:**695–702.
25. **Merkofer, T.** 1994. Position and configuration of the double bond in unsaturated lipid A-fatty acid in various bacteria. Ph.D. thesis. University of Freiburg, Freiburg, Germany.
26. **Morrison, D. C., and D. M. Jacobs.** 1976. Binding of polymyxin B to the lipid A portion of bacterial LPS. Immunochemistry **13:**813–818.
27. **Morrison, D. C., M. Lei, T. Kirikae, and T. Y. Chen.** 1993. Endotoxin receptors on mammalian cells. Immunobiology **187:**212–226.
28. **Morrison, D. C., and J. L. Ryan.** 1987. Endotoxins and disease mechanisms. Annu. Rev. Med. **38:**417–432.
29. **Obayashi, T., H. Tamura, S. Tanaka, M. Ohki, S. Takahashi, M. Arai, M. Masuda, and T. Kawai.** 1985. A new chromogenic endotoxin-specific assay using recombined limulus coagulation enzymes and its clinical applications. Clin. Chim. Acta **149:**55–65.
30. **Parness, J., and S. B. Horwit.** 1981. Taxol binds to polymerized tubulin in vitro. J. Cell Biol. **91:**479–487.
31. **Pugin, J., C. Schurer-Maly, D. Leturcq, A. Moriarty, R. J. Ulevitch, and P. S. Tobias.** 1993. Lipopolysaccharide activation of human endothelial and epithelial cells is mediated by lipopolysaccharide-binding protein and soluble CD14. Proc. Natl. Acad. Sci. USA **90:**2744–2748.
32. **Qureshi, N., J. P. Honovich, H. Hara, R. J. Cotter, and K. Takayama.** 1988. Location of fatty acids in lipid A obtained from lipopolysaccharide of *Rhodopseudomonas sphaeroides* ATCC 17023. J. Biol. Chem. **263:**5502–5504.
33. **Qureshi, N., K. Takayama, and R. Kurtz.** 1991. Diphosphoryl lipid A obtained from the nontoxic lipopolysaccharide of *Rhodoseudomonas sphaeroides* is an endotoxin antagonist in mice. Infect. Immun. **59:**441–444.
34. **Qureshi, N., K. Takayama, K. C. Meyers, T. N. Kirkland, C. A. Bush, L. Chen, R. Wang, and R. J. Cotter.** 1991. Chemical reduction of 3-oxo and unsaturated groups in fatty acids of diphosphoryl lipid A from the lipopolysaccharide of *Rhodopseudomonas sphaeroides*. Comparison of biological properties before and after reduction. J. Biol. Chem. **266:**6532–6538.
35. **Raetz, C. R. H., R. J. Ulevitch, S. D. Wright, C. H. Sibley, A. Ding, and C. F. Nathan.** 1991. Gram-negative endotoxin: an extraordinary lipid with profound effects on eukaryotic signal transduction. FASEB J. **5:**2652–2660.
36. **Rao, S., S. B. Horwitz, and I. Ringel.** 1992. Direct photoaffinity labeling of tubulin with taxol. J. Natl. Cancer Inst. **84:**785–788.
37. **Rietschel, E. T., T. Kirikae, F. U. Schade, U. Mamat, G. Schmidt, H. Loppnow, A. J. Ulmer, U. Zahringer, U. Seydel, F. di Padova, M. Schreier, and H. Brade.** 1994. Bacterial endotoxin: molecular relationships of structure to activity and function. FASEB J. **8:**217–225.
38. **Roeder, D. J., M. Lei, and D. C. Morrison.** 1989. Endotoxic-lipopolysaccharide-specific binding proteins on lymphoid cells of various animal species: association with endotoxin susceptibility. Infect. Immun. **57:**1054–1058.
39. **Ruco, L. P., M. S. Meltzer, and D. L. Rosenstreich.** 1987. Macrophage activation for tumor cytotoxicity: control of macrophage tumoricidal capacity by the LPS gene. J. Immunol. **121:**543–548.
40. **Ruff, M. R., and G. E. Gifford.** 1980. Purification and physiochemical characterization of rabbit tumor necrosis factor. J. Immunol. **125:**1671–1677.
41. **Salimath, P. V., J. Weckesser, W. Strittmatter, and H. Mayer.** 1983. Structural studies on the non-toxic lipid A from *Rhodopseudomonas sphaeroides* ATCC 17023. Eur. J. Biochem. **136:**195–200.
42. **Schiff, P. B., and S. B. Horwitz.** 1980. Taxol stabilizes microtubules in mouse fibroblast cells. Proc. Natl. Acad. Sci. USA **77:**1561–1565.
43. **Schiff, P. B., J. Fant, and S. B. Horwitz.** 1979. Promotion of microtubule assembly *in vitro* by taxol. Nature (London) **277:**665–667.
44. **Schumann, R. R., S. R. Leong, G. W. Flaggs, P. W. Gray, S. D. Wright, J. C. Mathison, P. S. Tobias, and R. J. Ulevitch.** 1990. Structure and function of lipopolysaccharide binding protein. Science **249:**1429–1431.
45. **Štefanová, I., V. Hořejší, I. J. Ansotegui, W. Knapp, and H. Stockinger.** 1991. GPI-anchored cell-surface molecules complexed to protein tyrosine kinases. Science **254:**1016–1019.
46. **Štefanová, I., M. L. Corcoran, E. M. Horak, L. M. Wahl, J. B. Bolen, and I. D. Horak.** 1993. Lipopolysaccharide induces activation of CD14-associated protein trysine kinase $p53/56^{lyn}$. J. Biol. Chem. **268:**20725–20728.
47. **Strittmatter, W., J. Weckesser, P. V. Salimath, and C. Galanos.** 1983. Nontoxic lipopolysaccharide from *Rhodopseudomonas sphaeroides* ATCC 17023. J. Bacteriol. **155:**153–158.
48. **Sultzer, B. M.** 1968. Genetic control of leukocyte responses to endotoxin. Nature (London) **219:**1253–1254.
49. **Takayama, K., N. Qureshi, B. Beutler, and T. N. Kirkland.** 1989. Diphosphoryl lipid A from *Rhodopseudomonas sphaeroides* ATCC 17023 blocks induction of cachectin in macrophages by lipopolysaccharide. Infect. Immun. **57:**1336–1338.
50. **Tamura, H., and Y. Arimoto.** 1994. Personal communication.
51. **Ulevitch, R. J.** 1994. Personal communication.
52. **Ulevitch, R. J.** 1978. The preparation and characterization of a radioiodinated bacterial lipopolysaccharide. Immunochemistry **15:**157–164.
53. **Wani, M. C., H. L. Taylor, M. E. Wall, P. Coggon, and A. T. McPhail.** 1971. Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. J. Am. Chem. Sci. **93:**2325–2327.
54. **Wilson, L.** 1975. Microtubules as drug receptors: pharmacological properties of microtubule protein. Ann. N. Y. Acad. Sci. **253:**213–231.
55. **Wright, S. D.** 1991. Multiple receptors for endotoxin. Curr. Opin. Immunol. **3:**83–90.
56. **Wright, S. D., and M. C. Jong.** 1986. Adhesion-promoting receptors on human macrophages recognize *Escherichia coli* by binding to lipopolysaccharide. J. Exp. Med. **164:**1876–1888.
57. **Wright, S. D., R. A. Ramos, M. Patel, and D. S. Miller.** 1992. Septin: a factor in plasma that opsonizes lipopolysaccharide-bearing particles for recognition by CD14 on phagocytes. J. Exp. Med. **176:**719–727.
58. **Wright, S. D., R. A. Ramos, P. S. Tobias, R. J. Ulevitch, and J. C. Mathison.** 1990. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science **249:**1431–1433.
59. **Wright, S. D., P. S. Tobias, R. J. Ulevitch, and R. A. Ramos.** 1989. Lipopolysaccharide-binding protein opsonizes LPS-bearing particles for recognition via novel receptor on macrophages. J. Exp. Med. **170:**1231–1241.