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Paclitaxel (Taxol)-Induced NF-κB Translocation in Murine Macrophages

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Interaction of bacterial lipopolysaccharide (LPS) with macrophages results in the induction of a cascade of cytokines that mediate the varied effects of LPS. An early intracellular signaling event that follows receptor engagement is the activation of transcription factor NF-κB. NF-κB has been shown to be important for the induction of many LPS-inducible cytokine genes, including tumor necrosis factor alpha, interleukin-1β, and interleukin-6. Previously, we and others have shown that the antitumor agent paclitaxel (Taxol) is able to mimic bacterial LPS in its ability to activate murine macrophages. In this report, we have extended these findings by demonstrating that paclitaxel, like LPS, is able to stimulate the translocation of primarily p50-p65 heterodimers of NF-κB to the nucleus. This activation is dose dependent and requires a concentration of ≥5 μM paclitaxel. The kinetics of NF-κB activation by paclitaxel are slower than those of LPS: by 15 min poststimulation, LPS-induced NF-κB activation was readily detected, whereas the paclitaxel-induced NF-κB activation was minimal. Moreover, paclitaxel- and protein-free LPS-induced translocation of NF-κB was seen only in macrophages derived from LPS-responsive C3H/OuJ mice and not from the LPS-hyporesponsive C3H/HeJ mice, a finding that is consistent with those of previous genetic studies linking paclitaxel responsiveness to the *Lps* gene. Finally, the LPS structural antagonist *Rhodobacter sphaeroides* diphosphoryl lipid A inhibited both LPS- and paclitaxel-induced NF-κB activation, suggesting a common receptor component in this activation.

Paclitaxel (Taxol), derived from the Pacific yew tree, is a prototypic member of a class of drugs with a broad range of antineoplastic effects. Paclitaxel's effectiveness as an antitumor agent is believed to reside in its ability to stabilize microtubules against depolymerization leading to mitotic arrest during cell division (29). Recent reports indicate that paclitaxel and bacterial lipopolysaccharide (LPS) induce a broad range of shared bioactivities in murine macrophages, although they are structurally dissimilar. These shared bioactivities include the ability to internalize tumor necrosis factor (TNF) receptors, to induce the secretion of the cytokine TNF, to stimulate the expression of a panel of immediate early genes, to provide a second signal for macrophage tumoricidal activity and nitric oxide production, to induce the phosphorylation of mitogen-activated protein (MAP) kinases, and to activate *lyn* kinase autophosphorylation (9, 13, 24, 27). Additionally, in murine macrophages, the responsiveness to both agents has been reported to be genetically linked to the *Lps* gene (9, 29, 44). The cellular responses to bacterial LPS are widely viewed to occur as a result of the binding of LPS–LPS-binding protein complexes to glycosyl-phosphatidylinositol-anchored CD14 receptors on the surface of macrophages, monocytes, and neutrophils (42). Since CD14 lacks an intracellular signal transducing region, it is believed that it associates with an as yet unidentified signaling molecule(s). However, the mechanism(s) by which paclitaxel stimulates murine macrophages is currently not well defined. The ability of a disaccharide LPS antagonist, *Rhodobacter sphaeroides* diphosphoryl lipid A (*Rs*DPLA), and a monosaccharide lipid X analog, SDZ 880.431, to inhibit both LPS- and paclitaxel-induced early gene expression, TNF secretion, and tyrosine phosphorylation suggests that an LPS receptor-dependent pathway is a possible mechanism for paclitaxel's LPS-mimetic effects (26, 28).

Nuclear factor κB (NF-κB) is an inducible eukaryotic transcription factor (reviewed in references 1, 40, and 41). This transcription factor has been shown to regulate the expression of numerous cellular and viral genes that participate in inflammatory, immune, and acute-phase responses (reviewed in references 1, 40, and 41). Cytokines, such as TNF-α, interferon-β, interleukin-1 (IL-1), IL-6, and IL-8, and the adhesion molecules ELAM-1, ICAM-1, and VCAM-1 are products of some of the genes that are regulated by NF-κB-dependent mechanisms (1). Active DNA binding forms of NF-κB are dimeric complexes composed of various combinations of members that constitute the *rel* family of protein complexes. Of these, the p50-p65 heterodimeric complex is the most abundant of all dimeric NF-κB complexes being expressed in almost all cell types. Characteristically, NF-κB complexes are found to preexist in the cytoplasm of many cell types in an inactive form, associated with an inhibitor subunit, IκB, which, in turn, is composed of a family of proteins. Most members (IκB-α, -β, and -γ and precursor proteins p100 and p105) of the IκB family of proteins, when associated with NF-κB dimers that possess a subunit with a *trans*-activating domain (p65 and c-rel), act as inhibitors by retaining these dimers in the cytoplasm (2, 19, 22). In contrast, IκB family member Bcl-3 has been shown to function as an activator protein within the nucleus, modulating the functions of p50 and p52 homodimers that lack a transactivating domain (2, 19). NF-κB dimers can be activated by a

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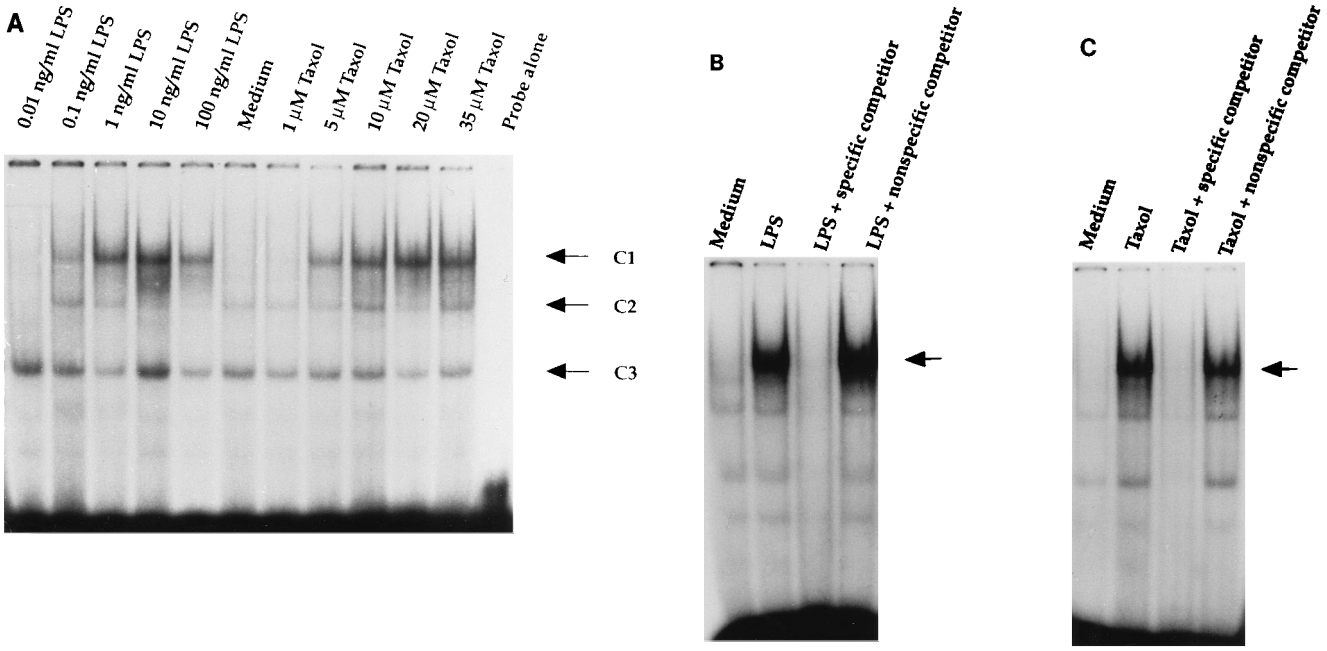

FIG. 1. (A) Dose-dependent translocation of DNA-protein complexes in response to paclitaxel (Taxol) and LPS in murine macrophages. Overnight cultures of C3H/OuJ macrophages were stimulated with medium alone or the indicated concentrations of LPS or paclitaxel for 1 h. Nuclear extracts were prepared, and EMSAs were performed in the presence of ^{32}P-labeled, double-stranded oligonucleotide NF-κB probe containing the consensus sequence from the immunoglobulin κ chain gene enhancer as described in Materials and Methods. Arrows indicate the positions of the three DNA-protein complexes. The figure represents one of three separate experiments performed. (B) Competition analyses of the DNA-protein complexes formed in response to LPS. Nuclear extracts of C3H/OuJ macrophages treated with 100 ng of LPS per ml for 1 h were analyzed by EMSAs in the presence of 25 molar excess of unlabeled specific or nonspecific oligonucleotide probe in the DNA binding reaction as described in Materials and Methods. The arrow indicates the position of the inducible complex. The figure represents one of four separate experiments performed. (C) Competition analyses of the DNA-protein complexes formed in response to paclitaxel (Taxol). Nuclear extracts of C3H/OuJ macrophages treated with 30 μM paclitaxel for 1 h were analyzed by EMSAs in the presence of 25 molar excess of unlabeled specific or nonspecific oligonucleotide probe in the DNA binding reaction as described in Materials and Methods. The arrow indicates the position of the inducible complex. The figure represents one of three separate experiments performed.

variety of stimulants. Among these are cytokines, such as IL-1, IL-2, and TNF, and phorbol esters, viruses, protein synthesis inhibitors, T-cell activators, and calcium ionophores (1). It was originally postulated that upon stimulation by various activators, the inhibitors IκB-α subunit, which binds most dimers (exceptions are p50 and p52 homodimers) and p50-p65 dimers in particular, is phosphorylated. This phosphorylation, in turn, was felt to cause the subunit to dissociate from the NF-κB dimer, thus freeing the active NF-κB dimer to translocate to the nucleus (1). More recently, it has been shown that following signal-induced phosphorylation of IκB-α, IκB-α remains complexed to the p50-p65 dimer until it is degraded by the ubiquitin-proteasome pathway of protein degradation (4, 6, 34). Upon proteolytic degradation of IκB-α, the active, free p50-p65 dimer translocates from the cytoplasm to the nucleus to interact with the promoter-enhancer region of target genes.

Bacterial LPS is also a potent stimulator of NF-κB translocation in macrophages, and it has been shown that NF-κB plays an important role in regulating many of the bioactivities induced by LPS (reviewed in reference 1). The data presented in this report extend our definition of paclitaxel as an LPS mimetic. Paclitaxel was able to stimulate predominantly the translocation of the p50-p65 subunits in a manner similar to that of bacterial LPS. This translocation was found to be dose dependent and transient in nature. The nuclear translocation of NF-κB dimers in response to both paclitaxel and protein-free LPS was observed in macrophages derived from LPS-responsive C3H/OuJ but not those from LPS-hyporesponsive C3H/HeJ mice. Moreover, the LPS antagonist *Rs*DPLA was able to inhibit both the paclitaxel- and LPS-induced activation of NF-κB, suggesting that there is a shared LPS receptor-dependent pathway for this activation.

MATERIALS AND METHODS

Cell culture conditions. Five- to 6-week-old female C3H/OuJ and C3H/HeJ mice (Jackson Laboratories, Bar Harbor, Maine) were injected intraperitoneally with 3 ml of 3% thioglycolate fluid. (Research was conducted according to the principles set forth in reference 16a.) Four days later, peritoneal exudate cells were extracted, washed, and resuspended in RPMI 1640 medium supplemented with 2% heat-inactivated fetal bovine serum, 2 mM glutamine, 30 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 0.3% $NaHCO_3$, 100 IU of penicillin per ml, and 100 μg of streptomycin per ml. Macrophages (5×10^6 per well) were cultured on six-well tissue culture dishes and incubated overnight at 37°C with 6% CO_2 before treatment.

Reagents. Protein-free (≤0.008% protein) phenol-water-extracted *Escherichia coli* K235 LPS (PW-LPS) was prepared according to the method of McIntire et al. (31). Protein-rich (18% protein) butanol-LPS (But-LPS) was prepared from *E. coli* K235 according to the method of Morrison and Leive (32). Paclitaxel was made available by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. Paclitaxel was solubilized in dimethyl sulfoxide to a stock concentration of 20 mM. The endotoxin content in the highest concentration of paclitaxel used in this study was determined by a *Limulus* amoebocyte lysate assay and was found to be <0.01 ng of endotoxin per ml (below the lower limit of detection of the assay). *Rs*DPLA was prepared by acid hydrolysis of *R. sphaeroides* LPS and purified with a DEAE-cellulose acetate column as previously described (36). Rabbit antibodies specific for p52, c-rel, p50, and p65 subunits were generously provided by Nancy Rice (National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, Md.). Calpain inhibitor I (*N*-acetyl-Leu-Leu-norleucinal) was purchased from Boehringer Mannheim Corporation, Indianapolis, Ind. TPCK (*N*-tosyl-L-phenylalanine chloromethyl ketone) was purchased from Sigma Chemical Company, St. Louis, Mo.

Preparation of nuclear extracts. Nuclear extracts were prepared according to the methods published by Dignam et al. (7) and Schreiber et al. (38), with some modifications as published elsewhere (5). The protein content of the nuclear

extracts was determined by the Bio-Rad protein assay according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, Calif.).

DNA-protein interactions and EMSA. Two synthetic oligonucleotides (sequences in boldface) corresponding to the prototypic NF-κB binding site in the murine immunoglobulin κ light chain gene enhancer or the human IL-6 gene promoter (20), flanked by identical sequences as shown below, were annealed in a buffer containing 10 mM Tris HCl (pH 8), 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol. The immunoglobulin κ light chain κB site oligonucleotide sequences were 5′ AGCTCAGAG**GGGACTTTCC**GAGAG 3′ and 3′ GTCTC **CCCTGAAAGG**CTCTCTCGA 5′. The IL-6 κB site oligonucleotide sequences were 5′ AGCTCAGAG**TGGGATTTTCCCA**GAGAG 3′ and 3′ GTCTC**AC CCTAAAAGGGT**CTCTCTCGA 5′. Fifty nanograms of the annealed oligonucleotides was labeled with the Pharmacia oligolabeling kit according to the manufacturer's instructions (Pharmacia Biotech Inc., Piscataway, N.J.). Following the labeling, unincorporated nucleotides were removed with a Bio-spin column (Bio-Rad Laboratories). For each DNA-protein binding reaction, 5 μg of nuclear extract (quantified as described above) was used in the presence of 0.2 ng of labeled probe in a 25-μl reaction mixture. The DNA-protein binding buffer contained 1 μg of (poly dI-dC) · (poly dI-dC) per ml, 10% glycerol, 10 mM Tris HCl (pH 8), 1 mM EDTA, 40 mM KCl, and 1 mM dithiothreitol. All DNA-protein binding reactions were allowed to proceed at room temperature for 30 min. At the end of the incubation period, samples were loaded onto a nondenaturing 4% polyacrylamide gel (acrylamide:bisacrylamide, 30:1, wt/wt) and electrophoresed in 0.25× Tris-borate running buffer (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA [pH 8]). Following electrophoresis, the gel was transferred onto 3-mm-thick Whatman chromatography paper and dried at 80°C for 45 min. The dried gel was then exposed for signal development to Kodak XAR-5 film in the presence of an intensifying screen at −70°C. Competition reactions were performed in the presence of 25 or 50 molar excess of unlabeled probe. Unlabeled NF-κB oligonucleotides were used as the specific competitor, and the NF-βA binding sequence from the promoter region of the IL-1β gene was used as the nonspecific competitor (15). Supershift assays were performed in the presence of rabbit antibody specific for p50, p65, c-rel, and p52 subunits. For these assays, nuclear extracts were preincubated for 10 min at room temperature with 1 μl of the antibody before the NF-κB probe was added, and the reaction was then allowed to proceed for a further 30 min before electrophoretic mobility shift assays (EMSAs) were performed. Unless specified otherwise, all EMSAs were performed with the labeled immunoglobulin κ light chain κB site oligonucleotide probe.

RESULTS

Induction of nuclear translocation of NF-κB by paclitaxel and LPS in murine macrophages. Previous studies in this laboratory and others have shown that paclitaxel and bacterial LPS share the ability to induce a series of similar bioactivities in murine macrophages, including the ability to induce tyrosine kinases that are found to participate in early intracellular signaling events (9, 13, 24, 27). The activation of the pleiotropic nuclear transactivating factor, NF-κB, is another early signaling event that has been shown to regulate a number of genes inducible by LPS. Since LPS has been shown to activate the translocation of NF-κB in pre-B cells, T cells, and macrophages (1), we sought to determine if paclitaxel, like LPS, could induce NF-κB translocation in primary murine macrophages. Peritoneal exudate macrophages from C3H/OuJ mice were treated with various concentrations of paclitaxel or LPS for 1 h (the optimum time as determined by preliminary experiments). Nuclear extracts were collected, and EMSAs were performed with a ^{32}P-labeled double-stranded oligonucleotide probe containing the NF-κB binding element. As can be seen in Fig. 1A, both LPS and paclitaxel treatment resulted in the formation of three DNA-protein complexes, C1, C2, and C3, of which only complex C1 was inducible in a dose-dependent manner. Competition reactions were next performed to ensure that the inducible DNA-protein complexes detected following LPS or paclitaxel treatment were specifically NF-κB complexes. Nuclear extracts from untreated and LPS- or paclitaxel-treated macrophages were incubated in the presence of labeled NF-κB probe and 25 molar excess of unlabeled NF-κB probe as a specific competitor or 25 molar excess of unlabeled NF-βA probe as a nonspecific competitor in the binding reaction. In the presence of excess unlabeled NF-κB-specific probe, both LPS- (Fig. 1B) and paclitaxel-induced (Fig. 1C) DNA-protein complex formation was inhibited. In contrast, no inhibition of DNA-protein complex formation was seen in the presence of excess nonspecific competitor, indicating that the complexes induced by paclitaxel and LPS were indeed NF-κB complexes.

Paclitaxel and LPS induce the translocation of p50-p65 heterodimeric subunits of NF-κB predominantly. Since the *rel* family of proteins consists of five members that are able to form homodimers (with the exception of RelB) or heterodimers with each other, supershift experiments were carried out to determine the subunit composition of the NF-κB complexes induced by LPS and paclitaxel (41). Nuclear extracts from untreated and LPS- or paclitaxel-treated macrophages were preincubated with a panel of rabbit antibodies specific for the p50, p65 (RelA), p52, and c-rel members of the *rel* family of proteins (RelB was not included in the panel because it has been shown not to be expressed significantly in macrophages [45]). NF-κB probe was then added, and EMSAs were performed. A supershift was seen in LPS- (Fig. 2A) and paclitaxel-treated (Fig. 2B) nuclear extracts, primarily in the presence of antibodies specific for p50 and p65 subunits, suggesting that the NF-κB complexes translocated in response to both activators are predominantly composed of p50 and p65 heterodimers. The low levels of supershifted species detected in the presence of antibodies specific for p52 and c-rel subunits, coupled with incomplete supershifts with anti-p50 and anti-p65 antibodies, suggest the possibility that various combinations of dimers composed of p50, p65, p52, and c-rel subunits are also induced by both activators. Because the degeneracy of the κB site sequences has provided a way by which different combinations of NF-κB dimers can be identified on the basis of the varied affinities of particular dimers for these sites, we next determined whether the subunits induced by LPS and paclitaxel in macrophages were of the same subunit composition by examining yet another κB site sequence used as a probe in the EMSA. For this determination, the κB binding site sequence of the human IL-6 promoter, which has been shown to preferentially bind c-rel homodimers, was selected, oligonucleotides flanked by sequences identical to those for the immunoglobulin κB binding site were synthesized and labeled as probe, and supershifts were performed as described above (20, 33). The pattern of the supershifted subunits seen with the immunoglobulin κ light chain κB site oligonucleotide probe (high levels of supershifted p50 and p65 and low levels of p52 and c-rel subunits) was similar to that seen with the IL-6 κB site oligonucleotide probe (data not shown). These data suggest that the stimulation of macrophages with LPS and paclitaxel indeed results in the activation of predominantly p50-p65 dimers and possibly low levels of dimers composed of various combinations of p50, p65, p52, and c-rel subunits.

The kinetics of paclitaxel- and LPS-stimulated translocation of NF-κB in macrophages. We next determined the time course of nuclear localization of NF-κB in response to both stimulants. Macrophages were treated with either 100 ng of LPS per ml or 35 μM paclitaxel for over a period of 2 h, nuclear extracts were prepared, and EMSAs were performed. While both LPS- and paclitaxel-induced nuclear translocation was detected maximally at 1 h, the initial rate of induction differed for the two activators (Fig. 3). There was no detectable translocation of NF-κB heterodimers 5 min after stimulation with either compound. By 15 min poststimulation, LPS-activated nuclear localization of NF-κB was well induced, whereas the paclitaxel-induced nuclear translocation was minimally detectable but reached detectable levels at 30 min poststimulation. The response to both activators showed a decline by 2 h posttreatment.

Macrophages from LPS-hyporesponsive C3H/HeJ mice do

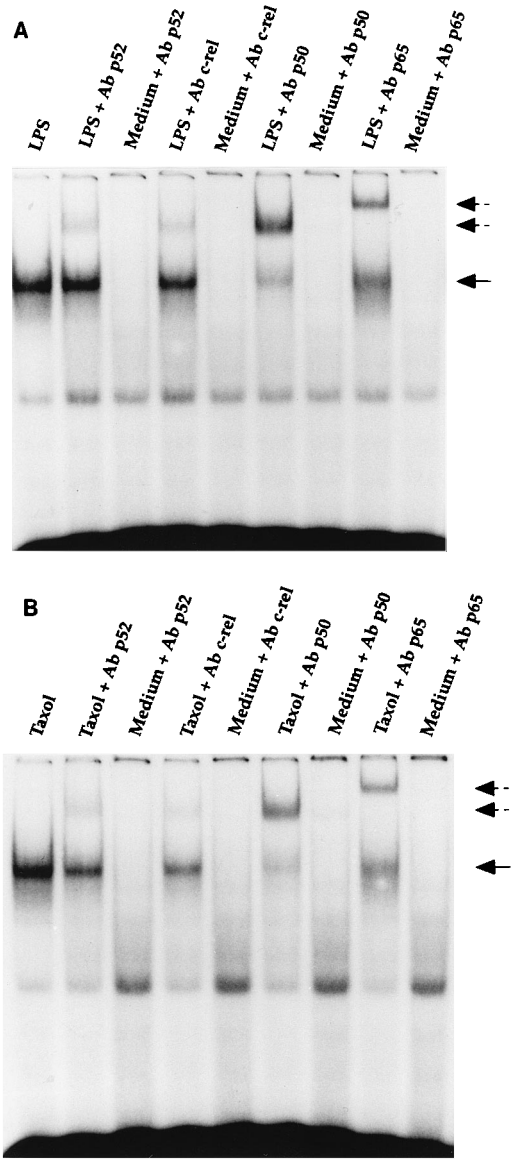

FIG. 2. (A) Analysis of the subunit composition of the NF-κB complexes formed in response to LPS. Nuclear extracts of C3H/OuJ macrophages treated with medium alone or 10 ng of LPS per ml for 1 h were analyzed in the presence of antibodies (Ab) specific for p52, c-rel, p50, and p65 (RelA) members of the *rel* family of proteins in the DNA binding reaction. The single arrow indicates the normal (unshifted) position of the NF-κB complex formed in response to LPS alone, and the supershifted complexes are indicated by double arrows. The figure represents one of three separate experiments performed. (B) Analysis of the subunit composition of the NF-κB complexes formed in response to paclitaxel (Taxol). Nuclear extracts of C3H/OuJ macrophages treated with medium alone or 35 μM paclitaxel for 1 h were analyzed in the presence of rabbit antibodies (Ab) specific for p52, c-rel, p50, and p65 (RelA) members of the *rel* family of proteins in the DNA binding reaction. The single arrow indicates the normal (unshifted) position of the NF-κB complex formed in response to paclitaxel alone, and the positions of the supershifted complexes are indicated by double arrows. The figure represents one of three separate experiments performed.

not respond to paclitaxel or PW-LPS. The response of murine macrophages to paclitaxel has been linked to the *Lps* gene (9, 29). In keeping with this finding, previous studies from this laboratory have shown that macrophages derived from LPS-hyporesponsive C3H/HeJ mice failed to respond to either paclitaxel or protein-free PW-LPS, to induce a panel of early genes, to activate gamma interferon-primed macrophages to a tumoricidal state, to induce MAP kinase isoforms, or to induce the secretion of TNF. In contrast, Ding et al. (8) recently reported that macrophages from C3H/HeJ mice responded to a commercial preparation of PW-LPS by activating the translocation of NF-κB complexes, implying that the hyporesponsiveness of the C3H/HeJ macrophages to LPS was due to a defect in signaling that occurs downstream of NF-κB activation. To reexamine this issue, we compared the capacities of paclitaxel and our highly purified PW-LPS to induce NF-κB translocation in C3H/HeJ macrophages and compared this effect to that induced by But-LPS, a known activator of C3H/HeJ macrophages. The ability of But-LPS to stimulate C3H/HeJ macrophages has been shown to be due to the presence of contaminating bacterial proteins (14, 25). As can be seen in Fig. 4, neither paclitaxel nor protein-free PW-LPS induced the nuclear translocation of NF-κB in the LPS-hyporesponsive C3H/HeJ macrophages, even at the highest concentrations tested. In contrast, protein-rich But-LPS readily activated the nuclear translocation of NF-κB in C3H/HeJ macrophages.

A peptide aldehyde blocks the activation of NF-κB by LPS and paclitaxel in murine macrophages. Recent reports implicate the multicatalytic protease complex (proteasome) in the proteolytic degradation of IκB-α following the stimulation of cells with a variety of stimulants, leading to the release and nuclear translocation of active NF-κB dimers (6, 21, 34). Several peptide aldehydes, including calpain inhibitor I (*N*-acetyl-Leu-Leu-norleucinal), that block the proteolytic activity of the proteasome have been used to demonstrate the involvement of the proteasome pathway in the degradation of IκB-α prior to the nuclear translocation of NF-κB dimers (6, 21, 34, 43). To examine whether the proteolytic activity of the proteasome was involved in the activation of NF-κB dimers in response to both LPS and paclitaxel in macrophages, cells were pretreated for 1 h with 50 μM calpain inhibitor I and then stimulated with either 1 ng of LPS or 5 μM paclitaxel for another hour before nuclear extracts were collected and EMSAs were performed. As can be seen in Fig. 5, calpain inhibitor I was able to block both LPS- and paclitaxel-induced nuclear translocation of

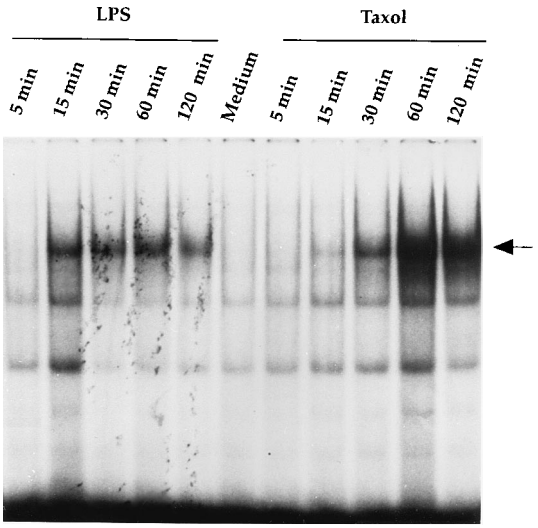

FIG. 3. The kinetics of NF-κB translocation in response to paclitaxel (Taxol) and LPS in murine macrophages. Overnight cultures of C3H/OuJ macrophages were treated with medium, 100 ng of LPS per ml, or 35 μM paclitaxel for the indicated times. Nuclear extracts were then prepared, and EMSAs were performed as described in Materials and Methods. The arrow indicates the inducible NF-κB complex. The figure represents one of five separate experiments performed.

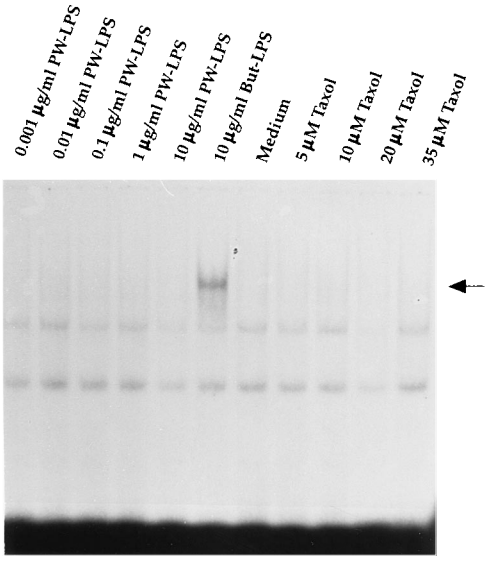

FIG. 4. Induction of NF-κB complexes in LPS-hyporesponsive C3H/HeJ macrophages. Macrophages from C3H/HeJ mice were treated with medium or the indicated concentrations of protein-free PW-LPS, protein-rich But-LPS, or paclitaxel (Taxol) for 1 h. Nuclear extracts were then prepared, and EMSAs were performed. The arrow indicates the inducible NF-κB complex. The figure represents one of two separate experiments performed.

NF-κB dimers, suggesting that the proteolytic activity of the proteasome is involved in the activation of NF-κB in murine macrophages in response to both LPS and paclitaxel.

The LPS antagonist, *Rs*DPLA, inhibits paclitaxel- and LPS-induced nuclear localization of NF-κB. *Rs*DPLA is a nontoxic disaccharide structural analog of LPS that exhibits very little agonist activity. Its ability to block LPS effects has established it as an important LPS structural antagonist (23). Because previous studies in this laboratory have shown that *Rs*DPLA is able to inhibit the induction of a panel of early genes, TNF secretion, and the activation of MAP kinase isoforms by both paclitaxel and LPS in macrophages (26, 28), we examined the ability of *Rs*DPLA to inhibit the nuclear localization of NF-κB in response to both activators. As can be seen in Fig. 6, at the highest concentration used in the study, *Rs*DPLA alone induced low levels of nuclear localization of NF-κB. Nevertheless, when *Rs*DPLA was added simultaneously with paclitaxel or LPS, this antagonist was able to inhibit markedly the paclitaxel- and LPS-induced NF-κB localization in macrophages in a dose-dependent manner.

DISCUSSION

Members of the *rel* family of transcription factors have been demonstrated to transduce extracellular signals received at the surface of cells to the nucleus, culminating in the expression of specific genes that are under their regulatory control. To date, five members have been identified and characterized in vertebrates: p50 (NF-κB1), p52 (NF-κB2), p65 (RelA), Rel (c-rel), and RelB. Recent studies employing the targeted disruption of p50 and RelB genes in mice have demonstrated distinct functions for the different members that cannot be fully compensated for by the other members of this closely related family (39, 45). The targeted disruption of the RelB gene indicated a role for this transcription factor in maintaining the immune system of adult mice, whereas the targeted disruption of the p50 gene indicated its importance as a transcription factor responsible for both innate and adaptive immune responses. In contrast to that of RelB and p50 genes, the disruption of the p65 gene has been shown to be lethal in mice (3).

Given the importance of the role of *rel* family members and macrophages to a fully functional immune system, we studied the ability of the antitumor agent paclitaxel to activate members of this family in murine macrophages. Our data clearly demonstrate that macrophages treated with paclitaxel induced the translocation of NF-κB complexes from their cytoplasmic location to the nucleus. The NF-κB subunits induced by paclitaxel were indistinguishable from those induced by LPS and were primarily p50 and p65 members of the *rel* family, with very low levels of p52 and c-rel subunits being induced in response to both activators. Since all four members of the *rel* family that were induced can form homo- or heterodimers with each other, it is possible that multiple combinations of dimers are induced, with the p50-p65 heterodimers being the predominant complex. Functionally, because of the presence of strong *trans*-activating domains in the p65 and c-rel subunits, only the dimers containing either p65 or c-rel as one of the subunits are likely to be of relevance as potent activators of gene expression (40). In addition, the times taken to induce the translocation of the NF-κB complexes maximally by both activators were the same, although the onset of induction was slightly slower for paclitaxel than for LPS (Fig. 3). Several studies have demonstrated a requirement for the degradation of IκB-α for the activation of NF-κB dimers in response to a number of stimuli in a variety of cells (4, 6, 21). These authors determined that IκB-α phosphorylation preceded but was not sufficient to cause the dissociation of the inhibitor subunit from NF-κB dimers. In addition, it was reported that the proteolytic activity of the proteasome was involved in the proteolytic degradation of IκB-α following phosphorylation, leading to the release and activation of p50-p65 NF-κB dimers (4, 6, 21). The peptide aldehyde calpain inhibitor I has been shown previously to inhibit the proteolytic activity of the proteasome on IκB-α deg-

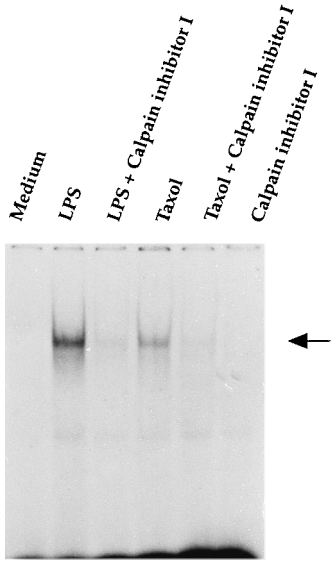

FIG. 5. Effect of the peptide aldehyde calpain inhibitor I on LPS- and paclitaxel (Taxol)-stimulated NF-κB translocation. Overnight cultures of C3H/OuJ macrophages were pretreated with 50 μM calpain inhibitor I for 1 h before 5 μM paclitaxel or 1 ng of LPS per ml was added for an additional hour. Nuclear extracts were prepared, and EMSAs were performed. The arrow indicates the inducible NF-κB complex. The figure represents one of two separate experiments performed.

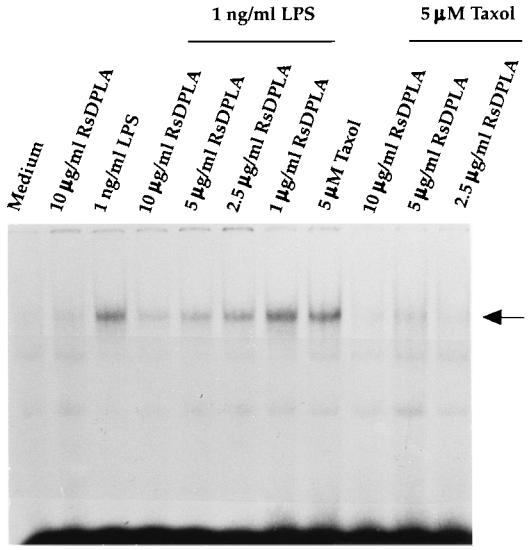

FIG. 6. The LPS structural antagonist *Rs*DPLA inhibits paclitaxel (Taxol)- and LPS-induced NF-κB translocation. Overnight cultures of macrophages were treated with medium alone, 10 μg of *Rs*DPLA per ml alone, or the indicated concentrations of *Rs*DPLA simultaneously with 5 μM paclitaxel or 1 ng of LPS per ml for 1 h. Nuclear extracts were then prepared, and EMSAs were performed. The arrow indicates the inducible NF-κB complex. The figure represents one of three separate experiments performed.

radation (6, 21, 34, 43). Our data with calpain inhibitor I support the hypothesis that the proteasome is likely to be involved in the activation of NF-κB dimers in response to both LPS and paclitaxel in murine macrophages. Moreover, the alkylating agent, TPCK, which is now believed to inhibit the phosphorylation of IκB-α (6), inhibited both LPS- and paclitaxel-induced NF-κB activation (data not shown). Similarly, an antioxidant, PDTC (pyrrolidine dithiocarbamate), which has been demonstrated to inhibit the activation of NF-κB by a number of stimulants, including paclitaxel, in 70Z/3 pre-B cells and macrophages, is now thought to inhibit the phosphorylation of IκB-α (6, 16, 37). Collectively, our data suggest that the mechanisms of activation of NF-κB by paclitaxel are similar to those of LPS in murine macrophages.

Both paclitaxel and protein-free PW-LPS failed to induce the nuclear translocation of NF-κB in macrophages derived from LPS-hyporesponsive C3H/HeJ mice, although the macrophages from these mice readily responded to the protein-rich But-LPS preparation. This finding is consistent with the observation that the response to paclitaxel is genetically linked to the *Lps* gene (9). The discrepancy between our findings and those of Ding et al. (8) is likely due to contaminants present in the commercial LPS preparation used in their study. However, we cannot account for the slight inducibility of NF-κB translocation observed by Hwang and Ding (16) in the C3H/HeJ macrophages in response to paclitaxel, although this response was minimal compared with that of the fully LPS-responsive C3H/HeN macrophages. Our results, together with the earlier findings that paclitaxel was able to mimic LPS in its capacity to activate macrophages to a tumoricidal state, to induce a set of early genes, and to induce isoforms of MAP kinases, strengthen the hypothesis of a shared pathway of activation for the two compounds.

*Rs*DPLA is a well-characterized disaccharide structural antagonist of bacterial LPS (23). Previous studies from this laboratory have established the capacity of this LPS antagonist to inhibit paclitaxel-induced early gene induction, TNF-α secretion, and the activation of MAP kinase isoforms (28). Consistent with these earlier findings, it was found that this LPS antagonist inhibits both paclitaxel- and LPS-induced NF-κB activation. Interestingly, the nuclear translocation of NF-κB by paclitaxel was more readily inhibited by *Rs*DPLA than that induced by LPS (Fig. 6). These results imply a shared mechanism of activation at the surface of macrophages, rather than shared signaling pathways following the convergence of post-receptor mechanisms leading to the common effects of paclitaxel and LPS. The CD14 glycoprotein is the best characterized of all the LPS-binding proteins that have been identified to date and is now regarded as a receptor for low concentrations of LPS (12, 17, 30, 42, 47). Since CD14-independent pathways are engaged at high concentrations of LPS (35, 47), it is very likely that other unidentified receptors or receptor complexes for LPS exist on cells. Proof that such receptors do exist have been provided by several authors (10, 11). Recently, using a CD14-deficient cell line, Kirikae et al. (18) reported that paclitaxel-induced TNF-α secretion and nitric oxide induction were CD14 independent. The simplest model to explain the LPS-mimetic action of paclitaxel would be to hypothesize that they bind to a common signaling receptor. The finding that *Rs*DPLA more readily inhibits paclitaxel-induced NF-κB translocation (Fig. 6) may suggest that the affinity of binding by paclitaxel for this putative receptor is low compared with that by LPS. Alternatively, paclitaxel and LPS may bind to distinct receptors that are in close proximity but share downstream signaling components. In this model, *Rs*DPLA binding of the LPS receptor might sterically hinder the binding of paclitaxel and thus preclude signaling. Another possibility is that both LPS- and paclitaxel-induced signaling requires the association of a common signaling molecule following receptor-ligand interaction that is inhibitable by *Rs*DPLA.

The data from this study strengthen the hypothesis that paclitaxel is able to bind to and/or share receptor components on macrophages that have evolved to recognize integral bacterial cell wall products and to induce the activation of transcription factor NF-κB that is important for the maintenance of innate and adaptive immunity in an LPS-like manner. Precedent for such a receptor is set by the CD14 molecule, which has been reported to bind such disparate structures as cell wall products from multiple bacteria and chitosans from arthropods (35, 46). The structurally diverse natures of the two compounds, their common and distinct effects on murine macrophages, and their abilities to bind to and/or share the same receptor complex should aid the future understanding of the complex mechanisms involved in LPS recognition and signaling.

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