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In the present study we used IL-6 knockout mice (IL-6KO) to evaluate the role of IL-6 in the inflammatory response caused by injection of carrageenan into the pleural space. Compared with carrageenan-treated IL-6 wild-type (IL-6WT) mice, carrageenan-treated IL-6KO mice exhibited a reduced degree of pleural exudation and polymorphonuclear cell migration. Lung myeloperoxidase activity and lipid peroxidation were significantly reduced in IL-6KO mice compared with those in IL-6WT mice treated with carrageenan. Immunohistochemical analysis for nitrotyrosine and poly(A)DP-ribose polymerase revealed a positive staining in lungs from carrageenan-treated IL-6WT mice. No positive staining for nitrotyrosine or PARS was found in the lungs of the carrageenan-treated IL-6KO mice. Staining of lung tissue sections obtained from carrageenan-treated IL-6WT mice with an anti-cyclo-oxygenase-2 Ab showed a diffuse staining of the inflamed tissue. Furthermore, expression of inducible nitric oxide synthase was found mainly in the macrophages of the inflamed lungs from carrageenan-treated IL-6WT mice. The intensity and degree of the staining for cyclo-oxygenase-2 and inducible nitric oxide synthase were markedly reduced in tissue sections obtained from carrageenan-treated IL-6KO mice. Most notably, the degree of lung injury caused by carrageenan was also reduced in IL-6KO mice. Treatment of IL-6WT mice with anti-IL-6 (5 µg/day/mouse at 24 and 1 h before carrageenan treatment) also significantly attenuated all the above indicators of lung inflammation. Taken together, our results clearly demonstrate that IL-6KO mice are more resistant to the acute inflammation of the lung caused by carrageenan injection into the pleural space than the corresponding WT mice.


The inflammatory process is invariably characterized by the production of PGs, leukotrienes, histamine, bradykinin, platelet-activating factor, and IL-1 and by the release of chemicals from tissues and migrating cells (1). Furthermore, there is a large amount of evidence that reactive oxygen species (ROS) play an important role in the tissue destruction associated with the inflammatory process (see Discussion). Proinflammatory cytokines, including TNF-α and IL-1 and possibly IL-6, contribute to the extension of the inflammatory process. IL-6 is a multifunctional cytokine that is produced and acts on a wide range of cells (2–4). An enhanced formation of IL-6 has been reported in patients with severe burns (5), after surgical operations (6), during bacterial infections (7), in the synovial fluid of patients with rheumatoid arthritis (8), and in the cerebrospinal fluid of patients with bacterial meningitis (9, 10). The role of IL-6 in the pathophysiology of inflammation is still controversial. Recently, mice in which the gene for IL-6 has been deleted (IL-6 knockout mice (IL-6KO mice)) have been used to investigate the role of this cytokine in various models of inflammation. In IL-6KO mice, the induction of acute phase proteins, the weight loss, and the hypoglycemia caused by injection of turpentine were dramatically reduced (11). The recruitment of polymorphonuclear cells (PMNs) caused by injection of carrageenan in a s.c. airpouch was also substantially reduced in IL-6KO mice compared with that in wild-type (WT) mice (12). Most notably, the arthritis (accumulation of PMNs in the knee joint and related tissue damage) caused by collagen was abolished in IL-6KO mice (13). IL-6-deficient mice are also protected against the bone loss caused by estrogen depletion (14). All these studies support a proinflammatory role of IL-6. There are, however, other studies that do not document a proinflammatory role of IL-6 or even suggest that IL-6 may be an anti-inflammatory cytokine. For instance, IL-6KO mice treated with LPS produced 3-fold more TNF-α than their WT controls suggesting that endogenous IL-6 may be important in the control of LPS-induced TNF-α synthesis. Similar to WT controls, LPS-treated IL-6 deficient mice develop anorexia, weight loss, and hypoglycemia, suggesting that IL-6 is not required to generate and inflammatory response to LPS and may, in fact, exert a protective effect during acute inflammation (15). In addition, IL-6 is not necessary for the suppression of the synthesis of proteoglycan caused by IL-1 and leukemia inhibitory factor (16). Most notably, the cartilage destruction caused by injection of zymosan into the knee joint is significantly enhanced in IL-6KO mice, while injection of IL-6 (into the knee joint) protects WT mice against this zymosan-induced cartilage degradation.

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3 Abbreviations used in this paper: ROS, reactive oxygen species; IL-6KO, IL-6 knockout mice; PMN, polymorphonuclear cells; IL-6WT, IL-6 wild-type mice; LTβ, leukotriene B2; PARP, poly(A)DP-ribose polymerase; COX-2, cyclo-oxygenase protein-2; iNOS, inducible NO synthase; NOx, nitrate plus nitrite; MPO, myeloperoxidase; MDA, malondialdehyde.
Thus, it appears that the role of IL-6 in acute inflammation depends on the stimulus and/or the model of inflammation used.

Injection of carrageenan into the pleural space leads to pleurisy, PMN infiltration, and lung injury. Models of carrageenan-induced pleurisy have been widely employed to investigate the pathophysiology of acute inflammation and to evaluate the efficacy of drugs in inflammation. Interestingly, IL-6KO mice have not been used to elucidate whether IL-6 plays a pro- or an anti-inflammatory role in this model. This is surprising, as the injection of carrageenan leads to a rapid and substantial rise in IL-6, which is maximal after 4 h, but IL-6 remains elevated for up to 16 h after injection of carrageenan. In this study we have investigated the role of IL-6 in a model of carrageenan-induced pleurisy using IL-6KO mice and IL-6WT mice. To characterize the role of IL-6 in this model of acute inflammation, we have determined the following end points of the inflammatory response in IL-6KO mice and in the corresponding WT mice: 1) exudate formation, 2) PMN infiltration, 3) peroxynitrite formation (immunohistochemistry), 4) activation of the nuclear enzyme poly(ADP-ribose) polymerase (PARP), 5) expression of cyclooxygenase-2 (COX-2) protein (immunohistochemistry) and activity, 6) formation of leukotriene B4, 7) expression of the inducible NO synthase (iNOS) protein (immunohistochemistry) and activity, 8) lipid peroxidation, and 9) lung injury. In addition, we have investigated the effects of the systemic administration (pretreatment) of an mAb against IL-6 on the above parameters of inflammation in WT mice subjected to carrageenan-induced pleurisy.

Materials and Methods

Animals

Male IL-6KO and IL-6WT mice (20–22 g) were used to assess the role of IL-6 in the pathogenesis of carrageenan-induced pleurisy. All animals were allowed access to food and water ad libitum. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purposes (D.M. 116/1992) as well as with the European Economic Community regulations (O.J. of E.C. L 358/1 12/18/1986).

Carrageenan-induced pleurisy

Mice were anesthetized with isoflurane and submitted to a skin incision at the level of the left sixth intercostal space. The underlying muscle was dissected, and saline (0.2 ml) or saline containing 1% λ-carrageenan (0.2 ml) was injected into the pleural cavity. The skin incision was closed with a suture, and the animals were allowed to recover. At 4 h after the injection of carrageenan, the animals were euthanized by inhalation of CO2. The chest was carefully opened, and the pleural cavity was rinsed with 2 ml of saline solution containing heparin (5 U/ml) and indomethacin (10 µg/ml). The exudate and washing solution were removed by aspiration, and the total volume was measured. Any exudate that was contaminated with blood was discarded. The amount of exudate was calculated by subtracting the volume injected (2 ml) from the total volume recovered. The leukocytes in the exudate were suspended in PBS and counted with an optical microscope in a Burker’s chamber after vital trypan blue staining. In a second experiment, the exudate were suspended in PBS and counted with an optical microscope. The leukocytes in the exudate were harvested by aspiration, and the total volume was measured. Any exudate that was contaminated with blood was discarded. The amount of exudate was calculated by subtracting the volume injected (2 ml) from the total volume recovered. The leukocytes in the exudate were suspended in PBS and counted with an optical microscope. In a second experiment, the exudate were suspended in PBS and counted with an optical microscope. The leukocytes in the exudate were harvested by aspiration, and the total volume was measured. Any exudate that was contaminated with blood was discarded. The amount of exudate was calculated by subtracting the volume injected (2 ml) from the total volume recovered.

Cell culture

Resident pleural cells macrophages were collected from mice 4 h after the carrageenan injection as previously described (3). The cells (10^6/ml), which were mainly macrophages (~70%), were cultured in DMEM supplemented with l-glutamine (3.5 mM), penicillin (50 U/ml), streptomycin (50 µg/ml), and heparin sodium (10 U/ml) in 12-well plates for 2 h and were allowed cells to adhere at 37°C in a humidified 5% CO2 incubator. Nonadherent cells were removed by rinsing the plates three times with 5% dextrose water. After removing nonadherent cells (~10%), adherent macrophages were scraped for the measurement of DNA strand breaks and cellular NAD+. Mitochondrial respiration and peroxynitrite formation were measured in the adherent cells in the subsequent 1-h period.

Measurement of nitrite/nitrate

Nitrite plus nitrate (NOx) production, an indicator of NO synthesis, was measured in the supernatant samples as previously described (18). Briefly, the nitrate in the supernatant was first reduced to nitrite by incubation with nitrate reductase (670 µM/ml) and NADPH (160 µM) at room temperature for 3 h. The nitrite concentration in the samples was then measured by the Griess reaction, by adding 100 µl of Griess reagent (0.1% naphthylene diamine dihydrochloride in H2O and 1% sulfanilamide in 5% concentrated H3PO4; 1/1, v/v) to 100-µM samples. The OD at 550 nm (OD550) was measured using an ELISA microplate reader (SLT-LabInstruments, Salzburg, Austria). Nitrate concentrations were calculated by comparison with OD530 of standard solutions of DMEM.

Determination of NOS activity

The calcium-independent conversion of L-arginine to L-citrulline in the homogenates of lungs (obtained 4 h after carrageenan treatment) served as an indicator of iNOS activity (18). Lungs were scraped into a homogenization buffer composed of 50 mM Tris-HCl, 0.1 mM EDTA, and 1 mM PMSF (pH 7.4) and homogenized in the buffer on ice using a tissue homogenizer. Conversion of L-[3H]arginine to L-[3H]citrulline was measured in the homogenates as previously described (3). Briefly, homogenates (30 µl) were incubated in the presence of L-[3H]arginine (10 µM, 5 KBq/tube), NADPH (1 mM), calmodulin (30 mM), tetrahydrobiopterin (5 µM), and EGTA (2 mM) for 20 min at 22°C. Reactions were stopped by dilution with 0.5 ml of ice-cold HEPES buffer (pH 5.5) containing EGTA (2 mM) and EDTA (2 mM). Reaction mixtures were applied to Dowex 50W (Na+ form) columns and the eluted L-[3H]citrulline activity was measured by a Beckman scintillation counter (Palo Alto, CA).

Measurement of peroxynitrite-induced oxidation of dihydrorhodamine 123

The formation of peroxynitrite was measured by the peroxynitrite-dependant oxidation of dihydrorhodamine 123 to rhodamine 123, as previously described (18). Cells were rinsed with PBS, and the medium was then replaced with PBS containing 5 µM dihydrorhodamine 123. After a 60-min incubation at 37°C, the fluorescence of rhodamine 123 was measured using a fluorometer at an excitation wavelength of 500 nm and an emission wavelength of 536 nm (slit widths, 2.5 and 3.0 nm, respectively). Thus, this method is an indirect measurement of peroxynitrite production because also other oxidant species can induce oxidation of dihydrorhodamine 123.

Measurement of mitochondrial respiration

Cell respiration was assessed by measuring the mitochondrial-dependent reduction of MTT to formazan (18). Cells in 96-well plates were incubated at 37°C with MTT (0.2 mg/ml) for 1 h. Culture medium was removed by aspiration, and the cells were solubilized in DMSO (100 µl). The extent of reduction of MTT to formazan within cells was quantified by the measurement of OD570. As previously discussed (18), the measurement of MTT reduction appears to be mainly by the mitochondrial complexes I and II, but may also involve NADH- and NADPH-dependent energetic processes that occur outside the mitochondrial inner membrane. Thus, this method cannot be used to separate the effects of free radicals, oxidants, or other factors on the individual enzymes in the mitochondrial respiratory chain, but is useful to monitor changes in the general energetic status of the cells.

Determination of DNA single-strand breaks

The formation of DNA strand breaks in dsDNA was determined by the alkaline unwinding methods as previously described (18). Cells in 12-well plates were scraped into 0.2 ml of solution A buffer (250 mM myo-inositol, 10 mM NaH2PO3, and 1 mM MgCl2, pH 7.2). The next day, transferred to plastic tubes designated T (maximum fluorescence), P (fluorescence in sample used to estimate extent of DNA unwinding), or B (background fluorescence). To each tube, 0.2 ml of solution B (alkaline lysis solution: 10 mM NaOH, 9 M urea, 2.5 mM EDTA, and 0.1% SDS) was added and incubated at 4°C for 10 min to allow cell lysis and chromatin disruption; 0.1 ml each of solutions C (0.45 vol of solution B in 0.2 N NaOH), and D (0.4 vol of NaOH) were then added to the P and B tubes, and 0.1 ml of solution E (neutralizing solution: 1 M glucose and 14 mM ME) was added to the T tubes before solutions C and D were added. From this point onward, all incubations were conducted in the dark. A 30-min incubation period at 0°C was then allowed, during which the alkali diffused into the viscous lysate. As the neutralizing solution C and D were added, the DNA in the T tubes was never exposed to a denaturing pH. At the end of the 30-min incubation, the contents of the B tubes were sonicated for 30 s to ensure rapid denaturation of DNA in the alkaline
solution. All tubes were then incubated at 15°C for 10 min. Denaturation was stopped by chilling to 0°C and adding 0.4 ml of solution E to the P and B tubes. Then, 1.5 ml of solution F (6.7 μg/ml ethidium bromide in 13.3 mM NaOH) was added to all the tubes, and fluorescence (excitation, 520 nm; emission, 590 nm) was measured by a fluorometer. Under the conditions used, in which ethidium bromide binds preferentially to dsDNA, the percentage of dsDNA (D) may be determined using the equation: % D = 100 X (F(P) - F(B))/[F(T) - F(B)], where F(P) is the fluorescence of the sample, F(B) is the background fluorescence, i.e., fluorescence due to all cell components other than dsDNA, and F(T) is the maximum fluorescence.

Measurement of cellular NAD+ levels

Cells in 12-well plates were extracted in 0.25 ml of 0.5 N HClO4, scraped, neutralized with 3 M KOH, and centrifuged for 2 min at 10,000 x g. The supernatant was assayed for NAD+ using a modification of the colorimetric method (18) in which NADH produced by enzymatic cycling with alcohol dehydrogenase reduces MTT to formazan through the intermediation of phenazine methosulfate. The rate of MTT reduction is proportional to the concentration of the coenzyme. The reaction mixture contained 10 μl of a solution of 2.5 mg/ml MTT, 20 μl of a solution of 4 mg/ml phenazine methosulfate, 10 μl of a solution of 0.6 mg/ml alcohol dehydrogenase (300 U/mg), and 190 μl of 0.065 M glycyglycine buffer, pH 7.4, that contained 0.1% Triton X-100 and 0.5 M ethanol. The mixture was warmed to 37°C for 10 min, and the reaction was started by the addition of 20 μl of the sample. The rate of increase in absorbance was read immediately after the addition of NAD+ samples and after 10- and 20-min incubation at 37°C against blank at 560 nm in the ELISA microplate reader (SLT-Lab Instruments).

Measurement of cytokines

TNF-α and IL-1β levels were evaluated in the exudate at 4 h after the induction of pleurisy by carrageenan injection. The assay was conducted by using a colorimetric, commercial kit (Calbiochem-Novabiochem, La Jolla, CA). The ELISA has a lower detection limit of 30 pg/ml.

Immunohistochemistry of iNOS

Lung biopsies were taken 4 h after the induction of pleurisy by carrageenan injection. The lungs were perfused for 15 min with fresh 3.5% cetyldeut-buffed paraformaldehyde, and cryostat sections were prepared from the fixed lung tissue. Endogenous peroxidase was quenched with 0.3% H2O2 in 60% methanol for 30 min. The sections were permeabilized with 0.1% Triton X-100 in PBS for 20 min. Nonspecific adsorption was minimized by incubating the section in 2% normal goat serum in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min each with avidin and biotin. The sections were then incubated overnight with a 1/1000 dilution of primary anti-iNOS Ab or with control solutions. Controls included buffer alone or nonspecific purified rabbit IgG.

Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex (DBA).

Measurement of PGE2 in the pleural exudate

The amount of PGE2 present in the pleural fluid was measured by RIA without prior extraction or purification (20).

Assessment of COX activity

Lungs were obtained at 4 h after the induction of pleurisy by carrageenan injection. The material was homogenized at 4°C in a buffer containing the following reagents in a ratio of 5/1 (v/w). The protein concentra-
tion in the homogenates was measured by the Bradford assay (21), with BSA used as the standard. Homogenates were incubated at 37°C for 30 min in the presence of excess arachidonic acid (30 μM). The samples were boiled and centrifuged at 10,000 x g for min. The concentration of 6-keto-PGF1α, present in the supernatant was measured by RIA as previously described (22).

Immunohistochemical localization of COX-1 and COX-2

Lung biopsies were fixed in 10% buffered formalin, and 8-μm sections were prepared from paraffin-embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% H2O2 in 60% methanol for 30 min. The sections were permeabilized with 0.1% Triton X-100 in PBS for 20 min. Nonspecific binding was minimized by incubating the section in 2% normal goat serum in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with avidin and biotin (DBA, Milan, Italy). The sections were then incubated overnight with a 1/1000 dilution of primary anti-COX-1 or anti-COX-2 Ab (DBA) or with con-trol solutions. Controls included buffer alone or nonspecific purified rabbit IgG. Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex (DBA).

Measurement of LTB4

LTB4 levels were evaluated in the exudate at 4 h after the induction of pleurisy by carrageenan injection. The assay was conducted by using a colorimetric commercial kit (Calbiochem-Novabiochem).

Immunohistochemical localization of nitrotyrosine

Tyrosine nitration, an index of the nitrosylation of proteins by peroxynitrite and/or oxygen-derived free radicals, was determined by immunohistochemistry as previously described (18). At the end of the experiment, the relevant organs were fixed in 4% buffered formalin, and 8-μm sections were prepared from paraffin-embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% H2O2 in 60% methanol for 30 min. The sections were permeabilized with 0.1% Triton X-100 in PBS for 20 min. Nonspecific adsorption was minimized by incubating the section in 2% normal goat serum in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min each with avidin and biotin. The sections were then incubated overnight with a 1/1000 dilution of primary anti-nitrotyrosine Ab or with control solutions. Controls included buffer alone or nonspecific purified rabbit IgG.

Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex (DBA).

Immunohistochemical localization of PARP

At 4 h after carrageenan injection, lung tissues were fixed in 10% buffered formalin, and 8-μm sections were prepared from paraffin-embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% H2O2 in 60% methanol for 30 min. The sections were permeabilized with 0.1% Triton X-100 in PBS for 20 min. Nonspecific adsorption was minimized by incubating the section in 2% normal goat serum in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with avidin and biotin (DBA). The sections were then incubated overnight with a 1/500 dilution of primary anti-PARP Ab (DBA) or with control solutions. Controls included buffer alone or nonspecific purified rabbit IgG.

Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex (DBA).

Light microscopy

Lung biopsies were taken at 4 h after injection of carrageenan. The biopsies were fixed for 1 wk in buffered formaldehyde solution (10% PBS) at room temperature, dehydrated by graded ethanol, and embedded in Paraplast (Sherwood Medical, Mahwah, NJ). Tissue sections (thickness, 7 μm) were deparaffinized with xylene, stained with trichromic Van Gieson, and studied using light microscopy (Diaphax 22, Leitz, Rockleigh, NJ).

Determination of myeloperoxidase activity

Myeloperoxidase (MPO) activity, an indicator of PMN accumulation, was determined as previously described (23). At 4 h after intrapleural injection of carrageenan, lung tissues were obtained and weighed. Each piece of tissue was homogenized in a solution containing 0.5% hexa-decyl-trimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged for 30 min at 20,000 x g at 4°C. An aliquot of the supernatant was then allowed to react with a solution of tetra-methyl-benzi-
zidine (1.6 mM) and 0.1 mM H2O2. The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1 μmol of peroxidase min at 37°C and was expressed in milliunits per gram weight of wet tissue.

Determination of malondialdehyde (MDA) levels

The levels of MDA in lung tissue were determined as an indicator of lipid peroxidation (24). Lung tissue, collected at the specified time, was homog-
enized in 1.15% KCl solution. An aliquot (100 μl) of the homogenate was added to a reaction mixture containing 200 μl of 8.1% SDS, 1500 μl of 20% acetic acid (pH 3.5), 1500 μl of 0.8% thiobarbituric acid, and 700 μl of distilled water. Samples were then boiled for 1 h at 95°C and centrifuged at 3000 x g for 10 min. The absorbance of the supernatant was measured by spectrophotometry at 650 nm.

Materials

Biotin blocking kit, biotin-conjugated goat anti-rabbit IgG, primary anti-nitrotyrosine, anti-COX-2, anti-polyclonal DP-ribose Ab, and avidin-biotin peroxidase complex were obtained from DBA. All other reagents and com-
pounds used were obtained from Sigma (Milan, Italy).
Data analysis

All values in the figures and text are expressed as the mean ± SEM of n observations. For the in vitro studies, data represent the number of wells studied (six to nine wells from two or three independent experiments). For the in vivo studies, n represents the number of animals studied. In the experiments involving histology or immunohistochemistry, the figures shown are representative of at least three experiments performed on different experimental days. The results were analyzed by one-way ANOVA followed by a Bonferroni post-hoc test for multiple comparisons. p, 0.05 was considered significant.

Results

The development of carrageenan-induced pleurisy is attenuated in IL-6KO mice

All WT mice that had received carrageenan developed acute pleurisy, producing 0.9 ± 0.02 ml of turbid exudate (Fig. 1a). Compared with the number of cells collected from the pleural space of sham-operated mice (0.9 ± 0.02 × 10⁶/mice; Fig. 1b), injection of carrageenan induced a significant increase in the number of PMNs (53 ± 1.5 × 10⁶/mice; Fig. 1b). Pretreatment of IL-6WT mice with anti-IL-6 Ab as well as IL-6KO mice showed a significant attenuation of the pleural exudate as well as the number of PMNs within the exudate (Fig. 1, a and b). No significant exudate and no significant increase in the number of PMNs were observed in the pleural cavity of sham-operated mice.

Cytokine production

The levels of TNF-α and IL-1β were significantly elevated in the exudate from IL-6WT mice at 4 h after carrageenan administration. In contrast, the levels of these cytokines were significantly lower in IL-6KO mice and IL-6WT mice treated with an Ab against IL-6 (Fig. 2). No significant cytokine increased was observed in the exudate of sham-operated mice.

MPO, MDA, and histological evaluation of lung injury

All IL-6WT mice that were treated with carrageenan exhibited a substantial increase in the activities of MPO and MDA in the lung (Fig. 3, a and b). Pretreatment of IL-6WT mice with anti-IL-6 Ab as well as IL-6KO mice showed a significant attenuation of the increases in MPO and MDA caused by carrageenan in the lung (Fig. 3, a and b). There was no increase in either MPO activity or MDA level in sham-operated animals. Histological examination of lung sections of IL-6WT mice treated with carrageenan showed edema, tissue injury, as well as infiltration of the tissue with PMNs, lymphocytes, and plasma cells (Fig. 4a). Pretreatment of IL-6WT mice with anti-IL-6 Ab as well as IL-6KO mice showed a significant reduction of the lung injury as well as the infiltration of the tissue with white blood cells (Fig. 4, b and c). No histological alteration was found in sham-operated mice (data not shown).
NO production

The levels of NOx were significantly ($p < 0.01$) increased in the exudate from carrageenan-treated WT mice (141 ± 9 vs 11 ± 2 nmol/sham mice; Fig. 5a). In contrast, the levels of NOx were significantly lower in the exudate of carrageenan-treated IL-6KO and carrageenan-treated IL-6WT mice treated with the anti-IL-6 Ab (Fig. 5a). In the lungs obtained from animals subjected to carrageenan-induced pleurisy, a significant increase in iNOS activity was detected at 4 h (Fig. 5b). The iNOS activity was significantly ($p < 0.01$) lower in IL-6KO mice as well as from IL-6WT mice treated with the anti-IL-6 Ab (Fig. 5b).

COX activity (PGE$_2$ and 6-keto-PGF$_{1α}$)

The COX activity in carrageenan-induced pleural exudate and lung homogenates was assessed by measuring the increase in the formation of PGE$_2$ in the exudate. The amounts of PGE$_2$ found in the pleural exudate of carrageenan-treated WT mice was 475 ± 19 pg/mice ($n = 6$). The amounts of PGE$_2$ were significantly lower in the exudate obtained from IL-6KO mice as well as from IL-6WT mice treated with the anti-IL-6 Ab (Fig. 7a). In lungs from carrageenan-treated WT mice, the amount of 6-keto-PGF$_{1α}$ was 271 ± 30 pg/mg/tissue (Fig. 7b). The amount of 6-keto-PGF$_{1α}$ was significantly reduced in the lungs from carrageenan-treated IL-6KO mice as well as from IL-6WT mice treated with the anti-IL-6 Ab (Fig. 7b). Immunohistochemical analysis of lung sections obtained from IL-6WT mice treated with carrageenan revealed a positive staining for COX-2, which was primarily localized in alveolar macrophages (Fig. 8a). In contrast, no positive COX-2 staining was found in the lungs of carrageenan-treated IL-6KO mice and IL-6WT mice treated with the anti-IL-6 Ab (Fig. 8, b and c).
Staining was absent in tissue obtained from sham-operated control animals (data not shown).

COX-1 was also detected by immunohistochemistry analysis in the lung sections obtained from IL-6WT mice treated with carrageenan, but its positive staining was almost the same as that in the tissue obtained from sham-operated control animals (data not shown). The lungs of carrageenan-treated IL-6KO showed a similar amount of positive staining for COX-1 as that in the IL-6WT mice (data not shown).

LTB₄ production

The levels of LTB₄ were significantly elevated in the exudate from IL-6WT mice at 4 h after carrageenan administration. In contrast, the levels of LTB₄ were significantly (p < 0.05) lower in IL-6KO mice and IL-6WT mice treated with an Ab against IL-6 (Fig. 9).

Nitrotyrosine and PARP formation

Immunohistochemical analysis of lung sections obtained from IL-6WT mice treated with carrageenan revealed positive staining for nitrotyrosine, which was primarily localized in alveolar macrophages and airway epithelial cells (Fig. 10a). In contrast, no positive nitrotyrosine staining was found in the lungs of carrageenan-treated IL-6KO mice and IL-6WT mice treated with the anti-IL-6 Ab (Fig. 10, b and c). Immunohistochemical analysis of lung sections obtained from IL-6WT mice treated with carrageenan revealed positive staining for PARP (Fig. 11a). In contrast, no positive PARP staining was found in the lungs of carrageenan-treated IL-6KO mice or in IL-6WT mice treated with the anti-IL-6 Ab (Fig. 12, b and c). Note that there was no staining for either nitrotyrosine or PARP in lungs obtained from sham-operated mice (data not shown).

Effect of IL-6 inhibition on the increase in peroxynitrite formation, iNOS induction, DNA damage, and injury of macrophages obtained from the pleural cavity of carrageenan-treated mice

Compared with the supernatant of macrophages collected from the pleural cavity of sham-operated animals, the supernatant of
macrophages obtained from carrageenan-treated IL-6WT mice showed a significant increase in the concentration of NOx (Fig. 13a). This was associated with a significant increase in iNOS activity in these cells (Fig. 13b). The amount of NOx found in the supernatant of macrophages obtained from IL-6WT mice that had been pretreated with anti-IL-6 Ab or from IL-6KO mice was significantly lower. Similarly, the iNOS activity measured in the macrophages from carrageenan-treated IL-6KO mice and IL-6WT mice pretreated with anti-IL-6 Ab was significantly lower (Fig. 13, a and b). Compared with the supernatant of macrophages collected from the pleural cavity of sham-operated animals, the supernatant of macrophages obtained from carrageenan-treated IL-6WT mice showed a significant increase in the concentration of peroxynitrite (Fig. 13c). This was associated with a significant increase in the occurrence of single-strand breaks in the DNA (Fig. 13d), a reduction in mitochondrial respiration (Fig. 13e), as well as a significant fall in the intracellular levels of NAD (Fig. 13f) in these cells. Macrophages obtained from IL-6WT mice that had been pretreated with anti-IL-6 Ab or from IL-6KO mice show an attenuation of the formation of peroxynitrite (Fig. 13c) as well as the associated DNA damage (Fig. 13d), impairment of mitochondrial respiration (Fig. 13e), as well as a fall in NAD levels (Fig. 13f).

**Discussion**

The inflammatory process is invariably characterized by the production of PGs, leukotrienes, histamine, bradykinin, and platelet-activating factor and the release of chemicals from tissues and migrating cells (1, 22). Local or systemic inflammation is also associated with an enhanced formation of the proinflammatory cytokines TNF-α, IL-1, and IL-6. Using mice in which the gene for IL-6 was deleted (IL-6KO mice), we have investigated the role of IL-6 in the inflammation associated with the injection of carrageenan in the pleural cavity. We demonstrate that 1) the development of carrageenan-induced pleurisy, 2) the infiltration of the lung with PMNs (histology and MPO activity), 3) the degree of lipid peroxidation in the lung, and 4) the degree of lung injury (histology) caused by injection of carrageenan are significantly attenuated in IL-6KO mice (compared with those in wild-type...
mice treated with carrageenan). All these findings suggest that endogenous IL-6 augments the inflammation associated with carrageenan-induced pleurisy. To support the hypothesis that endogenous IL-6 plays a proinflammatory role in the model of inflammation used here, we have also pretreated wild-type mice with an Ab against IL-6 and subsequently subjected these animals to injection of carrageenan into the pleural space. Interestingly, pretreatment of wild-type mice with an Ab against IL-6 also attenuated the development of carrageenan-induced pleurisy, the infiltration of the lung with PMNs, the degree of lipid peroxidation, and the degree of lung injury caused by injection of carrageenan.

Taken together, these two studies strongly support the view that IL-6 plays a proinflammatory role in carrageenan-induced pleurisy in the rat. What, then, is the mechanism by which IL-6 augments the inflammation caused by injection of carrageenan into the pleural cavity of the rat?
There is evidence that the proinflammatory cytokines TNF-\(\alpha\) and IL-1 help to propagate the extension of a local or systemic inflammatory process (11, 12, 25). We confirm here that the inflammatory process caused by injection of carrageenan into the pleural cavity leads to a substantial increase in the levels of both TNF-\(\alpha\) and IL-1 in the exudate. Interestingly, the levels of these two proinflammatory cytokines are significantly lower in the exudate obtained from animals that are unable to produce endogenous IL-6 (IL-6KO mice) or from WT mice in which the effects of endogenous IL-6 have been attenuated by an Ab against this cytokine. These findings suggest that in the presence of endogenous IL-6, the degree of inflammation and, hence, the formation of TNF-\(\alpha\) and IL-1 are significantly enhanced (e.g., by positive feedback).

There is a large amount of evidence that the production of ROS such as hydrogen peroxide, superoxide, and hydroxyl radicals at the site of inflammation contributes to tissue damage (18, 26–29). Inhibitors of NOS activity reduce the development of carrageenan-induced inflammation and support a role for NO in the pathophysiology associated with this model of inflammation (17, 29–32). We demonstrate here that the formation of nitrite and nitrate (metabolites of NO in water) as well as the induction of iNOS protein in macrophages and airway epithelial cells caused by injection of carrageenan into the pleural cavity are reduced in lungs of IL-6KO mice as well as in WT mice pretreated with an Ab against IL-6. This finding suggests that endogenous IL-6 amplifies the induction of iNOS caused by carrageenan in the lung. The induction of iNOS caused, e.g., by injection of endotoxin in rodents in vivo is mediated by endogenous TNF-\(\alpha\), as polyclonal Abs against this cytokine abolish the induction of iNOS caused by endotoxin in the rat (33). Like TNF-\(\alpha\), endogenous IL-1 also plays an important role in the induction of iNOS, as the endogenous IL-1R antagonist also reduces the degree of iNOS induction caused by LPS in rodents (34). As the levels of TNF-\(\alpha\) and IL-1 are significantly lower in the exudate obtained from IL-6KO mice and from WT mice pretreated with an Ab against IL-6, we propose that the attenuation of the induction of iNOS protein and activity observed in mice that are unable to produce endogenous IL-6 (IL-6KO mice) are secondary to reduced formation of endogenous TNF-\(\alpha\) and IL-1. Like iNOS, the expression of COX-2 is also mediated by TNF-\(\alpha\) and IL-1. As the levels of TNF-\(\alpha\) and IL-1 as well as the induction of iNOS are significantly lower in the exudate obtained from IL-6KO mice (and from WT mice pretreated with an Ab against IL-6), it is not surprising that the degree of COX-2 induction is also significantly attenuated in macrophages located in the lungs of IL-6KO mice subjected to carrageenan-induced pleurisy. There is good evidence...
In conclusion, this study demonstrates that the degree of inflammation caused by injection of carrageenan in the pleural cavity of the mouse is significantly attenuated in IL-6KO mice. Similarly, pretreatment of WT mice with an Ab against IL-6 reduced the inflammatory response caused by subsequent injection of carrageenan. These findings support the view that endogenous IL-6 contributes to the extension of inflammation in the model of carrageenan-induced pleurisy used here. The mechanisms of the proinflammatory effect of IL-6 are not entirely clear. It appears that IL-6 augments (positive feedback; Fig. 12) the formation of other proinflammatory PG metabolites. In conclusion, this study demonstrates that the degree of inflammation caused by injection of carrageenan in the pleural cavity of the mouse is significantly attenuated in IL-6KO mice. Similarly, pretreatment of WT mice with an Ab against IL-6 reduced the inflammatory response caused by subsequent injection of carrageenan. These findings support the view that endogenous IL-6 contributes to the extension of inflammation in the model of carrageenan-induced pleurisy used here. The mechanisms of the proinflammatory effect of IL-6 are not entirely clear. It appears that IL-6 augments (positive feedback; Fig. 12) the formation of other in this and other models of inflammation that enhanced formation of prostanoids following the induction of COX-2 contributes to the pathophysiology of local inflammation (35, 36) and that selective inhibitors of COX-2 exert potent anti-inflammatory effects (37–39). We demonstrate here that the increase in the levels of PGE2 is secondary to the expression of COX-2 protein, as 1) there was no increase in the expression of COX-1 protein (detected by immunohistochemistry) after carrageenan injection; and 2) selective inhibitors of COX-2 activity, including NS-398 (nimesulide) and SC-58125 (Celecoxib), abolished the increase in PGE2 caused by injection of carrageenan into the pleural space (38) Thus, we propose that IL-6 amplifies the expression of COX-2 protein and activity caused by injection of carrageenan in the lung and that the subsequent enhanced formation of PGE2 (and other COX-2 metabolites) contributes to the observed inflammatory process. This conclusion is supported by the previous finding that selective inhibitors of COX-2 activity reduced the inflammation caused by carrageenan in the rat (37–39).

In addition to PGs and NO, peroxynitrite is also generated in carrageenan-induced inflammation (18, 27, 29). The biological activity and decomposition of peroxynitrite are very much dependent on the cellular or chemical environment (presence of proteins, thiols, and glucose; ratio of NO and superoxide; carbon dioxide levels; and other factors), and these factors influence its toxic potential (40–42). We demonstrate here that injection of carrageenan into the pleural cavity of WT mice leads to a substantial increase in the degree of nitrosylation of proteins in the lung. In contrast, the degree of staining for nitrotyrosine was significantly reduced in IL-6KO mice or in WT mice pretreated with an Ab against IL-6. Nitrotyrosine formation along with its detection by immunostaining were initially proposed as relatively specific markers for the detection of the endogenous formation (footprint) of peroxynitrite (43). There is, however, recent evidence that certain other reactions can also induce tyrosine nitration; e.g., the reaction of nitrate with hypochlorous acid and the reaction of myeloperoxidase with hydrogen peroxide can lead to the formation of nitrotyrosine (44). Increased nitrotyrosine staining is considered, therefore, an indication of increased nitrosative stress rather than a specific marker of the generation of peroxynitrite. Thus, our results suggest that the degree of nitrosative stress caused by injection of carrageenan is reduced in lungs from animals that are unable to produce endogenous IL-6 or those in which the effects of endogenous IL-6 have been attenuated/abolished by an Ab against this cytokine.

ROS and peroxynitrite produce cellular injury and necrosis via several mechanisms, including peroxidation of membrane lipids, protein denaturation, and DNA damage. ROS produce strand breaks in DNA, which triggers energy-consuming DNA repair mechanisms and activates the nuclear enzyme PARS, resulting in the depletion of its substrate NAD in vitro and a reduction in the rate of glycolysis. As NAD functions as a cofactor in glycolysis and the tricarboxylic acid cycle, NAD depletion leads to a rapid fall in intracellular ATP. This process has been termed the PARS suicide hypothesis (45, 46). There is recent evidence that the activation of PARS may also play an important role in inflammation (45–48). We also demonstrate here that the increase in PARS activity caused by injection of carrageenan in the lung is attenuated in lungs of IL-6KO mice as well as in lungs of WT mice pretreated with an Ab against IL-6.

In conclusion, this study demonstrates that the degree of inflammation caused by injection of carrageenan in the pleural cavity of the mouse is significantly attenuated in IL-6KO mice. Similarly, pretreatment of WT mice with an Ab against IL-6 reduced the inflammatory response caused by subsequent injection of carrageenan. These findings support the view that endogenous IL-6 contributes to the extension of inflammation in the model of carrageenan-induced pleurisy used here. The mechanisms of the proinflammatory effect of IL-6 are not entirely clear. It appears that IL-6 augments (positive feedback; Fig. 12) the formation of other...
proinflammatory cytokines, such as TNF-α and IL-1, which, in turn, may augment the recruitment of neutrophils, the expression of iNOS and COX-2 protein and activity, and ultimately the degree of peroxynitrite formation and tissue injury. In addition, IL-6 appears to enhance the formation of LTB₄, which, in turn, may contribute to the recruitment of PMNs. Finally, our findings suggest that interventions that may reduce the generation or the effects of IL-6 may be useful in conditions associated with local or systemic inflammation.

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