Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 4885–4890, May 1996
Immunology

Elevated interleukin 6 is induced by prostaglandin E_2 in a murine model of inflammation: Possible role of cyclooxygenase-2

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Communicated by Michael Potter, National Institutes of Health, Bethesda, MD, January 2, 1996 (received for review July 25, 1995)

ABSTRACT **Injection of mineral oils such as pristane into the peritoneal cavities of BALB/c mice results in a chronic peritonitis associated with high tissue levels of interleukin 6 (IL-6). Here we show that increased prostaglandin E_2 (PGE_2) synthesis causes induction of IL-6 and that expression of an inducible cyclooxygenase, Cox-2, may mediate this process. Levels of both PGE_2 and IL-6 are elevated in inflammatory exudates from pristane-treated mice compared with lavage samples from untreated mice. The *Cox-2* gene is induced in the peritoneal macrophage fraction isolated from the mice. A cause and effect relationship between increased macrophage PGE_2 and IL-6 production is shown *in vitro*. When peritoneal macrophages are activated with an inflammatory stimulus (polymerized albumin), the *Cox-2* gene is induced and secretion of PGE_2 and IL-6 increases, with elevated PGE_2 appearing before IL-6. Cotreatment with 1 μM indomethacin inhibits PGE_2 production by the cells and reduces the induction of IL-6 mRNA but has no effect on Cox-2 mRNA, consistent with the fact that the drug inhibits catalytic activity of the cyclooxygenase but does not affect expression of the gene. Addition of exogenous PGE_2 to macrophages induces IL-6 protein and mRNA synthesis, indicating that the eicosanoid stimulates IL-6 production at the level of gene expression. PGE_2-stimulated IL-6 production is unaffected by addition of indomethacin. Taken together with the earlier finding that indomethacin diminishes the elevation of IL-6 in pristane-treated mice, the results show that PGE_2 can induce IL-6 production *in vivo* and implicate expression of the *Cox-2* gene in the regulation of this cytokine.**

Interleukin 6 (IL-6) is a multifunctional cytokine that is produced in a variety of inflammatory conditions *in vivo* (1, 2). It stimulates activation and differentiation of B and T lymphocytes, induces fever, and regulates acute phase protein synthesis (3–6). Because of its central role in modulating immunity, IL-6 production is normally tightly regulated. On exposure to an inflammatory stimulus (e.g., infectious agent, endotoxin, wounding), IL-6 levels *in vivo* rise transiently and then return to background levels on resolution of the insult (7–10). Dysregulation of IL-6 production or turnover can lead to chronic elevations of the cytokine level and may have pathologic consequences (5). For example, IL-6 is an important growth factor for multiple myeloma (11) and levels are correlated with severity of disease (12). Similarly, synovial fluids from patients with rheumatoid arthritis, a chronic inflammatory disease, contain high levels of IL-6 (13) that may be responsible for some of the systemic abnormalities common in these patients. In most conditions involving dysregulated IL-6, the mechanisms responsible for maintaining the high cytokine levels are not known.

The goal of our work is to identify the pathways responsible for inducing IL-6 production during chronic inflammation. We have used a murine model that involves injection of mineral oils such as pristane (2,6,10,14-tetramethylpentadecane) into the peritoneal cavities of BALB/c mice (14). This treatment induces a chronic peritonitis that is associated with prolonged high levels of IL-6 (15). A high percentage of the mice go on to develop IL-6-dependent (16) plasmacytomas (17, 18). The prolonged abundance of macrophages in the peritoneal cavity and the constitutive secretion of IL-6 by these cells (15) suggest that macrophages are the primary source of IL-6 in the pristane model. The mechanism by which IL-6 production is stimulated *in vivo* has not been determined.

A clue to identifying the pathway that controls IL-6 synthesis *in vivo* derives from the observation that chronic administration of the nonsteroidal anti-inflammatory drug indomethacin markedly reduces the levels of IL-6 induced by pristane (15). Indomethacin also inhibits pristane-induced plasmacytomagenesis (19). Because the main bioactivity of indomethacin is the inhibition of prostaglandin synthesis (20), this finding raises the possibility that prostaglandin E_2 (PGE_2) and/or other cyclooxygenase products may be involved in stimulating IL-6 synthesis *in vivo*. However, because indomethacin can have biological activities separate from its effects on prostaglandin metabolism (21–23), the role of prostaglandins needs to be established directly.

Two gene products, Cox-1 and Cox-2, have been identified as having prostaglandin synthase (EC 1.14.99.1) activity (24, 25). Cox-1 is expressed constitutively in most mammalian tissues (26) and is thought to be responsible for housekeeping functions of prostaglandins such as regulation of gastric acid secretion (27). Cox-2 is an inducible enzyme that is thought to give rise to the increased prostaglandin levels produced during inflammation (28). Cox-2 expression is known to be induced in macrophages exposed to inflammatory stimuli such as lipopolysaccharide (LPS) (29–31). This report provides evidence that prostaglandins serve as the predominant stimulus for IL-6 production in the pristane model. Elevated PGE_2 probably derives from induction of Cox-2 expression.

EXPERIMENTAL PROCEDURES

Mice. BALB/c mice were from a conventional mouse colony at Organon Teknika–BRL (National Cancer Institute contract N01CB21075) or were purchased from Charles River Breeding Laboratories and fed Purina laboratory chow and water ad lib. Animals were cared for in accordance with the National Institutes of Health Animal Care Guidelines. Female mice (8 to 16 week old) received a single 0.5-ml i.p. injection of pristane.

Preparation of Peritoneal Macrophages. Peritoneal lavage samples were collected 2–4 months after pristane treatment (15). The peritoneal macrophage population was isolated as described (15). Briefly, neutrophils and macrophages were

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Abbreviations: IL-6, interleukin 6; LPS, lipopolysaccharide; pAlb, polymerized albumin; PGE_2, prostaglandin E_2; bicyclo-PGE_2, 11-deoxy-13,14-dihydro-15-keto-11β,16ξ-cyclo-PGE_2; IBMX, 3-isobutyl-1-methylxanthine.

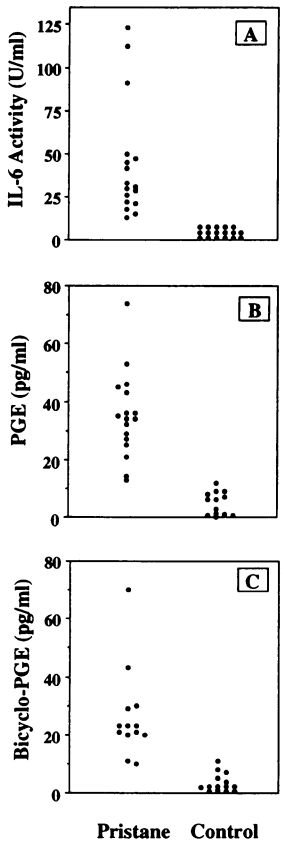

FIG. 1. Measurement of IL-6 and prostaglandins in inflammatory exudates from pristane-treated mice. Pristane-primed BALB/c mice were lavaged with PBS containing 1 μM indomethacin (to inhibit induction of prostaglandin production during the lavage procedure). (*A*) IL-6 in each lavage sample was measured by the B9 bioassay, which was validated by ELISA. (*B* and *C*) PGE_2 and bicyclo-PGE_2 were measured in freshly collected and extracted lavage samples. Values represent the concentrations present in each lavage sample and are not corrected for dilution by the lavage process. The levels of PGE_2 and bicyclo-PGE_2 found in control mice are below or near the limits of detection of the assays. IL-6 was undetectable in all of the peritoneal fluids collected from control mice.

separated by density gradient centrifugation (32). Macrophages were purified further by plating at a concentration of 2.5×10^6 cells per ml (0.5 ml per well) in 24-well plastic tissue culture plates in DMEM containing antibiotics and allowing cells to adhere at 37°C for 1–4 hr. Nonadherent cells were removed by washing with DMEM. Resident macrophages from control (untreated) mice were collected by peritoneal lavage as described previously (15). Cell numbers and viability were quantified by hemocytometry with trypan blue. Cell differentials were analyzed from Diff-Quick stained cytocentrifuge slides.

Macrophage Treatments *in Vitro*. BSA preparations that contain polymerized albumin (pAlb) stimulate IL-6 and PGE_2 production when added to macrophages *in vitro* and also act as acute inflammatory stimuli *in vivo*, similar to LPS (33). pAlb is somewhat more potent and less toxic to the cells than LPS and thus was used in the studies described in this report. However, most of these experiments have been confirmed using LPS as stimulant, indicating that the results are not unique to treatment with pAlb.

Macrophages were cultured in DMEM at 37°C in a humidified atmosphere containing 5% CO_2. pAlb (10–200 μg/ml; ref. 33) or PGE_2 (0.001–1 μM) was added to washed cells in fresh DMEM. Where indicated, indomethacin was added to the cell cultures 30 min before adding the stimulus. 3-Isobutyl-1-methylxanthine (IBMX; 0.5 mM), a phosphodiesterase inhibitor, was added to inhibit breakdown of cAMP in some experiments with exogenously added PGE_2. In experiments that contained no added protein, ultrapure BSA (Boehringer Mannheim; ref. 33) was added to each macrophage supernatant at the time of collection to decrease the adsorption of IL-6 to the walls of the collection tube. After various times of incubation, the culture medium was removed from each well and centrifuged. Supernatants were stored frozen until assayed for the presence IL-6 or PGE_2.

Purification of RNA and Northern Blot Analysis. Total RNA was purified from *in vitro*-plated macrophages after various times of incubation using TRIzol (GIBCO/BRL) following the protocol recommended by the manufacturer. For examination of *in vivo* expression of Cox-2, RNA was purified from freshly isolated peritoneal cells (the macrophage fraction of pristane-elicited cells and the total resident cell population from control mice) without plating. RNA samples (4 or 15 μg each) were run on 1% agarose/0.7% formaldehyde gels containing ethidium bromide and transferred to nitrocellulose. ^{32}P-labeled IL-6 and actin cDNA probes were prepared using a random priming system (GIBCO/BRL) and [α-^{32}P]dCTP (Amersham). Blots were hybridized overnight with probe (1.2×10^6 cpm/ml, 5×10^8 cpm/μg) at 65°C in Hybrisol II solution (Oncor) and then washed by standard procedures. Autoradiography was performed using Kodak XAR film. Films were scanned (Microtek Scanmaker) and analyzed using the Macintosh densitometry program NIH IMAGE (National Institutes of Health, Bethesda).

Bioassay for IL-6. IL-6-dependent B9 hybridoma cells (34) were cultured in a serum-free medium (35) supplemented with 5% heat-inactivated fetal calf serum and IL-6. Before the assay, the B9 cells were washed to remove IL-6 and were then cultured in flat-bottom 96-well plates at 3000 cells per well. Samples to be assayed for IL-6 activity were added at serial 2-fold dilutions. After 3 days of culture at 37°C, the number of viable cells was assayed using the colorimetric reagent MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 0.55 mg/ml] (15, 36). Standard curves with recombinant murine IL-6 were run to control for interassay variation. The data were calculated using a four-parameter fit analysis. A unit of activity is defined as the dilution that gives half-maximal B9 cell growth in 3 days and corresponds to $\approx$2 pg/ml of homogeneous murine recombinant IL-6. Specificity of the assay was confirmed by blocking all activity with a polyclonal anti-IL-6 antibody. Results of the B9 bioassay were verified using an

Table 1. Levels of PGE_2 and bicyclo-PGE_2 in peritoneal fluids isolated from pristane-treated and control (untreated) mice

Mice	Level in lavage fluid		
	PGE_2, pg/ml	Bicyclo-PGE_2, pg/ml	IL-6, units/ml
Pristane-treated	35.2 ± 3.5 (n = 17)	25.5 ± 4.1 (n = 14)	44.0 ± 7.4 (n = 19)
Control	2.7 ± 1.2 (n = 17)	3.3 ± 0.7 (n = 16)	0 ± 0 (n = 19)

Peritoneal fluids were collected by lavage and eicosanoids extracted as described in *Experimental Procedures*. The data reflect the mean ± sem for PGE_2, bicyclo-PGE_2, and IL-6 detected in "n" individual mice. The data are taken from Fig. 1.

ELISA that was specific for mouse IL-6 (Endogen, Cambridge, MA).

Extraction and Analysis of Prostaglandins. Prostaglandins were extracted from freshly isolated lavage fluids using Sep-Pak (Millipore–Waters) column chromatography according to procedures outlined by Powell (37). Recovery was determined using samples of reagent PGE_2 (Sigma). PGE_2 was assayed using a polyclonal antibody/enzyme immunoassay kit from PerSeptive Diagnostics (Cambridge, MA). The linear range of the assay was from 10 to 1000 pg/ml. Bicyclo-PGE_2 (11-deoxy-13,14-dihydro-15-keto-11β,16ξ-cyclo-PGE_2; ref. 38) was assayed by ELISA using a kit from Cayman Chemicals (Ann Arbor, MI). The linear range of this assay was from 1 to 25 pg/ml. Extracts were diluted or concentrated to achieve values that fall within the linear ranges of the assays.

Materials. pAlb is a component of fatty acid-free BSA (Boehringer Mannheim catalog no. 100 069). Indomethacin, MTT, IBMX, and PGE_2 were from Sigma. NS-398 was purchased from Biomol (Plymouth Meeting, PA). Pristane was from Aldrich. A cDNA probe for murine IL-6 (0.65 kb) was a gift from D. Pluznik (Center for Biologics Evaluation and Research, Food and Drug Administration). A cDNA probe for murine Cox-2 (1.7 kb; ref. 30) was the gift of J. Farber (National Institute of Allergy and Infectious Diseases, Bethesda). A cDNA probe for murine β-actin (2 kb) was provided by K. Huppi (National Cancer Institute). Probes were purified from plasmids according to standard procedures. Murine recombinant IL-6 purified to homogeneity was a gift of B. Brown (National Cancer Institute).

RESULTS AND DISCUSSION

Intraperitoneal PGE_2 and *Cox-2* Gene Expression Are Elevated in Pristane-Treated Mice. To determine whether prostaglandins could be responsible for elevating IL-6 levels *in vivo* in the murine pristane model, we measured PGE_2 levels in the peritoneal exudates induced in the mice. This has not previously been measured. Since prostaglandins are rapidly metabolized *in vivo* (38), assays were carried out for both PGE_2 and bicyclo-PGE_2, a stable breakdown product of PGE_2. Because of the spuriousness of the data obtained with unextracted or stored samples, only freshly collected and extracted lavage fluids were assayed. The results obtained are shown in Fig. 1. Statistical analysis of the data is presented in Table 1. Both PGE_2 and bicyclo-PGE_2 are elevated in pristane-treated mice over the levels observed in untreated (control) mice. The levels of IL-6 found in these same mice are also presented. As described (15), IL-6 is elevated in the inflammatory exudates induced in pristane-treated mice and is undetectable in the peritoneal fluids of control mice.

The main source of elevated prostaglandins in inflamed tissues is thought to derive from increased expression of Cox-2 (27, 28) such as is observed in synovial tissue from patients with rheumatoid arthritis (39, 40). To determine whether this enzyme might be responsible for the increased levels of PGE_2 observed in pristane-induced inflammation, total RNA was isolated from resident and inflammatory peritoneal macrophages and tested for the presence of Cox-2 transcripts. As shown in Fig. 2, *Cox-2* gene expression is elevated in the peritoneal cells elicited by pristane. Further experiments were performed *in vitro* to establish whether there is a correlation between elevated prostaglandin synthesis, Cox-2 expression, and secretion of IL-6 by the peritoneal macrophages elicited by pristane.

Kinetics of Induction of PGE_2 and IL-6 Production in Inflammatory Macrophages. Treatment of peritoneal macrophages with inflammatory stimuli such as pAlb or LPS leads to production of both PGE_2 and IL-6 by the cells (15, 33). The kinetics of the early stages of this response are shown in Fig. 3. Elevated PGE_2 can be seen as early as 40 min after addition

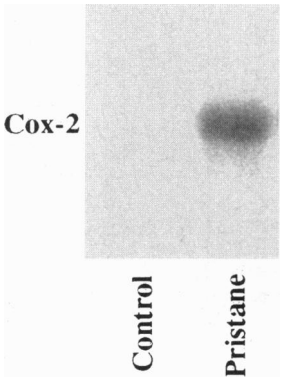

FIG. 2. Expression of Cox-2 mRNA in inflammatory peritoneal macrophages. Total RNA was purified from freshly-isolated pristane-elicited macrophages (2 months postpristane) and from resident peritoneal cells from control (untreated) mice as described in *Experimental Procedures*. Cox-2 mRNA was detected by Northern blot analysis (15 μg mRNA per lane) using a ^{32}P-labeled murine cDNA probe. The blot was analyzed on a PhosphorImager.

of pAlb to the cells and appears to precede IL-6 production, which is not detected until ≈100 min after addition of the stimulus. The result supports the hypothesis that PGE_2 synthesis leads to IL-6 production by the cells. The lag in IL-6 secretion is not due to inadequate sensitivity of the assay since as little as 4 units/ml IL-6 can be detected by the B9 assay (accounting for dilution of the sample in the assay), which is well below the 50 units/ml IL-6 found in the first time point at which elevated IL-6 is detected.

The dependence of IL-6 synthesis on PGE_2 production in these cells is shown by the effect of indomethacin on pAlb-stimulated IL-6 mRNA expression. IL-6 mRNA is elevated within 2 h after addition of pAlb to macrophages in culture and peaks at ≈4 h (Fig. 4). However, when the macrophages are stimulated with pAlb in the presence of 1 μM indomethacin, which can inhibit both Cox-1 and Cox-2 activity (41), induction of IL-6 mRNA expression is inhibited by ≈75% (Fig. 5). A similar level of inhibition is achieved using a Cox-2 specific inhibitor, NS-398 (27, 42) (Fig. 5), suggesting that PGE_2 derived from Cox-2 is responsible for the induction of IL-6. *Cox-2* gene expression is induced by treatment with pAlb and, in contrast to IL-6, mRNA levels are unaffected by addition of indomethacin (Fig. 6). Both indomethacin and NS-398 inhibit

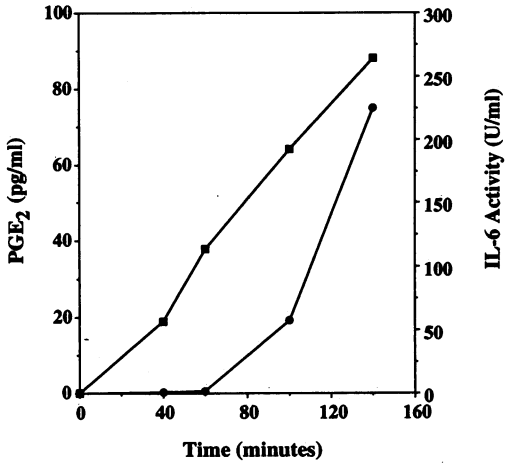

FIG. 3. Kinetics of induction of PGE_2 and IL-6 in inflammatory macrophages. Pristane-elicited macrophages were cultured for the indicated times in DMEM in the presence of pAlb (200 μg/ml). IL-6 (●) and PGE_2 (■) secreted into the culture medium were assayed. The data represent the mean of the results obtained with duplicate wells from a representative experiment.

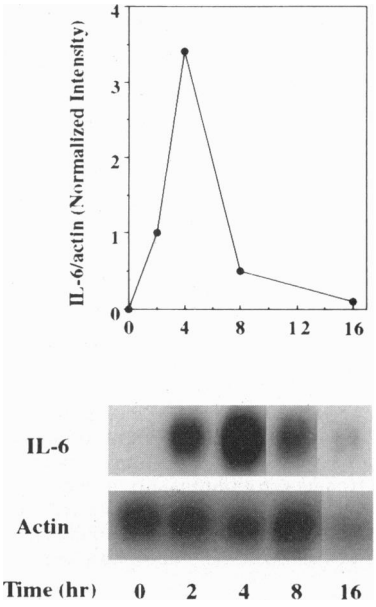

FIG. 4. Time course of induction of IL-6 mRNA in peritoneal macrophages stimulated with pAlb. Pristane-elicited macrophages were cultured in DMEM in the presence of 200 μg/ml pAlb. At the times indicated, total mRNA was isolated and analyzed by Northern blotting (*Lower*). β-actin mRNA was measured to control for differences in sample loading and membrane transfer. IL-6 band intensities were quantified using densitometric imaging software and normalized to the respective actin band intensities (*Upper*).

the secretion of PGE_2 by ≈95%, decreasing the cumulative overnight levels of production from 1.8 ng/ml to an average of 0.09 ng/ml in the presence of either agent.

PGE_2 Stimulates IL-6 Production at the Level of Gene Transcription. To establish whether PGE_2 can act directly as a stimulus for IL-6 production, the effect of adding exogenous

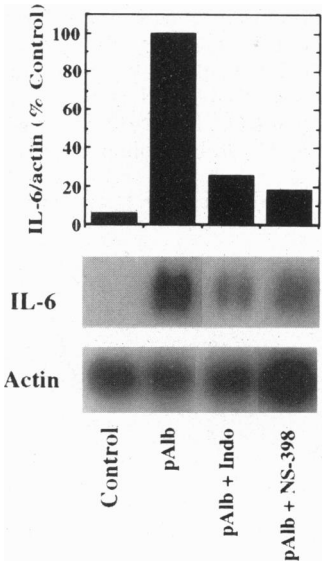

FIG. 5. Inhibition of IL-6 mRNA expression by nonsteroidal anti-inflammatory drugs. Pristane-elicited macrophages were stimulated by pAlb (200 μg/ml) in culture in the presence and absence of indomethacin (1 μM) or NS-398 (10 μM) as indicated. Total RNA was isolated after a 4-h incubation and probed for IL-6 and actin mRNAs. Band intensities were quantified using densitometric imaging software and normalized for load based on actin levels. The bar graph shows the normalized levels of steady-state IL-6 mRNA as a percent of the maximum level induced by pAlb (lane 2). The results were reproduced in three separate macrophage experiments.

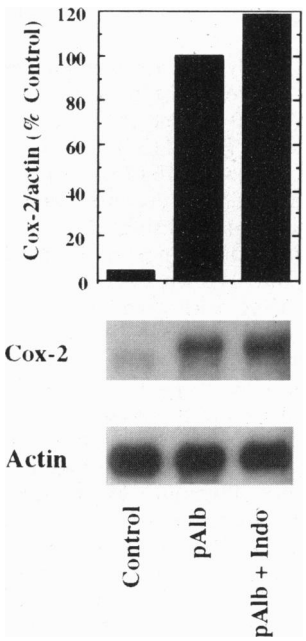

FIG. 6. Induction of Cox-2 mRNA by pAlb *in vitro*. Total RNA was purified from pristane-elicited macrophages 4 h after stimulation with pAlb (200 μg/ml) and probed for Cox-2 and actin mRNAs. The bar graph shows the normalized levels of steady-state Cox-2 mRNA as a percent of the maximum level induced by pAlb (average of two separate experiments).

PGE_2 to macrophage cultures was tested. As shown in Fig. 7, IL-6 production is stimulated in a concentration-dependent manner. A 1 μM dose induces a 4-fold increase in IL-6 secretion after an overnight incubation. Similar results are obtained using resident peritoneal macrophages from control (untreated) mice, indicating that this effect of PGE_2 is not restricted to pristane-elicited macrophages. When IBMX (0.5 mM) is added to inhibit breakdown of the cAMP induced by 1 μM PGE_2, the level of stimulation increases to 15-fold (data not shown), consistent with the view that the PGE_2 effect is mediated by cAMP. Ethanol at concentrations identical to

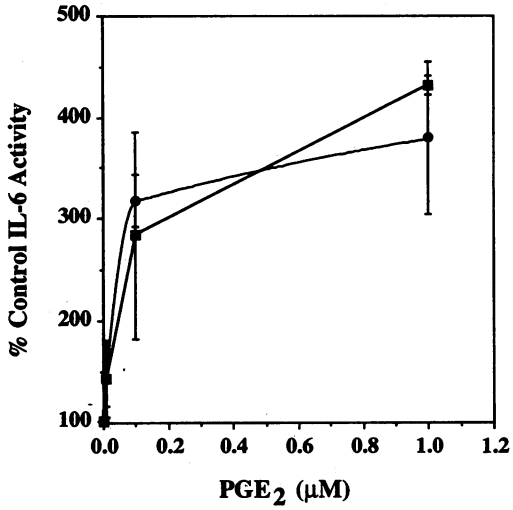

FIG. 7. Effect of exogenous PGE_2 on IL-6 protein secretion in peritoneal macrophages. Pristane-elicited (●) and resident (■) macrophages were cultured overnight in DMEM with the indicated concentrations of PGE_2. Culture supernatants were collected after an overnight incubation and assayed for the presence of IL-6 protein. The data represent the mean ± SD of two separate macrophage experiments with 2–4 wells per experiment.

those used as solvent for PGE_2 did not stimulate IL-6 production (data not shown).

The mechanism of PGE_2 action in peritoneal macrophages involves stimulation of transcription of the IL-6 gene. Addition of PGE_2 to macrophage cultures induces a 12-fold increase in IL-6 mRNA levels in the presence of IBMX (Fig. 8), comparable with the level of IL-6 protein induced. Indomethacin did not affect PGE_2-stimulated IL-6 mRNA expression; i.e., it acts by inhibiting cyclooxygenase-catalyzed PGE_2 synthesis but has no direct effect on the activity of the prostaglandin. Thus, indomethacin reduces macrophage IL-6 production through its effect on cellular prostaglandin metabolism and not through a secondary effect on IL-6 gene expression. This is in contrast to the actions of steroidal anti-inflammatory drugs such as dexamethasone that can directly inhibit IL-6 gene expression (43) in addition to inhibiting prostaglandin production (44).

There is controversy in the literature as to whether PGE_2 is stimulatory or inhibitory to cellular IL-6 production. Most of the confusion comes from trying to compare results obtained with different cell types, from comparing cell lines to primary cells, or from using different agents and conditions to stimulate cytokine and eicosanoid production. Production of IL-6 in a fibroblast cell line (45) and in peritoneal mast cells (46) is stimulated by addition of exogenous prostaglandin, but PGE_2 inhibits IL-6 synthesis in resident liver macrophages (Kupfer cells) (47). Similarly, indomethacin inhibits IL-6 production by rat bone marrow macrophages (48) but not human articular chondrocytes (49), or bovine aortic endothelial cells (50). Discrepant results have been reported for human peripheral blood monocytes (51, 52). Finally, PGE_2 appears to inhibit LPS stimulation of IL-6 production by murine and human peritoneal macrophages (53, 54).

We have focused on investigating IL-6 production in a mouse model of inflammation and have used primary cultures of peritoneal macrophages so that the *in vivo* processes that control IL-6 may be elucidated by the *in vitro* studies. The experiments described in this report and by Shacter *et al.* (15) show that PGE_2 synthesis is likely responsible for most of the elevation of IL-6 found when mice are subjected to a chronic inflammatory stimulus such as pristane. PGE_2 is capable of stimulating IL-6 gene expression in resident and inflammatory macrophages *in vitro* and is present in the peritoneal fluid surrounding these cells *in vivo*. IL-6 levels are lowered by ≈75% when pristane-treated mice are cotreated with indomethacin (15). A similar level of inhibition by indomethacin is observed when cells are treated with an acute stimulus *in vitro* (e.g., pAlb, LPS). Unlike corticosteroids (55, 56), indomethacin does not prevent the inflammatory response to pristane (15) and has no direct effect on IL-6 gene expression. Rather, it acts by inhibiting a biochemical pathway(s) associated with the inflammation induced by pristane: prostaglandin synthesis. It is tempting to speculate that the inhibition of IL-6 production by indomethacin is responsible for its inhibitory effect on tumorigenesis in this system because IL-6 is necessary for plasmacytoma growth (16, 57).

The results also show that the *Cox-2* gene is expressed in pristane-elicited macrophages *in vivo* and is induced in macrophages *in vitro* by the same agents that stimulate IL-6 production. Thus, Cox-2 may modulate IL-6 levels. To our knowledge, this marks the first implication that the *Cox-2* gene regulates expression of an inflammatory cytokine. More definitive experiments *in vitro* will confirm this association (J.A.W., R.M.H., and E.S., manuscript in preparation). The role of prostaglandins in elevating IL-6 levels in pristane-treated BALB/c mice may be analagous to some human chronic inflammatory diseases in which both prostaglandins and IL-6 are chronically elevated, such as rheumatoid arthritis (58, 59) and Crohn disease (60, 61). A key to reducing IL-6 levels in these conditions may lie in control of the factors responsible for dysregulated prostaglandin metabolism.

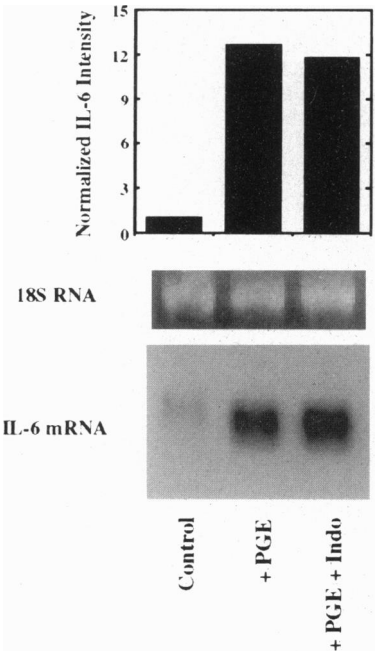

FIG. 8. PGE_2 directly stimulates IL-6 gene expression. Pristane-elicited macrophages were cultured for 4 h in DMEM in the presence or absence of 1 μM PGE_2 and 0.5 mM IBMX with or without indomethacin (1 μM). Total RNA was isolated and probed for IL-6 mRNA. Band intensities were normalized for load based on ethidium bromide staining of 18S RNA and then quantified using densitometric imaging software. The result was reproduced in two separate macrophage experiments.

We thank Giovanna Tosato for careful reading of the manuscript and many useful suggestions. We are grateful to Michael Potter for his thoughtful comments and for providing some of the mice used in these studies. We also thank Josh Farber, Konrad Huppi, and Dov Pluznik for supplying key probes. This work was supported by the Department of Clinical Investigation, Walter Reed Army Medical Center (Washington, DC) and Wyeth Pediatrics.

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