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Regulatory Effects of Interleukin-6 in Immunoglobulin G Immune-Complex-Induced Lung Injury

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Interleukin-6 (IL-6) is a cytokine produced in response to a variety of inflammatory stimuli. Although IL-6 is often observed in increased amounts in acute respiratory distress syndrome, its role in the development of lung injury is unclear. The role of IL-6 was studied in the rat model of lung injury induced by the intra-alveolar deposition of IgG immune complexes. IL-6 induction, as determined by Northern blot analysis and bioactivity, was found as a function of time during the course of development of injury. Recombinant IL-6 instilled intratracheally at commencement of injury led to substantial reductions in lung vascular permeability, neutrophil accumulation, and levels of tumor necrosis factor (TNF)-α and macrophage inflammatory protein (MIP)-2 in bronchoalveolar lavage fluids. Conversely, blocking of intrinsic IL-6 by a neutralizing antibody resulted in increases in lung vascular permeability, neutrophil content, and TNF-α levels in bronchoalveolar lavage fluids. Rat alveolar macrophages stimulated in vitro with lipopolysaccharide in the presence of IL-6 showed a significant reduction in TNF-α expression. Together, these findings suggest that IL-6 acts as an intrinsic regulator of lung inflammatory injury after deposition of IgG immune complexes and that the protective effects of exogenously administered IL-6 may be in part linked to suppressed TNF-α production. (Am J Pathol 1997, 151:193–203)

Interleukin-6 (IL-6) is a multifunctional cytokine that has been demonstrated to interact with numerous target cells, inducing a variety of responses (reviewed in Refs. 1 and 2). For example, IL-6 stimulates terminal B cell differentiation into plasma cells with secretion of IgG,[3] induces the differentiation of cytolytic T lymphocytes,[4] and synergizes with interleukin (IL)-3 to promote hematopoietic colony formation.[5] In the context of the inflammatory response associated with adult respiratory distress syndrome (ARDS), sepsis, trauma, and bacterial infections, increases in serum levels of IL-6 have been found.[6–14] Plasma levels of IL-6 are persistently elevated in ARDS.[7] Thus, several experimental and human clinical studies have suggested an important role for cytokines such as IL-6 in the development of ARDS; however, despite this evidence, the relationship between IL-6 and development of organ dysfunction remains to be defined. *In vitro* and *in vivo* studies have suggested that IL-6 may function as an anti-inflammatory cytokine.[15–18]

Lung inflammation caused by the intratracheal deposition of IgG immune complexes is characterized by increases in pulmonary vascular permeability as well as development of alveolar hemorrhage and neutrophil accumulation.[19] This model of acute lung injury is also characterized by an eventual resolution of the inflammatory response, suggesting the presence of autoregulatory molecules that counteract the effects of pro-inflammatory molecules such as tumor necrosis factor (TNF)-α and IL-1. We sought to determine whether IL-6 may function as a regulator of inflammation in this model. On the basis of Northern blot analysis and IL-6 bioassay, induc-

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tion of IL-6 has been demonstrated in the lung. Treatment with human recombinant IL-6 resulted in a dose-dependent decrease in permeability and neutrophils in bronchoalveolar lavage (BAL) fluids, with a concomitant decrease in both BAL TNF-α and macrophage inflammatory protein (MIP)-2. *In vitro* studies have demonstrated that IL-6 decreases TNF-α production from rat alveolar macrophages stimulated with lipopolysaccharide (LPS), suggesting one mechanism by which IL-6 may function as an autoregulatory cytokine. Consistent with the hypothesis that intrinsic IL-6 functions as a down-regulator of inflammation in this model, it was found that administration of anti-IL-6 resulted in significant increases in lung permeability and BAL neutrophil content. Furthermore, treatment of rats with anti-IL-6 resulted in a significant increase in the levels of TNF-α in BAL fluids. These findings were concomitant with a decrease in levels of bioactive IL-6 in BAL fluids recovered from rats treated with anti-IL-6. Together, these data support the conclusion that IL-6 is an important regulatory cytokine during the development of acute lung injury after intrapulmonary deposition of IgG immune complexes.

Materials and Methods

Reagents

Except where noted, all reagents were purchased from the Sigma Chemical Co. (St. Louis, MO).

Model of IgG Immune-Complex-Induced Alveolitis

For all studies, adult male, 275- to 300-g (specific-pathogen-free) Long-Evans rats (Harlan Co., Indianapolis, IN) were used. Intraperitoneal ketamine was administered for sedation and anesthesia. Rabbit polyclonal (2.5 mg) rich in IgG antibody to bovine serum albumin (BSA) in a volume of 300 μl of phosphate-buffered saline (PBS) was instilled via an intratracheal catheter during inspiration. Immediately thereafter, 10 mg of BSA together with trace amounts of ^{125}I-labeled BSA (as quantitative marker of permeability) were injected intravenously, as described elsewhere.[19] Rats were sacrificed 4 hours later, the pulmonary circulation was flushed with 10 ml of sterile saline, the lungs were surgically dissected, and the amount of radioactivity (^{125}I-labeled BSA) remaining in the lungs was determined by scintillation counting. Negative control animals received anti-BSA intratracheally in the absence of intravenously infused BSA. For calculations of the permeability index, the amount of radioactivity (^{125}I-labeled BSA) remaining in the lungs in which the vasculature was perfused with saline was divided by the amount of radioactivity present in 1.0 ml of blood obtained from the inferior vena cava at the time of sacrifice. Where indicated, either recombinant human IL-6 or anti-IL-6 (see below) were co-administered intratracheally with anti-BSA antibody.

Recombinant IL-6

Recombinant human interleukin-6 was obtained from AMGEN (Thousand Oaks, CA). This protein was expressed in a bacterial expression system and purified to homogeneity as reflected by Coomassie-stained sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of this recombinant protein (data not shown). Previous reports have confirmed the activity of this protein in rodent models.[15]

Anti-IL-6 Antibody

Polyclonal goat anti-murine IL-6 antibody (R&D Systems, Minneapolis, MN) containing <10 ng of endotoxin/mg of protein was resuspended from lyophilized form to an initial concentration of 1 mg/200 μl of sterile PBS (pH 7.4) and used for all studies. Where indicated, either 0.5, 5, or 50 μg of anti-IL-6 were co-instilled intratracheally (constant volume of 10 μl) with anti-BSA at the commencement of injury. The ability of this antibody to react with rat IL-6 was demonstrated by Western blot analysis and *in vitro* inhibition studies as shown below. In addition, other investigators have demonstrated reactivity of this antibody preparation *in vivo* in rat models of inflammation.[20]

Northern Blot Analysis

After IgG immune complex deposition, rats were sacrificed at 2-hour intervals from 0 to 8 hours. Whole lungs were dissected and frozen in liquid nitrogen for Northern blot analysis of IL-6 mRNA. RNA was extracted using a guanidinium-isothiocyanate method as described previously.[21] Twelve micrograms of cytoplasmic RNA was fractionated electrophoretically in a 1% formaldehyde gel and transferred to a nylon blot (Zetabind, CUNO Laboratories, Meriden, CT). Equal loading of samples was confirmed by methylene blue staining of 18 and 28 S rRNA bands. Full-length cDNA for rat IL-6 was polymerase chain reaction (PCR) cloned by reverse transcription of mRNA isolated from IgG immune-complex-injured lungs. The PCR reaction was primed using the following oligonucleotides: 5′ primer, 5′-ATG AAG TTT

CTC TCC GCA-3′; 3′ primer, 5′-CTA GGT TTG CCG AGT AGA-3′. The cDNA product was sequenced and confirmed to be rat IL-6 cDNA by comparison with the published sequence.[22] The cDNA for rat IL-6 was [^{32}P]dCTP-radiolabeled (NEN-DuPont, Boston, MA) by PCR to generate the cDNA probe that was applied to the Northern blot. Hybridization was performed at 65°C for 18 hours, and the autoradiogram was developed on Kodak X-Omat film (Rochester, NY).

IL-6 Bioactivity in BAL Fluids

BAL fluid samples were collected after induction of IgG immune-complex-induced lung injury from 0 to 8 hours. Eight milliliters of PBS was three times instilled and withdrawn from the lungs via an intratracheal cannula. These samples were centrifuged at 1500 × *g* for 10 minutes, and the supernatant fluids were evaluated for IL-6 bioactivity using a B9 cell proliferation assay as previously reported.[23] Briefly, B9 cells (10^5/well in 96-well microtiter plates) were incubated in RPMI 1640 medium for 72 hours in the presence of serial dilutions of BAL fluid samples or recombinant murine IL-6 standards. Twenty-five microliters of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolinium bromide (MTT) was added to each well. After 4 hours of incubation, acidified isopropanol was added, and optical density was read at 550 nm. IL-6 levels were calculated according to a standard curve generated with IL-6 standards.

BAL Neutrophil Counts

At 4 hours after induction of IgG immune complex, BAL fluid samples were collected as described above to determine the effect of either neutralization of endogenous IL-6 (achieved by administration of anti-IL-6) or of exogenous IL-6 administration on lung neutrophil accumulation. Total white cell counts in BAL fluids were determined using a Coulter counter (Coulter Electronics, Hialeah, FL). Cell differentials were determined using cytospin centrifugation (700 × *g* for 7 minutes) on BAL fluids. Specimens were fixed and stained with Diff-Quik products (Baxter Scientific, Miami, FL) for determination of percentage of neutrophils and macrophages/monocytes. The total number of neutrophils for each BAL sample was then calculated by multiplying the total white blood cell number times the percentage of neutrophils times the volume of BAL fluid recovered. The average volume recovered was 7.1 ± 0.1 ml for all samples collected.

TNF-α and MIP-2 in BAL Fluids

Remaining BAL samples were centrifuged at 1500 × *g* for 10 minutes, and the supernatant fluids were evaluated for TNF-α bioactivity using a standard cell cytotoxicity (WEHI clone) assay as previously reported[24] to determine the effect of either exogenous IL-6 administration or neutralization of intrinsic IL-6 on TNF-α expression. Similarly, these BAL fluid samples were tested for MIP-2 content employing a rat MIP-2-specific immunoassay (Biosource, Camarillo, CA). The assay's minimal detectable dose of MIP-2 was less than 1 pg/ml and demonstrated no cross-reactivity with rat MCP-1, CINC, or interferon-γ.

In Vitro *Determination of IL-6 Suppression of TNF-α Expression*

Adult male, Long-Evans rats (275 to 330 g) were anesthetized with intraperitoneal ketamine and sacrificed by exsanguination from the inferior vena cava. Rat alveolar macrophages were isolated by exhaustive BAL using 10-ml aliquots of PBS (without Ca^{2+} or Mg^{2+}). Recovered cells were washed twice in PBS, pelleted by centrifugation (700 × *g* for 10 minutes), and resuspended in 10 ml of Dulbecco's modified Eagle's medium (Gibco BRL, Gaithersburg, MD) with 10% fetal bovine serum (DMEM 10%), 1% non-essential amino acids, and 1% glutamine. Purity of ≥98% macrophages was confirmed by Diff-Quik (Baxter) staining of cytospin preparations. Cell viability was ≥98% as determined by trypan blue exclusion. Cells were diluted to 10^6 cells/ml of DMEM 10%, and 200-μl aliquots were plated on a 96-well tissue culture plate (Sarstedt, Newton, NC). Varying doses of recombinant human IL-6 (in a constant volume of 10 μl) were added to appropriate wells, and the plate was incubated at 37°C with 5% CO_2 for 4 hours before macrophage stimulation. After this pretreatment, LPS (serotype 0111:B4, Sigma) was added at a final concentration of 200 ng/ml. Cells were incubated for an additional 4 hours. Supernatants from each cell suspension were harvested by centrifugation (700 × *g* for 10 minutes) to remove any contaminating cells and frozen at −20°C before testing in the Wehi TNF-α bioassay.

Western Blot Analysis

BAL fluids from rats undergoing IgG immune-complex-induced lung injury were collected at 4 and 6 hours and subjected to SDS-polyacrylamide gel electrophoresis (15%) according to the method of

Laemmli.[25] At each time point, BAL fluid volume between 16 and 20 μl was loaded per lane under nonreducing conditions. The separated proteins were transblotted to polyvinylidene difluoride membrane (Westran, Schleicher and Schuell, Keene, NH) for 1.5 hours at 10 V. After transfer, the membrane was blocked with 20 mmol/L Tris/HCl, pH 7.5, 500 mmol/L NaCl, 0.05% Tween 20 (T-TBS) (v/v) containing 5% dry milk for 2 hours at room temperature. The blot was next incubated for 1 hour with the primary antibody (goat anti-murine IL-6 antibody, 5 μg/ml dilution). After washing, secondary antibody (donkey anti-goat IgG horseradish-peroxidase-conjugated antibody; Jackson ImmunoResearch Laboratories, West Grand, PA) was added at a final dilution of 1:40,000 in T-TBS and incubated for 30 minutes. After washing, the membrane was developed by enhanced chemiluminescence technique according to the manufacturer's protocol (Amersham, Little Chalfont, UK). Rainbow molecular weight markers (Amersham, Arlington Heights, IL) were used to estimate the size of the immunoreactive bands (2,350 to 46,000 Da).

Anti-IL-6 Antibody Titered Against BAL Fluid IL-6 Activity

To confirm the ability of the antibody to neutralize IL-6, BAL fluid samples were collected from IgG immune-complex-injured rats. These samples were diluted to a standard IL-6 concentration of 500 pg/ml as determined by B9 assay. Serial dilutions of anti-IL-6 (0 to 50 μg/ml) were then co-incubated (37°C for 1 hour) with samples at the standard concentration across a 96-well plate. IL-6 bioactivity was then repeated to determine the neutralizing effect of the antibody preparation.

Statistical Analysis

All values are expressed as mean ± SEM. For *in vivo* studies, all data points reflect $n \geq 6$. Data sets were examined with one- and two-way analysis of variance, and individual group means were then compared using either Scheffé's or Tukey's procedure. For calculation of percentage change in permeability index, mean negative control values were first subtracted from values in positive control groups and in each treatment group.

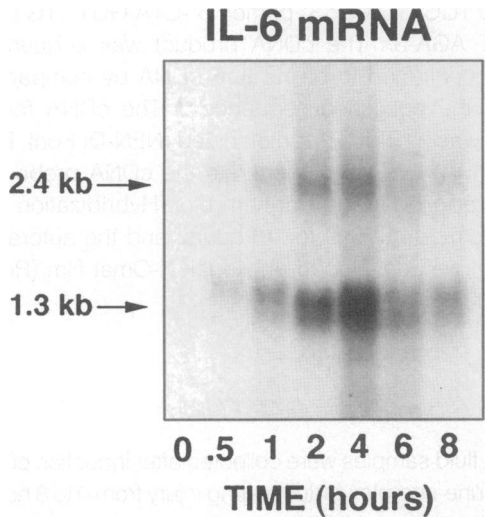

Figure 1. *Time course in appearance of IL-6 mRNA in lung extracts obtained during intrapulmonary deposition of IgG immune complexes in rats. These data are representative of three separate Northern blots (obtained from separate animal experiments) probed with ^{32}P-labeled cDNA for rat IL-6. Equal loading was confirmed by methylene blue staining of 18 and 28 S rRNA bands (not shown). Two transcriptional forms of rat IL-6 mRNA were identified at ~2.4 and ~1.3 kb, respectively.*

Results

IL-6 mRNA Time Course in IgG Immune Complex Alveolitis

Expression of IL-6 mRNA in lung extracts from animals undergoing IgG immune-complex-induced lung injury was determined as a function of time (0 to 8 hours) after induction of the lung inflammatory reaction. mRNA was analyzed by Northern blots that were probed with [^{32}P]cDNA for rat IL-6 (Figure 1). Equal loading was confirmed by methylene blue staining of 18 and 28 S bands (not shown). Very little mRNA was found at time 0 hour. Messenger RNA could be detected by 1 hour, with a progressive increase in IL-6 mRNA until 4 hours, followed by a decline thereafter. Two transcriptional forms (~1.3 and ~2.4 kb) of IL-6 mRNA were identified as had been previously described for rat IL-6.[21]

IL-6 Content in BAL Fluids during IgG Immune-Complex-Induced Lung Injury

Recoverable IL-6 from BAL fluids was determined by bioassay as described above. IL-6 content at each time point represents the average value based on BAL fluids obtained from three animals. IL-6 protein was found to be expressed in a pattern similar to mRNA. Very little IL-6 was detected at the initiation of injury (0 hour, 19 ± 9 pg/ml), but there was a rapid increase in IL-6 by 1 hour (7524 ± 1893 pg/ml), with

highest expression at 2 hours (42,858 ± 12,338 pg/ml), 4 hours (41,117 ± 9,249 pg/ml), and 6 hours (20,982 ± 5,931 pg/ml), followed by a rapid decline at 8 hours (26 ± 8 pg/ml). The increases in IL-6 at 1, 2, 4, and 6 hours were statistically significant when compared with the 0 time value ($P < 0.01$), whereas this was not the case at 8 hours.

Effect of IL-6 on IgG Immune-Complex-Mediated Lung Injury

The ability of exogenous IL-6 to function as an anti-inflammatory cytokine was investigated. The effects of exogenous IL-6 (administered intratracheally) after intrapulmonary deposition of IgG immune complexes on lung permeability, BAL neutrophils, and BAL TNF-α were assessed. Because of lack of availability of sufficient amounts of rat or mouse IL-6, recombinant human IL-6 was administered to rats. This recombinant human IL-6 has been demonstrated to have activity *in vivo* in rats in another model of lung inflammation.[15,16] Lung injury was induced as described above, and the permeability index was measured at 4 hours. To control for IL-6 treatment, positive control animals received 60 μg of heat-inactivated (95°C for 30 minutes) human IL-6. The mean permeability values in the negative and positive (treated with heat-inactivated IL-6) controls were 0.17 ± 0.03 and 0.56 ± 0.04, respectively (Figure 2A). The co-administration of either 10 or 60 μg of recombinant human IL-6 resulted in a reduction in lung permeability (0.49 ± 0.03, 18% decrease; and 0.38 ± 0.03, 57% decrease, respectively) as compared with positive control rats (Figure 2A). Only at the 60-μg dose did this reduction reach statistical significance ($P < 0.05$; n = 6 in each group). Of note, in a single study employing recombinant mouse IL-6, 1 μg given intratracheally at the commencement of injury resulted in a 33% decrease in permeability when compared with positive controls (data not shown), suggesting a log-fold improvement in the bioactivity of recombinant murine IL-6 in rats as compared with recombinant human IL-6 in this experimental animal model.

The effect of IL-6 on neutrophil accumulation after intrapulmonary deposition of IgG immune complexes was also assessed. Human IL-6 was administered in the same doses (10 and 60 μg). In this series of experiments, the mean neutrophil number increased from 683 ± 53 × 10^3 in negative control animals receiving anti-BSA only (negative controls, Figure 2B) to 15,305 ± 3,323 × 10^3 in positive control animals treated with heat-inactivated IL-6 (positive). Treatment of rats with either 10 or 60 μg of human IL-6 resulted in significant decreases (to 9474 ± 2723 × 10^3, $P < 0.05$ and 5241 ± 998 × 10^3, $P < 0.01$, respectively) in BAL neutrophils (Figure 2B). To determine any independent effects of IL-6 *per se* on rats, a set of four animals received IL-6 (60 μg) alone intratracheally. This dose of IL-6 resulted in neither an increase in BAL neutrophils (26 ± 24 × 10^3 *versus* 18 ± 16 × 10^3) nor an alteration in the number of lavagable alveolar macrophages (2011 ±

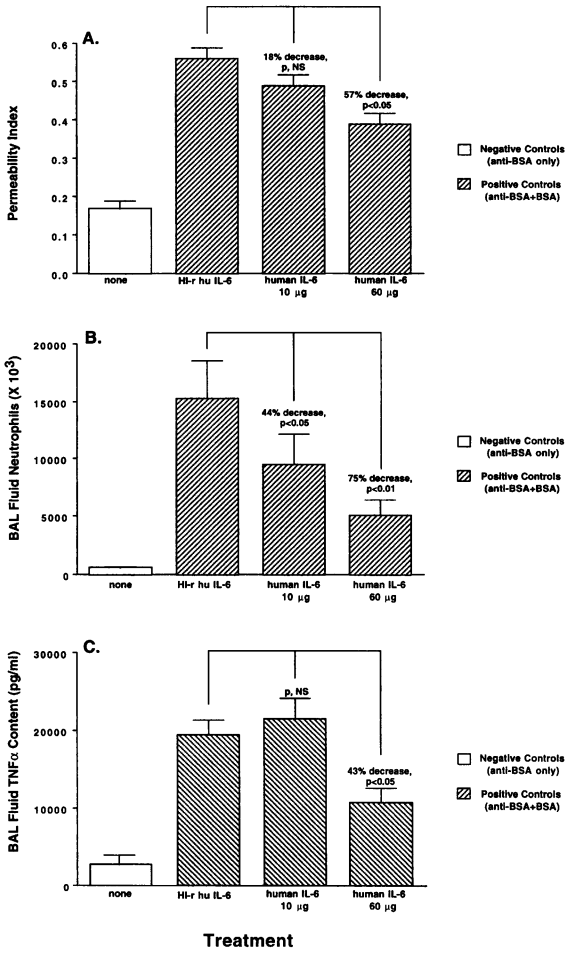

Figure 2. A: *Effect of recombinant human IL-6 on lung injury (as defined by leakage of ^{125}I-labeled albumin) 4 hours after intrapulmonary deposition of IgG immune complexes. Positive controls (anti-BSA plus BSA) were treated with either 10 or 60 µg of recombinant human IL-6 intratracheally or heat-inactivated recombinant human IL-6 (HI-r hu IL-6) at commencement of injury. Negative controls (anti-BSA only) received anti-BSA intratracheally, but the 10-mg dose of BSA was omitted. n = 6 for all groups. NS, not significant (P > 0.05).* B: *Effect of recombinant human IL-6 on BAL neutrophil accumulation 4 hours after intrapulmonary deposition of IgG immune complexes in rats. Positive and negative control groups are companion to those described in* A. *Values were reported as BAL PMN counts × 10^3. n = 6 for all groups.* C: *Effect of recombinant human IL-6 on BAL TNF-α content 4 hours after intrapulmonary deposition of IgG immune complexes. Positive and negative control groups are companion to those described in* A. *n = 6 for all groups.*

252 × 10^3 *versus* 1899 ± 163 × 10^3) when compared with untreated rats (data not shown).

BAL fluid levels of TNF-α from animals undergoing intrapulmonary deposition of IgG immune complexes were also determined. Mean TNF-α content in positive control animals was 19,370 ± 1,919 pg/ml (Figure 2C). Although treatment of rats with 10 μg of human IL-6 did not alter this TNF-α content (21,524 ± 2,609; *P* value not significant), administration of 60 μg of human IL-6 resulted in a significant decrease in levels of BAL TNF-α (11,034 ± 1,941, 43% decrease, $P < 0.05$; Figure 2C). (Consistent with its lack of effect on BAL fluid cell content, 60 μg of IL-6 alone administered intratracheally resulted in no significant difference (26 ± 18 pg/ml) in BAL TNF-α levels as compared with untreated rats (19 ± 15 pg/ml; data not shown)).

The C-X-C chemokine MIP-2 had been shown previously to play an important role in both the recruitment and activation of neutrophils in acute lung injury in the rat,[26] similar to the role of IL-8 in humans. Because MIP-2 is derived from macrophages, as is TNF-α, we determined the effect of IL-6 on BAL fluid levels of MIP-2 from animals undergoing intrapulmonary deposition of IgG immune complexes. Although MIP-2 content in negative control animals was low (24 ± 8 pg/ml), mean MIP-2 content in positive control animals increased to 295 ± 33 pg/ml. Treatment of positive control rats with either 10 or 60 μg of human IL-6 resulted in a decrease in levels of BAL MIP-2 (208 ± 24 pg/ml and 173 ± 16 pg/ml, respectively); however, only at the 60-μg dose was this finding statistically significant ($P < 0.05$).

To confirm the presence of bioactive IL-6 in rat BAL fluids after exogenous administration of recombinant IL-6, bioassays were performed on the same BAL fluids described in Figure 2C. In the positive control animals treated with heat-inactivated IL-6, mean IL-6 levels were 67.1 ± 4.9 ng/ml, not significantly different from those values obtained in the time-course study. Substantially increased bioactivity in BAL fluids of IL-6-treated animals was confirmed with IL-6 levels increasing to 3600 ± 500 ng/ml in rats treated with 10 μg of human IL-6 and to 50,000 ± 20,000 ng/ml in rats treated with 60 μg of human IL-6. These high levels of IL-6 as a result of exogenous administration, combined with the limitations of the bioassay at the upper end of the scale required substantial dilutions of these BAL fluids before assaying for IL-6; therefore, the exact values of IL-6 content may not be precise. However, these data confirm a significant increase in IL-6 activity as a result of exogenous IL-6 administration.

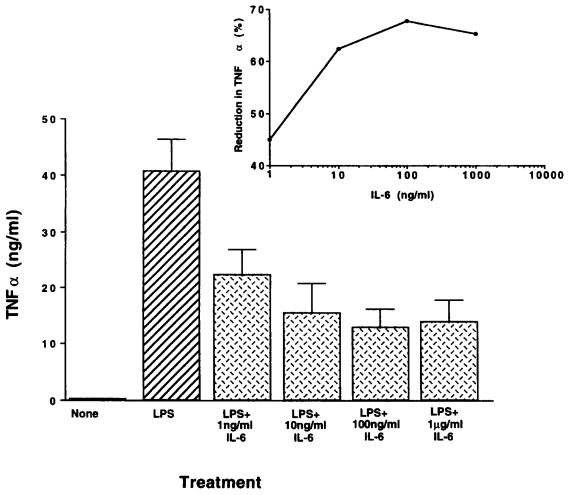

Figure 3. *Effect of recombinant human IL-6 on TNF-α production from LPS-stimulated rat alveolar macrophages* in vitro. *Dose-dependent (0.001 to 1 μg) decreases in TNF-α were observed.* **Inset:** *Percentage reduction in TNF-α content as compared with positive control (LPS).*

In Vitro *Effect of IL-6 on Isolated Alveolar Macrophage Production of TNF-α*

To assess the *in vitro* effects of human IL-6 on TNF-α production, IL-6 was added to isolated rat alveolar macrophages stimulated with LPS. At the time of harvesting supernatant fluids for TNF-α content (6 hours after plating the macrophages), cell viability under all treatment conditions, which was determined using trypan blue exclusion, averaged >98%. Isolated, unstimulated alveolar macrophages incubated for 6 hours *in vitro* produced minimal amounts of TNF-α (0.15 ng/ml; Figure 3). In contrast, alveolar macrophages stimulated with LPS produced substantial amounts of TNF-α (40.5 ± 8.1 ng/ml; Figure 3). Recombinant human IL-6 at doses from 1 ng/ml to 1 μg/ml resulted in significant reductions in TNF-α content (45 to 68%; Figure 3, inset). Alveolar macrophages treated with human IL-6 alone released no more TNF-α than unstimulated macrophages (0.07 ng/ml *versus* 0.15 ng/ml; data not shown). Thus, IL-6 *in vitro* can directly suppress production of TNF-α by LPS-stimulated rat alveolar macrophages, confirming a similar biological effect as seen *in vivo*.

Reactivity of Anti-Murine IL-6 with BAL Fluid Rat IL-6

BAL fluids from IgG immune-complex-injured rats sacrificed at 4 and 6 hours were subjected to Western Blot analysis using the goat anti-murine antibody as the primary antibody as described in Materials and Methods. A band (approximately 21 kd) was observed in the BAL fluids, confirming reactivity be-

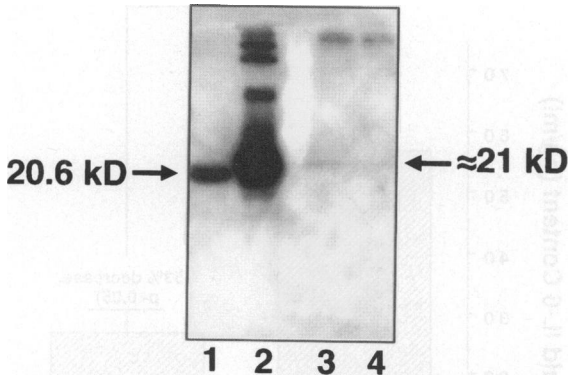

Figure 4. *Reactivity between anti-IL-6 and rat IL-6. Recombinant human IL-6* (lane 1), *mouse IL-6* (lane 2), *and BAL fluids from IgG immune-complex-injured rats* (lane 3, *6 hours;* lane 4, *4 hours*) *were subjected to Western blot analysis using a primary goat anti-murine IL-6 antibody as described in Materials and Methods. As demonstrated, reactions at the predicted sizes for human and mouse IL-6 (20.6 kd) were observed. In addition, a band at ~21 kd was observed in the BAL fluids of IgG immune-complex-injured rats. The larger bands in* lanes 3 *and* 4 *at ~40 kd likely represent dimeric forms of rat IL-6 as they did not appear when samples were subjected to reducing conditions (data not shown).*

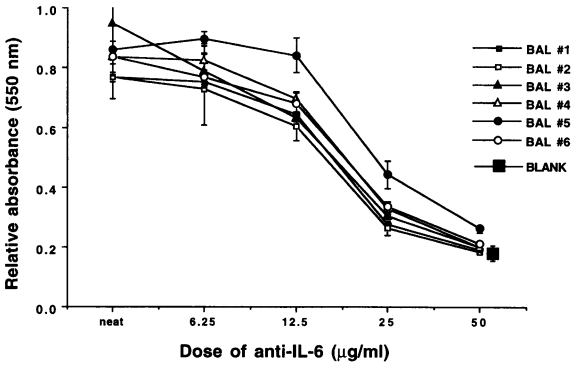

Figure 5. In vitro *neutralization of rat IL-6 bioactivity. BAL fluids from injured rats at 4 hours (n = 6) were diluted to a standard concentration of 500 pg/ml as determined by B9 bioassay. Serial samples were then co-incubated with increasing amounts of anti-IL-6 (0 to 50 μg/ml). IL-6 bioassays were subsequently performed in triplicate on each sample after the co-incubation and mean fluoresence (550 nm) were recorded. The mean fluoresence (±SEM) of each sample is graphed against the dose of anti-IL-6. The mean fluoresence of blank wells was 0.187 ± 0.004, indicating nearly complete neutralization at an antibody dose of 50 μg/ml.*

tween the anti-IL-6 antibody and a protein in BAL fluids (lanes 3 and 4), consistent with the estimated size of rat IL-6 (Figure 4). This band was in the identical positions for both human (lane 1) and murine (lane 2) IL-6 (Figure 4). A band at approximately 40 kd was also observed, suggestive of a dimeric form of rat IL-6 in BAL fluids. This finding is consistent with previous investigators' description of dimeric IL-6 in humans.[27]

Effect of Anti-Murine IL-6 on IL-6 Biological Activity in BAL Fluids

To confirm the ability of this antibody to neutralize intrinsic rat IL-6, BAL fluids from six rats were diluted to a standard concentration of approximately 500 pg/ml as determined by B9 bioassay. Serial samples were then co-incubated with increasing amounts of anti-IL-6 (0 to 50 μg/ml). To titrate the neutralization capability of the antibody, subsequent IL-6 bioassays were performed in triplicate on each sample after co-incubation, and the mean fluoresence (550 nm) was reported (Figure 5). As demonstrated in Figure 5, the anti-IL-6 antibody neutralized the IL-6 bioactivity in a dose-dependent manner. The mean fluoresence of blank wells was 0.187, indicating nearly complete neutralization at an antibody dose of 50 μg/ml. With these data and the Western blot results (Figure 4), it was concluded that the anti-IL-6 antibody used in these studies reacts with and neutralizes rat IL-6.

Effects of Anti-IL-6 on IgG Immune-Complex-Mediated Lung Injury

Having demonstrated reactivity between this antibody and rat IL-6, we determined whether intrinsic IL-6 could regulate the lung inflammatory response triggered by IgG immune complexes. Lung injury was induced as described above under conditions of anti-IL-6 treatment, and the parameters of injury were determined at 4 hours. Positive control rats received 50 μg of goat preimmune IgG antibody (Lampire Biologicals, Everett, PA) or a dose range of anti-IL-6. The dose of anti-IL-6 was based upon the results of the IL-6 bioactivity time course (above) that showed a concentration of ~50 ng/ml at 4 hours. With an estimated lung volume of 10 ml, the total IL-6 activity was estimated to be 0.5 μg. Therefore, doses of 0.5, 5, and 50 μg of anti-IL-6 were co-administered with the anti-BSA intratracheally at the commencement of injury. The mean permeability values in the negative and positive controls (preimmune IgG) were 0.17 ± 0.03 and 0.63 ± 0.09, respectively (Figure 6A). Intratracheal administration at time 0 hour of increasing doses of anti-IL-6 resulted in a dose-response increase in lung permeability index at the time of sacrifice (4 hours) (to 0.58 ± 0.04, 0.75 ± 0.10, and 0.93 ± 0.11 for 0.5, 5, and 50 μg of anti-IL-6, respectively; Figure 6A). Only at the 50-μg dose did this result reach statistical significance ($P < 0.05$).

Under similar conditions, the effects of anti-IL-6 treatment on BAL neutrophils was assessed. The mean neutrophil number increased from 638 ± 53 × 10^3 in negative control animals receiving anti-BSA

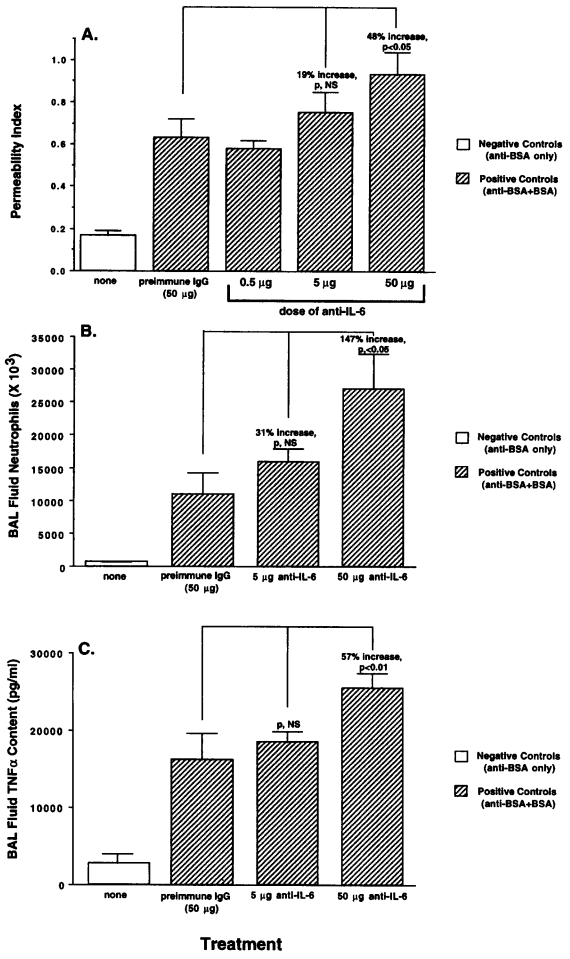

Figure 6. A: *Augmentation in lung injury (as defined by leakage of ^{125}I-labeled albumin) 4 hours after intrapulmonary deposition of IgG immune complexes with anti-IL-6. Positive controls (anti-BSA plus BSA) were administered either goat preimmune IgG (preimmune IgG) or anti-IL-6 intratracheally at the commencement of injury. Negative controls received anti-BSA intratracheally, but the 10-mg dose of BSA was omitted (anti-BSA only). n = 6 for all groups.* B: *Effect of anti-IL-6 on BAL neutrophils 4 hours after intrapulmonary deposition of IgG immune complexes. Positive and negative control groups are companion to those described in* A. *Values were reported as BAL PMN counts × 10³. n = 6 for all groups.* C: *Effect of anti-IL-6 on BAL TNF-α 4 hours after intrapulmonary deposition of IgG immune complexes. Positive and negative control groups are companion to those described in* A. *n = 6 for all groups.*

alone (negative) to 10,934 ± 3,242 × 10^3 in positive control animals treated with goat preimmune IgG (Figure 6B). Treatment of rats with either 5 or 50 µg of anti-IL-6 resulted in significant increases in BAL neutrophils (to 15,942 ± 2,007 × 10^3 and 27,080 ± 5,302 × 10^3, respectively; Figure 6B). Again, only at the 50-µg dose was the increase significant ($P < 0.05$).

Levels of TNF-α in BAL fluids under similar experimental conditions were determined. Negative control animals had low levels of TNF-α (2641 ± 512 pg/ml; Figure 6C). Injury initiated by IgG immune complex deposition was associated with a substantial increase in BAL TNF-α levels in positive controls treated with preimmune IgG (to 16,260 ± 3,355 pg/ml; Figure 6C), consistent with previous studies.[24] In animals treated with either 5 or 50 µg of anti-IL-6, there was an increase in BAL TNF-α (to 18,574 ± 1,342 pg/ml and 25,546 ± 1,918, respectively; Figure 6C), which correlated with increased neutrophils as described in Figure 6B. This finding reached statistical significance only at the 50-µg dose ($P < 0.05$).

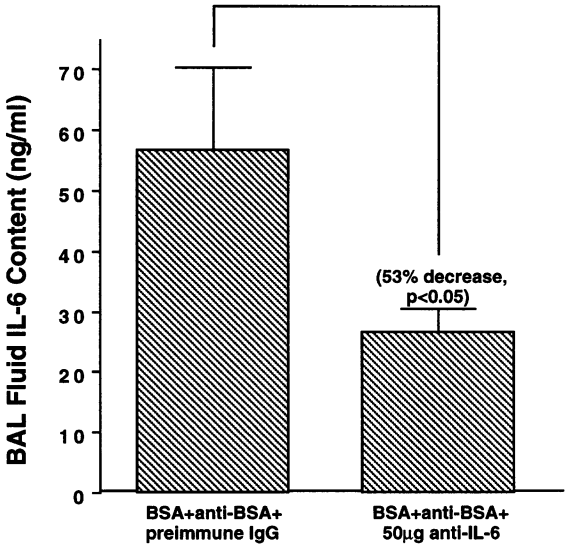

Figure 7. *BAL fluid IL-6 content as determined by bioassay after administration of 50 µg of anti-IL-6. Values are reported in ng/ml. n = 6 for both groups.*

Effect of Anti-IL-6 on BAL IL-6 Bioactivity

To determine whether these findings correlated with IL-6 neutralization, bioassays were performed on BAL fluids obtained from the rats treated with 50 µg of anti-IL-6. In the positive control rats receiving preimmune goat IgG, IL-6 levels were 56.8 ± 13.5 ng/ml (Figure 7). This value decreased to 26.6 ± 4.0 ng/ml (53% decrease; $P < 0.05$), confirming the ability of this antibody to neutralize endogenous rat IL-6 (Figure 7).

Discussion

IL-6 is a multifunctional cytokine that has often been identified in the setting of acute inflammation, especially in the context of causing liver production of acute phase reactants.[6–14] Despite these observations, the functional role of IL-6 in the setting of acute lung injury has not been fully characterized. We sought to determine whether IL-6 might function as a

regulatory cytokine during development of acute lung injury under the conditions described above. The present studies demonstrate that intrapulmonary up-regulation of IL-6 occurs at both the mRNA and protein levels during development of lung injury after IgG immune complex deposition. In this model, it is known that the inflammatory response is in some manner regulated, as vascular leakage of albumin and accumulation of neutrophils (as defined by myeloperoxidase content) rapidly increase between 2 and 4 hours and then show a decline, suggesting that the inflammatory response is somehow self-regulated. IL-6 may be an important regulatory cytokine expressed during acute inflammatory responses, at least under the experimental conditions described in this report. Our studies show that exogenously administered IL-6 has anti-inflammatory effects, whereas neutralization of intrinsic IL-6 *in vivo*, as defined by reductions in IL-6 biological activity, results in significant increases in lung permeability and BAL neutrophils. In the latter case, this augmentation of injury was companion to an increase in TNF-α activity found in the BAL fluids, suggesting that intrinsic IL-6 may be an important *in vivo* regulator of TNF-α production, which in turn leads to up-regulation of lung vascular ICAM-1 and E-selectin.

The observation that anti-IL-6 increased the levels of TNF-α in BAL fluids is compatible with the role of endogenous IL-6 to suppress production of TNF-α both *in vivo* (Figure 2C) and *in vitro* (Figure 3) and is consistent with other reports. *In vivo*, administration of IL-6 into the airways resulted in a 66% decrease in BAL TNF-α levels in response to intrapulmonary deposition of LPS.[15] *In vitro*, IL-6 treatment of either granulocyte/macrophage colony-stimulating factor (GM-CSF)-primed U937 cells or peripheral blood mononuclear cells reduced TNF-α production by 50 to 95% after LPS-induced stimulation.[18] In the same study, IL-6 treatment also resulted in reduced levels of serum TNF-α in mice challenged with LPS intraperitoneally.[18] Expression of the adhesion molecules E-selectin and ICAM-1 is required for full development of injury in the IgG immune complex model[28,29] and is dependent on the presence of TNF-α.[30] Therefore, increased TNF-α levels observed in the anti-IL-6-treated animals likely results in enhanced expression of vascular adhesion molecules and in increased emigration of neutrophils (quantitated as BAL neutrophils). Thus, the increased pulmonary vascular endothelial cell injury (reflected as increased permeability) in the current studies is likely the result of increased numbers of neutrophils and their consequent release of toxic oxygen radical species and proteases.

The results obtained in other animal models aimed at defining the functional role of IL-6 have also suggested an anti-inflammatory role. For example, IL-6 administered concurrently with either intrapulmonary[15] or intravenous[16] LPS in rats resulted in significant decreases in BAL neutrophils and TNF-α production similar to the finding in the present study. In a murine model of sepsis induced by LPS in the presence of D-galactosamine, IL-6 protected mice against mortality and antibody to IL-6 resulted in increased mortality, suggesting that IL-6 might play a protective role against septic shock.[31] In a model of murine hypersensitivity pneumonitis, treatment with a monoclonal anti-IL-6 antibody resulted in higher numbers of lung leukocytes and a greater degree of pulmonary fibrosis; in contrast, exogenously administered IL-6 decreased both white cell recruitment and the fibrotic response.[17] Finally, in a mouse model of staphylococcal enterotoxin-induced toxic shock, administration of anti-IL-6 increased mortality from 55 to 90% whereas pretreatment of mice with recombinant human IL-6 reduced mortality.[32] Thus, in studies aimed at defining a potential role for IL-6, anti-inflammatory properties have been observed.

The concept that IL-6 functions as an anti-inflammatory molecule might appear to be inconsistent with clinical findings in which increased levels of IL-6 are associated with clinical inflammatory states of ARDS,[6,7] sepsis,[8,9] trauma,[10–13] and infection.[14] In some instances, IL-6 levels have been shown to be reliable predictors of mortality.[7] Although these studies have described elevated IL-6 levels, there is frequently a concurrent increase in pro-inflammatory cytokines such as TNF-α, IL-1β, or IL-8. Thus, it is possible that these findings reflect an attempt by the host to induce homeostatic balance to a dysregulated pro-inflammatory response by increasing the production of an anti-inflammatory molecule such as IL-6.

Our findings are similar to those from recent studies aimed at defining the role of intrinsic IL-10 in the same model of lung injury.[33] IL-10 was demonstrated to be up-regulated during the course of development of injury, and the neutralization of intrinsically expressed IL-10 resulted in both increased lung permeability and BAL fluid neutrophils that was commensurate with an increase in BAL TNF-α. Taken together, these studies suggest that there may be a set of cytokines the function of which appears to be regulation of the acute inflammatory response, especially in the context of the lung. That

TNF-α can drive production of IL-6[34,35] suggests that there may be a negative feedback mechanism that operates to regulate expression of pro-inflammatory cytokines, especially TNF-α. The exact mechanisms and signaling pathways by which regulatory cytokines such as IL-4, IL-6, and IL-10 effect TNF-α expression in this model are the subject of ongoing studies.

IL-6 is also associated with the acute-phase response, and it has been suggested that IL-6 may drive the acute-phase response.[36–38] This response is characterized by production of a variety of plasma proteins including C-reactive protein, α_1-proteinase inhibitor, and cysteine proteinase inhibitor.[35,36] There are accumulating data to suggest that the acute-phase response provides protection from an acute inflammatory response. For example, the subcutaneous administration of turpentine, which induces an acute-phase response, protected mice from D-galactosamine/LPS-induced lethality.[39] Recently, it has been found that mice transgenically expressing human C-reactive protein were protected from a lethal infection with *Streptococcus pneumoniae*,[40] suggesting this protein may also possess anti-inflammatory properties. In the model of lung injury employed in the current studies, administration of proteinase inhibitors such as tissue inhibitor of metalloproteinases-2 (TIMP-2) and secretory leukoproteinase inhibitor (SLPI) have provided protection against the increased lung vascular permeability and neutrophil accumulation.[41] Thus, in addition to decreasing the production of TNF-α, IL-6 may induce acute-phase proteins the function of which may in part be anti-inflammatory. These results are compatible with the concept that, in IgG immune-complex-induced acute lung injury, IL-6 functions as an intrinsic down-regulator of inflammation and contribute to furthering our understanding of the role of IL-6 in acute lung inflammation.

The experimental design we chose to determine the role of IL-6 in this model of acute lung inflammation was by antibody neutralization as opposed to genetic knockout. Because of the multifunctional activities attributed to IL-6, we were concerned about the immunological development of an animal with IL-6 genetic deletion. We therefore tested our hypothesis in a genetically and immunologically intact animal. It is likely that employing IL-6 genetically deficient animals will also contribute to a greater understanding of the full role of IL-6 in inflammatory responses.

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