

# Chemokine Expression during Hepatic Ischemia/Reperfusion-induced Lung Injury in the Rat

## The Role of Epithelial Neutrophil Activating Protein

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### Abstract

The liver is highly susceptible to a number of pathological insults, including ischemia/reperfusion injury. One of the striking consequences of liver injury is the associated pulmonary dysfunction that may be related to the release of hepatic-derived cytokines. We have previously employed an animal model of hepatic ischemia/reperfusion injury, and demonstrated that this injury causes the production and release of hepatic-derived TNF, which mediates a neutrophil-dependent pulmonary microvascular injury. In this study, we have extended these previous observations to assess whether an interrelationship between TNF and the neutrophil chemoattractant/activating factor, epithelial neutrophil activating protein-78 (ENA-78), exists that may be accountable for the pathology of lung injury found in this model. In the context of hepatic ischemia/reperfusion injury, we demonstrated the following alterations in lung pathophysiology: (a) an increase in pulmonary microvascular permeability, lung neutrophil sequestration, and production of pulmonary-derived ENA-78; (b) passive immunization with neutralizing TNF antiserum resulted in a significant suppression of pulmonary-derived ENA-78; and (c) passive immunization with neutralizing ENA-78 antiserum resulted in a significant attenuation of pulmonary neutrophil sequestration and microvascular permeability similar to our previous studies with anti-TNF. These findings support the notion that pulmonary ENA-78 produced in response to hepatic-derived TNF is an important mediator of lung injury. (*J. Clin. Invest.* 1995. 95:134–141.) Key words: neutrophils • chemotaxins • adult respiratory distress syndrome • cytokines • liver

### Introduction

Ischemia/reperfusion injury is involved in the pathophysiology of many clinical disorders including myocardial infarction, stroke, mesenteric ischemia, peripheral vascular disease, organ

transplantation, and circulatory shock. This latter condition followed by resuscitation precipitates a systemic response resembling ischemia/reperfusion injury, and often leads to the adult respiratory distress syndrome and multiple organ system failure syndrome (1). Clinically, the liver is highly susceptible to hypovolemic shock, and since it has the largest fixed macrophage population in the body, the association of pulmonary dysfunction with liver injury may be related to the release of hepatic-derived inflammatory cytokines.

Our laboratory has previously employed an animal model of hepatic ischemia/reperfusion injury, and has demonstrated that hepatic ischemia/reperfusion injury causes the production and release of tumor necrosis factor- $\alpha$  (TNF), which in turn mediates a neutrophil-dependent microvascular lung injury (2, 3). While TNF was initially reported to be a neutrophil chemotaxin (4), recent studies have shown that recombinant TNF is not directly chemotactic for neutrophils (5, 6). These earlier findings suggest that cytokine networks may be operative in hepatic ischemia/reperfusion-induced lung injury, such that the initial expression of liver-derived early response cytokines, i.e., TNF, is followed by the generation of more distal neutrophil chemotactic/activating factors.

Recently a group of small proteins with specific neutrophil chemotactic properties, the C-X-C chemokines, have been isolated, cloned, and expressed. These chemokines share significant homology with the conservation of four cysteine amino acid residues, the first pair of cysteines separated by one amino acid residue (C-X-C) (7). This family includes neutrophil activating peptide-2 (NAP-2),<sup>1</sup> growth related oncogene (GRO)  $\alpha$ , GRO- $\beta$ , GRO- $\gamma$ , interleukin 8 (IL-8), and epithelial neutrophil activating protein (ENA-78) (8–11). This latter chemokine, ENA-78, has been found to be produced by variety of immune and nonimmune cells in response to TNF or interleukin 1 (IL-1) (12). Thus, both immune and nonimmune cells of the lung may be involved in cytokine cascades with the end result being the expression of appropriate chemokines that enhance neutrophil-dependent inflammatory responses.

We postulated that ENA-78 may account for neutrophil recruitment and extravasation into the lung following hepatic ischemia/reperfusion, resulting in acute pulmonary microvascular injury. In this study, we demonstrate the following: (a) hepatic ischemia/reperfusion injury leads to increased pulmonary mi-

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Received for publication 18 October 1993 and in revised form 1 September 1994.

*J. Clin. Invest.*

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0021-9738/95/01/0134/08 \$2.00

Volume 95, January 1995, 134–141

1. Abbreviations used in this paper: ACM, alveolar-capillary membrane; ENA-78, epithelial neutrophil activating protein-78; GRO, growth-related oncogene; HPF, high power field; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; MPO, myeloperoxidase; NAP, neutrophil activating protein.

crovascular permeability, neutrophil sequestration, and production of ENA-78; (b) neutralization of TNF by passive immunization in the context of hepatic ischemia/reperfusion injury results in the attenuation of the production of pulmonary-derived ENA-78; and (c) neutralization of ENA-78 by passive immunization in the context of hepatic ischemia/reperfusion injury results in significant reduction of pulmonary microvascular permeability and neutrophil sequestration. These findings support the notion that ENA-78 produced in response to hepatic-derived TNF is an important mediator of pulmonary microvascular injury and neutrophil sequestration.

## Methods

### *Hepatic ischemia/reperfusion injury model*

Adult male specific pathogen-free Sprague-Dawley rats (Charles River Laboratories, Portage, MI) weighing 250–300 g were used in all experiments. A rat model of lobar, rather than total, hepatic ischemia/reperfusion was used as previously described (2, 3, 13). This model induces a severe ischemic insult to the liver without inducing mesenteric venous hypertension and subsequent bacterial translocation into the portal venous blood (2, 14). Briefly, anesthesia was induced with intramuscular ketamine hydrochloride (100 mg/kg) and maintained with inhalation of methoxyflurane. After intravenous heparinization (200 United States Pharmacopeia U/kg), midline laparotomy was performed and an atraumatic microaneurysm (Heifitz; W. Lorenz, Jacksonville, FL) clip was used to interrupt the portal venous and hepatic arterial blood supply to the cephalad three lobes of the liver. The three caudal lobes retain an intact portal and arterial inflow and venous outflow, preventing intestinal venous congestion. Hepatic ischemia was maintained for 90 min; the Heifitz clip was then removed at a second laparotomy, initiating reperfusion. Previous experiments in our laboratory have demonstrated that 90 min of lobar hepatic ischemia followed by reperfusion induces a severe, isolated, and reproducible liver and associated lung injury without excessive animal mortality at 12 h of reperfusion (2, 3). However, mortality was 33% at 24 h (2, 3). Intravenous lactated Ringer's solution (40 ml/kg) was administered at the conclusion of the second laparotomy to replace operative fluid and blood losses. Sham-operated control animals were treated in an identical fashion with the omission of vascular occlusion. We have previously demonstrated that animals remain normotensive throughout this procedure and that there is no portal venous endotoxemia (2). Animals were killed at specified times and plasma and tissue processed as described below.

### *Peripheral blood and tissue procurement*

At the time of killing, anticoagulated blood (heparin 50 United States Pharmacopeia U/ml) was obtained via the animal's right ventricle. Plasma was removed after the blood was centrifuged at 2,000 g for 10 min and stored at  $-70^{\circ}\text{C}$  for later ENA-78 analysis. The lungs were perfused free of blood with 20 ml of sterile 0.9% normal saline infused via the spontaneously beating right ventricle. The lungs were then removed from the thoracic cavity and separated from the surrounding mediastinal structures. The right lung was homogenized in 5 ml of PBS, pH 7.5, on ice, using a tissue homogenizer (Polytron; Brinkmann Instruments, Westbury, NY). The lung homogenate was centrifuged at 2,000 g for 15 min at  $4^{\circ}\text{C}$ . The aqueous extract was then filtered through a 1.2- $\mu\text{m}$  filter (Gelman Inc., Ann Arbor, MI) and stored at  $-70^{\circ}\text{C}$  for later ENA-78 analysis.

### *ENA-78 ELISA*

Immunoreactive ENA-78 was quantitated using a modification of the double ligand method as previously described (12, 15). Briefly, flat bottomed 96-well microtiter plates (plate I 96-F; Nunc Immuno, Denmark, Netherlands) were coated with 50  $\mu\text{l}$ /well of rabbit anti-ENA-78 antibody (1  $\mu\text{g}/\text{ml}$  in 0.6 M NaCl, 0.26 M  $\text{H}_3\text{BO}_4$ , and 0.08 N NaOH, pH 9.6), incubated for 16 h at  $4^{\circ}\text{C}$ , and then washed with PBS, pH 7.5,

0.05% Tween-20 (wash buffer). Microtiter plate nonspecific binding sites were blocked with 2% BSA in PBS and incubated for 90 min at  $37^{\circ}\text{C}$ . Plates were rinsed four times with wash buffer and diluted (1:2 and 1:10) plasma or tissue aqueous extracts (50  $\mu\text{l}$ ) in duplicate were added, followed by incubation for 1 h at  $37^{\circ}\text{C}$ . Plates were washed four times, followed by the addition of 50  $\mu\text{l}$ /well biotinylated rabbit anti-ENA-78 antibody (3.5  $\mu\text{g}/\text{ml}$  in PBS, pH 7.5, 0.05% Tween-20, and 2% fetal calf serum), and plates were then incubated for 30 min at  $37^{\circ}\text{C}$ . Plates were washed four times, streptavidin-peroxidase conjugate (Bio Rad Laboratories, Richmond, CA) was added, and the plates were incubated for 30 min at  $37^{\circ}\text{C}$ . Plates were again washed four times and chromogen substrate (orthophenylenediamine; Bio Rad Laboratories) was added. The plates were then incubated at room temperature to the desired extinction, the reaction terminated with 50  $\mu\text{l}$ /well of 3 M  $\text{H}_2\text{SO}_4$  solution, and the plates were read at 490 nm in an ELISA reader. Standards were 1/2 log dilutions of recombinant ENA-78 protein from 1 pg/ml to 100 ng/ml. This ELISA method consistently detected ENA-78 concentrations above 10 pg/ml (12), and did not cross-react with other cytokines, including murine and human IL-1 alpha and beta, murine and human TNF alpha and beta, murine and human IL-2, murine and human IL-4, murine and human IL-6, human IL-7, human IL-8, murine and human monocyte chemoattractant protein (MCP-1) murine and human macrophage inflammatory protein (MIP-1) alpha and beta, murine and human MIP-2, murine, rat, and human GRO alpha, human NAP-2, human CTAP-III, human beta-thromboglobulin, human regulated upon activation, normal T cell expressed and presumably secreted (RANTES), human platelet factor-4, human gamma IP-10, and TGF beta. The concentration of ENA-78 antigen in lung tissue homogenates was standardized to the total protein content as measured by the BCA protein assay reagent (Pierce, Rockford, IL).

### *ENA-78 immunohistochemistry*

Pulmonary tissue immunohistochemical analysis was performed using a modification of a previously described technique (16). Briefly, lungs were fixed overnight in 4% paraformaldehyde in PBS and transferred to 70% ethanol until embedded in paraffin. Three micron thick paraffin sections (10 total) of left lung were prepared and mounted on poly-L-lysine-coated glass slides. The slides were deparaffinized in xylene, rehydrated in graded ethanol and PBS, and blocked with 1:50 normal goat serum for 30 min at  $37^{\circ}\text{C}$ , followed by exposure to optimal dilutions of rabbit anti-ENA-78 antibody or similar dilutions of control sera. After a 20-min incubation at  $37^{\circ}\text{C}$ , the slides were rinsed with PBS, overlaid with goat anti-rabbit biotinylated secondary antibodies (1:100; Vector Laboratories, Burlingame, CA), incubated for 30 min at  $37^{\circ}\text{C}$ , and rinsed with PBS. At this point, sections were then treated with conjugated streptavidin-horseradish peroxidase (Vector Laboratories) and incubated for 30 min at room temperature. The slides were then overlaid with substrate chromogen (3,3'-diaminobenzidine; Vector Laboratories), incubated for 10 min at room temperature to allow for color development, and rinsed with distilled water to stop the reaction. Mayer's hematoxylin was used as a counterstain.

### *Isolation and RT-PCR amplification of whole-lung mRNA*

The right lower lobe of lungs from animals subjected to hepatic ischemia and 2 h of reperfusion or sham laparotomy were snap frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$  until further analyzed by RT-PCR as previously described (17). Briefly, total cellular RNA from the lungs was isolated by homogenizing the lungs with a tissue homogenizer in a solution containing 25 mM Tris, pH 8.0, 4.2 M guanidine isothiocyanate, 0.5% sarkosyl, and 0.1 M 2-mercaptoethanol. After homogenization, the suspension was added to a solution containing an equal volume of 100 mM Tris, pH 8.0, 10 mM EDTA, and 1.0% SDS. The mixture was then extracted two times each with phenol-chloroform and chloroform-isoamyl alcohol. The RNA was alcohol precipitated and the pellet dissolved in diethyl pyrocarbonate water (Sigma Immunochemicals, St. Louis, MO). Total RNA was determined by spectrophotometric analysis at 260 nm wavelength. 5  $\mu\text{g}$  of total RNA was reverse transcribed into cDNA using an RT kit (GIBCO BRL, Gaithersburg, MD) and oligo

(dT)12–18 primers. The cDNA was then amplified using specific primers for cyclophilin as a control and ENA-78. The primers used were ENA-78 sense: 5'-AAT-CTG-CAA-GTG-TTC-GCC-ATA-GGC-CCA-CAG-TGC-3'; ENA-78 anti-sense: 5'-GCA-CTG-TGG-GCC-TAT-GGC-GAA-CAC-TTG-CAG-ATT-3'; cyclophilin sense: 5'-CAT-CTG-CAC-TGC-CAA-GAC-TG-3'; cyclophilin anti-sense: 5'-CTG-CAA-TCC-AGC-TAG-GCA-TG-3'. The amplification buffer contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), and 2.4 mM MgCl. The specific oligonucleotide primer was added (200 ng/sample) to the buffer, along with 1  $\mu$ l of the reverse transcribed cDNA samples. The cDNA was amplified after determining the optimal number of cycles. The mixture was first incubated for 5 min at 94°C, and then cycled 27 times at 95°C for 30 s, 58°C for 45 s, and elongated at 72°C for 75 s. This format allowed optimal amplification with little or no nonspecific amplification of contaminating DNA. After amplification, the sample (20  $\mu$ l) was separated on a 2% agarose gel containing 0.3  $\mu$ g/ml (0.003%) of ethidium bromide, and bands were visualized and photographed using ultraviolet transillumination.

### Lung permeability analysis

Pulmonary microvascular permeability was measured using a modification of the Evans blue dye extravasation technique as previously described (18–20). Extravasation of Evans blue (Sigma Immunochemicals) into the extravascular pulmonary tissue compartment was used as a quantitative measure of changes in pulmonary microvascular permeability. Briefly, animals received 20 mg/kg of Evans blue (pH 7.34) by tail vein injection 3 h before they were killed. At the time of killing, a heparinized sample of blood was taken from the right ventricle and plasma removed by centrifugation. The lungs were then perfused free of blood with 20 ml of 0.9% normal saline. The lungs were removed from the thoracic cavity and surrounding mediastinal structures and weighed. Evans blue was extracted from pulmonary tissues after homogenization in 3 ml of 0.9% normal saline. This volume was added to 2 vol of deionized formamide and incubated at 60°C for 12 h. The supernatant was separated by centrifugation at 2,000 g for 30 min. Evans blue in the plasma and lung tissue was quantitated by dual wavelength spectrophotometric analysis as described by Linderkamp et al. (19). This method corrects the specimen's absorbance at 620 nm for the absorbance of contaminating heme pigments, and is calculated by the following formula: corrected absorbance at 620 nm = actual absorbance at 620 nm - [1.426(absorbance at 740 nm) + 0.03]. The measured Evans blue in the pulmonary tissues was then normalized to tissue weight. A permeability index (PI) was calculated by dividing the corrected pulmonary tissue Evans blue absorbance at 620 nm/g of lung tissue by the corrected plasma Evans blue absorbance at 620 nm, and reflects the degree of extravasation of Evans blue into the extravascular pulmonary tissue compartment.

### Pulmonary neutrophil sequestration

Pulmonary neutrophil infiltration was quantitated using both a myeloperoxidase (MPO) assay and pulmonary tissue morphometric analysis.

**Myeloperoxidase assay.** The MPO assay was performed as previously described (21). Briefly, at the time the animals were killed the lungs were perfused free of blood with 20 ml of 0.9% normal saline via the spontaneously beating right ventricle. The left lung was excised and placed in a 50-mM potassium phosphate buffer solution (pH 6.0) with 5% hexadecyltrimethyl ammonium bromide (Sigma Immunochemicals). The pulmonary tissue was homogenized, sonicated, and centrifuged at 12,000 g for 15 min at 4°C. The supernatant was then assayed for MPO activity using a spectrophotometric reaction with *O*-dianisidine hydrochloride (Sigma Immunochemicals) at 460 nm.

**Pulmonary tissue neutrophil morphometrics.** Morphometric analysis was performed using a modification of a previously described technique (22). Briefly, the right lung was inflated and fixed with 4% glutaraldehyde buffered with 0.1 M cacodylate (pH 7.4) and postfixed in 2% OsO<sub>4</sub> in 0.1 M cacodylate (pH 7.4) for 1 h at 4°C. The specimens were then dehydrated in graded ethanol, infiltrated with increasing mixtures of epon/propylene oxide, and embedded in epon. 1- $\mu$ m plastic sections

were stained with 2% toluidine blue and tissue morphometrics were then performed using light microscopy (22). Five random 1- $\mu$ m plastic embedded sections were obtained from each of three animals and stained with 2% toluidine blue. Each tissue section was examined by light microscopy with a total of 100 high power fields (HPF;  $\times$ 400) viewed. All neutrophils (PMN) were counted and the results expressed as mean PMN per HPF.

### Passive immunization and neutralization of TNF or ENA-78

The effects of neutralization of TNF or ENA-78 in our model were assessed by the administration of a polyclonal rabbit anti-murine TNF or a polyclonal rabbit anti-human ENA-78. The TNF antiserum neutralizes both natural and recombinant TNF, cross-reacts with rat TNF, and has no cross-reactivity with interleukin 1 (23). The ENA-78 antiserum neutralizes both natural and recombinant ENA-78, cross-reacts with rat ENA-78, and has no cross-reactivity with other members of the C-X-C or C-C chemokine family (12). A dilution of 1:1000 of ENA-78 antiserum neutralizes 30 ng/ml of recombinant human ENA-78 in an *in vitro* neutrophil chemotaxis assay. A total of 1 ml of control rabbit serum or high titer (1:10<sup>6</sup>) neutralizing anti-TNF or anti-ENA-78 serum was administered intravenously to each animal immediately before induction of hepatic ischemia.

### Statistical analysis

The *in vivo* studies involved a minimum of five rats or as otherwise specified at each time point or for each manipulation. Groups of data were evaluated by analysis of variance to indicate groups with significant differences. The Bonferroni procedure was used for the comparison of the means of multiple groups. Data that appeared statistically significant were further compared by *t* test, and were considered significant if *P* values were less than 0.05. Results are presented as means  $\pm$  SEM. Data were analyzed by Macintosh IIfx computer using Statview II or 4.01 statistical software package (Abacus Concepts, Inc. Berkeley, CA).

## Results

**Time course and immunolocalization of pulmonary ENA-78 after hepatic ischemia/reperfusion injury.** Using a rat model of lobar hepatic ischemia/reperfusion, we have previously identified a specific neutrophil-mediated pulmonary injury that is associated with the release of hepatic-derived TNF (2, 3, 13). Since TNF does not have direct neutrophil chemotactic/activating properties (5, 6), we were interested in whether ENA-78 produced in the context of hepatic ischemia/reperfusion could account for the neutrophil-mediated lung injury. Using our lobar hepatic ischemia/reperfusion model, animals were subjected to 90 min of hepatic ischemia followed by reperfusion. Hepatic ischemia/reperfusion animals and sham-operated control animals were killed at 1, 6, 12, and 24 h after hepatic reperfusion or after laparotomy, respectively. Hepatic ischemia/reperfusion-injured animals demonstrated significantly elevated plasma levels of ENA-78, as compared to sham controls, at both 12 h and 24 h post-hepatic reperfusion (0.3  $\pm$  0.03 ng/ml vs 0.1  $\pm$  0.02 ng/ml at 1 h, 0.6  $\pm$  0.6 ng/ml vs 1.9  $\pm$  1.1 at 6 h, 5.1  $\pm$  0.6 ng/ml vs 3.6  $\pm$  0.3 ng/ml at 12 h, and 4.4  $\pm$  1.8 ng/ml vs 2.6  $\pm$  0.4 ng/ml at 24 h). Before the 12 h time-point, ENA-78 plasma levels were not statistically different between experimental and control animals. In contrast, pulmonary tissue ENA-78 levels from hepatic ischemia/reperfusion animals were significantly elevated as early as 1 h after reperfusion (1.4  $\pm$  0.6 ng/mg total protein), rising to 6.1  $\pm$  1.5 ng/mg total protein by 6 h of reperfusion, plateauing at 12 h at 5.23  $\pm$  1.13 ng/mg total protein, and peaking at 8.0  $\pm$  2.6 ng/mg total protein by 24 h of reperfusion (Fig. 1). By contrast, ENA-78 levels from the lungs of sham-operated

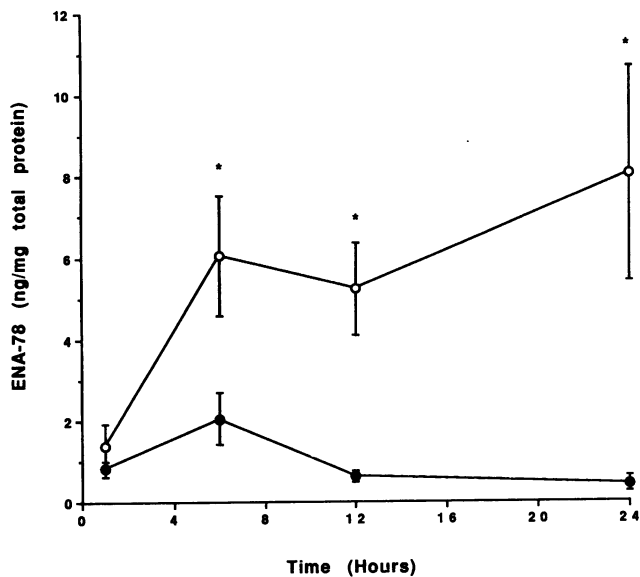


Figure 1. Pulmonary ENA-78 kinetics after hepatic ischemia/reperfusion. Pulmonary ENA-78 antigen levels become significantly elevated after 6 h of hepatic reperfusion as compared to sham-operated control animals, and remain significantly elevated at 24 h of hepatic reperfusion (\* $P < 0.005$ ). —○—, I/R; —●—, Sham.

control animals peaked at  $2.0 \pm 0.6$  ng/mg total protein at 6 h and declined to  $0.4 \pm 0.2$  ng/mg total protein by 24 h. These findings were interesting in light of our previous demonstration of peak TNF levels in the plasma of these animals within 30 min of hepatic reperfusion and a gradual decline to a nadir at 4 h after hepatic reperfusion (2).

We next determined the potential cellular source of ENA-78 using immunohistochemical localization. Immunolocalization of ENA-78 from the lungs of animals 6 h after hepatic reperfusion and 6 h after sham laparotomy are shown in Fig. 2, C and A, respectively, while Fig. 2, B and D are representative photomicrographs of lung sections treated with control antibodies. Interestingly, ENA-78 protein appeared to be diffusely localized to the entire alveolar-capillary membrane (ACM) in the lungs of animals that had undergone hepatic ischemia/reperfusion injury (Fig. 2 C). Sham-operated control lungs failed to demonstrate significant immunolocalization of ENA-78 protein (Fig. 2 A). Immunolocalization of ENA-78 to the cellular constituents of the ACM is not only consistent with staining of specific cells, but could also be consistent with immunolocalization of ENA-78 to the extracellular matrix components of the ACM. Preabsorption of anti-ENA-78 antibodies to recombinant human ENA-78 protein before exposure to lung tissue completely attenuated the immunolocalization of the anti-ENA-78 antibodies to the lungs of animals that had undergone hepatic ischemia/reperfusion injury (data not shown).

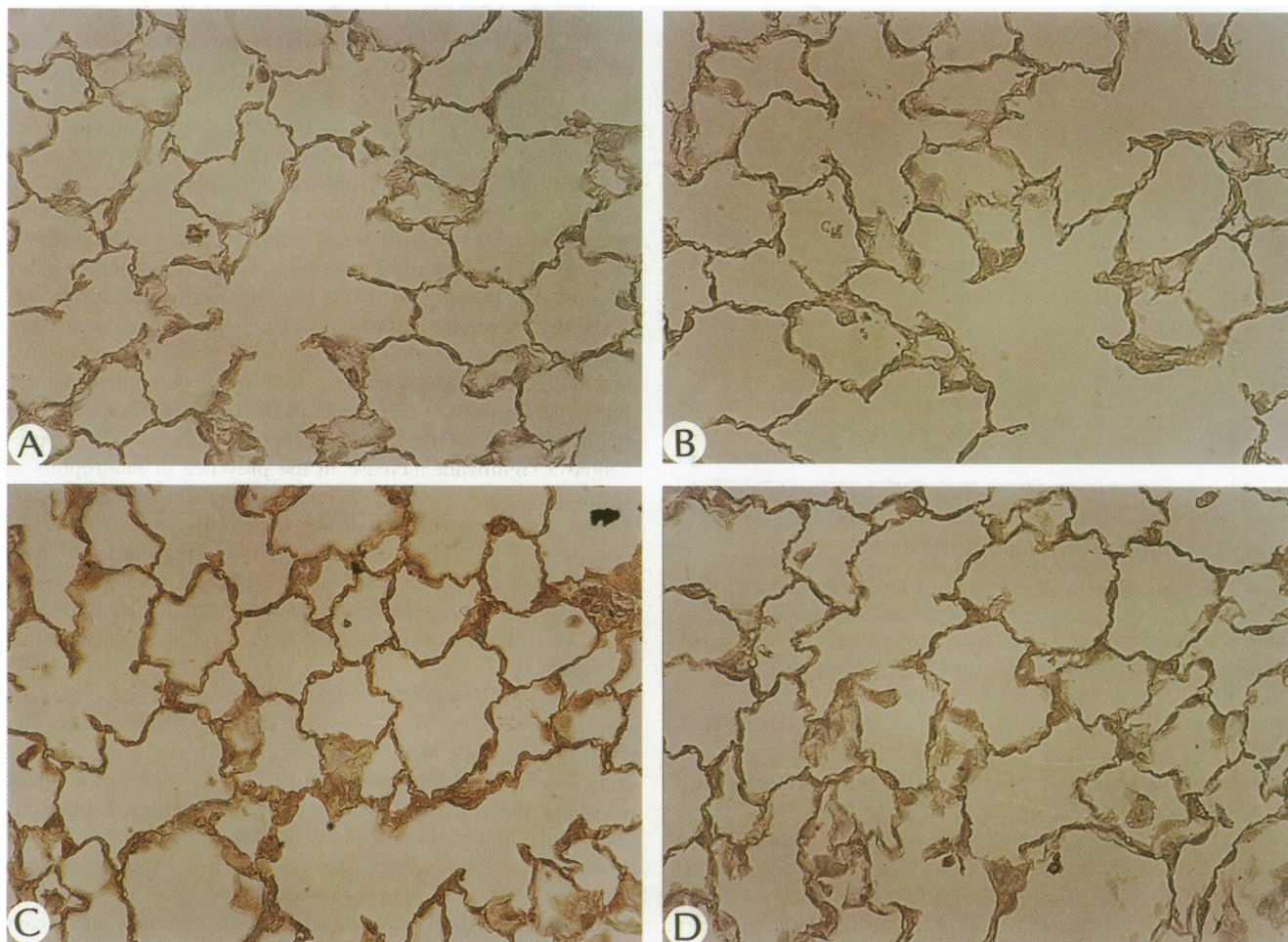
To further establish that ENA-78 was lung derived, lungs from animals subjected to hepatic ischemia followed by 2 h of reperfusion or sham laparotomy were snap frozen and total RNA isolated and subjected to RT-PCR for the detection of ENA-78 mRNA (Fig. 3). Lanes 1 and 4 are control (cyclophilin mRNA) and ENA-78 mRNA, respectively, from the lung of an animal subjected to hepatic ischemia followed by 2 h of reperfusion, while lanes 2 and 5 are control (cyclophilin mRNA) and ENA-78 mRNA, respectively, from the lung of

an animal subjected to sham laparotomy. ENA-78 mRNA was significantly expressed in the animal subjected to hepatic ischemia followed by 2 h of reperfusion. These data support that ENA-78 is generated in the lung.

*Lung injury and pulmonary neutrophil infiltration after hepatic ischemia/reperfusion injury.* 12 h after reperfusion the permeability index (PI) from the lungs of animals that had undergone hepatic ischemia/reperfusion was significantly greater ( $1.4 \pm 0.16$ ) than lungs from sham operated animals ( $0.8 \pm 0.1$ ), representing a 1.75-fold greater permeability index (Fig. 4 A). In addition, MPO activity from these lungs was markedly elevated ( $328 \pm 55$  mOD/min per g tissue) as compared to sham laparotomy animals ( $120 \pm 45$  mOD/min per tissue) (Fig. 4 B). Morphometric analysis of pulmonary neutrophils demonstrated  $3.26 \pm 0.2$  PMN/HPF, as compared to  $0.70 \pm 0.08$  PMN/HPF from sham-operated animals. This represented a significant increase in the presence of neutrophils in the lungs of animals that had undergone hepatic ischemia/reperfusion injury coincident with the increased presence of ENA-78 (Fig. 4 C). While this degree of lung injury did not result in excessive animal mortality at 12 h of reperfusion, in experiments in which the reperfusion period was lengthened to 24 h, 4 of 10 animals expired at 24 h with a resultant 40% animal mortality.

*Neutralization of TNF attenuates the production of pulmonary-derived ENA-78.* In previous studies (2), we found that TNF may mediate significant pathophysiologic changes in the lung of animals undergoing hepatic ischemia/reperfusion injury (2). The presence of TNF was associated with a neutrophil-mediated pulmonary microvascular injury, similar to our current findings (2). Similar experiments in the current study confirmed these findings. Treatment with neutralizing anti-TNF serum before the initiation of hepatic ischemia resulted in a significant reduction in pulmonary neutrophil sequestration as measured by lung MPO levels. MPO levels in animals passively immunized with neutralizing anti-TNF serum and subjected to 12 h of reperfusion were  $210.7 \pm 9$  mOD/min per g of tissue, as compared to  $350.4 \pm 20.3$  mOD/min per g of tissue in animals treated with control serum ( $P < 0.001$ ). We postulated that TNF may act as an early response cytokine, leading to the production of pulmonary-derived ENA-78. To test this hypothesis, we passively immunized rats with either control rabbit serum or neutralizing anti-TNF serum immediately before hepatic ischemia and assessed pulmonary ENA-78 levels 6 h after hepatic reperfusion. Animals treated with control serum demonstrated elevated levels of ENA-78 ( $4.7 \pm 0.1$  ng/mg total protein), as compared to animals passively immunized with neutralizing TNF antibodies ( $2.3 \pm 0.4$  ng/mg total protein), whereas sham-operated animal lungs demonstrated only  $1.3 \pm 0.1$  ng/mg total protein of ENA-78 (Fig. 5). These results suggested that TNF had a significant impact in the lung with respect to the induction of pulmonary-derived ENA-78, as neutralization of TNF resulted in a twofold reduction in pulmonary ENA-78.

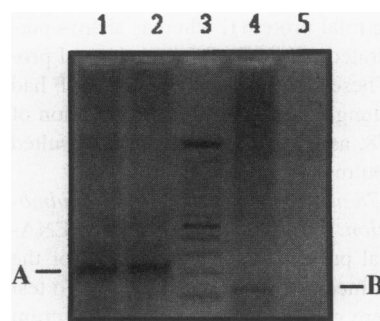
*Neutralization of ENA-78 attenuates lung injury and pulmonary neutrophil sequestration.* We next postulated that ENA-78 may act as a more distal proinflammatory cytokine for the induction of this neutrophil-mediated pulmonary injury. To test this hypothesis, animals were given either control rabbit serum or neutralizing anti-ENA-78 serum immediately before hepatic ischemia. The pulmonary effects of neutralizing ENA-78 are presented in Fig. 6. Fig. 6, A, B, and C are the histopathological changes in the lungs of sham-operated controls, hepatic isch-



**Figure 2.** Pulmonary tissue ENA-78 immunohistochemistry. *A* and *C* demonstrate immunolocalization of ENA-78 protein in the lungs of animals 6 h after sham laparotomy and hepatic reperfusion, respectively. Staining of the cellular constituents of the alveolar capillary membrane is demonstrated in *C*; no staining is evident in *A*. *B* and *D* are representative photomicrographs of lung sections treated with control antibodies from animals 6 h after sham laparotomy and hepatic reperfusion, respectively. These showed no evidence of nonspecific staining. ( $\times 400$ ).

emia/reperfusion animals who had received control serum, and hepatic ischemia/reperfusion animals who were passively immunized with neutralizing antibodies to ENA-78, respectively. Lung permeability, MPO activity, and neutrophil morphometric analysis were also assessed after 12 h of hepatic reperfusion. As

shown in Fig. 7 *A*, neutralizing anti-ENA-78 serum significantly attenuated (2.7-fold) the lung permeability index from  $2.54 \pm 0.45$  to  $0.94 \pm 0.07$ , equivalent to sham-operated animal lungs. In a comparable manner, MPO activity ( $344.5 \pm 20$  mOD/min per g tissue to  $260 \pm 9.7$  mOD/min per g tissue) and neutrophil morphometrical analysis ( $3.3 \pm 0.25$  PMN/HPF to  $1.7 \pm 0.16$  PMN/HPF) demonstrated similar changes in response to neutralizing anti-ENA-78 serum (Fig. 7 *B* and *C*, respectively). Interestingly, neutralizing anti-ENA-78 serum failed to attenuate MPO activity measured in the lungs at 1 h after hepatic reperfusion (data not shown). These findings support the notion that pulmonary-derived ENA-78 produced in the context of hepatic ischemia/reperfusion injury is a major mediator of subsequent lung injury.

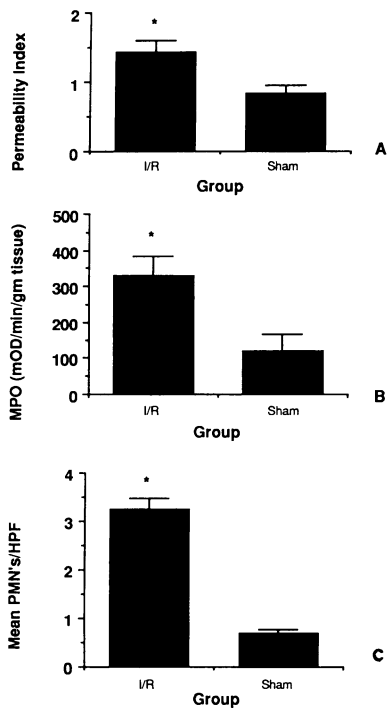


**Figure 3.** Expression of ENA-78 mRNA by RT-PCR. A representative photograph (negative) of RT-PCR of control (cyclophilin mRNA) and ENA-78. Lanes 1 and 2 are of cyclophilin cDNA (mRNA) corresponding to lanes 4 and 5 of ENA-78 cDNA (mRNA). Lane 3 is the DNA molecular mass

markers at increments of 100 bases, beginning at 200 bases. *A* points to the DNA band for cyclophilin with a molecular mass of approximately 300 bases. *B* points to the DNA band for ENA-78 at the predicted level of 205 bases.

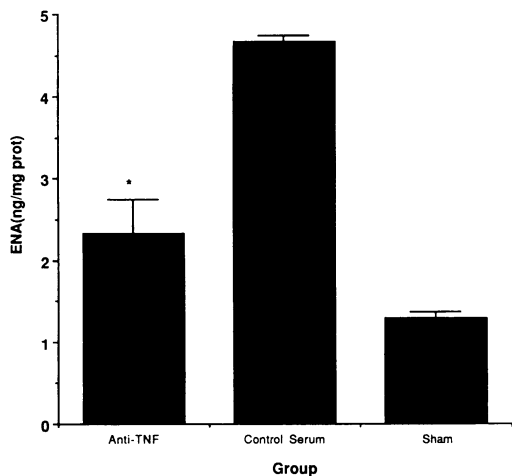
## Discussion

One of the salient features of acute pulmonary microvascular injury precipitated by hepatic ischemia/reperfusion appears to be related to pulmonary neutrophil sequestration (2, 3). Activated neutrophils are one of the primary effector cells mediating the development of adult respiratory distress syndrome and mul-

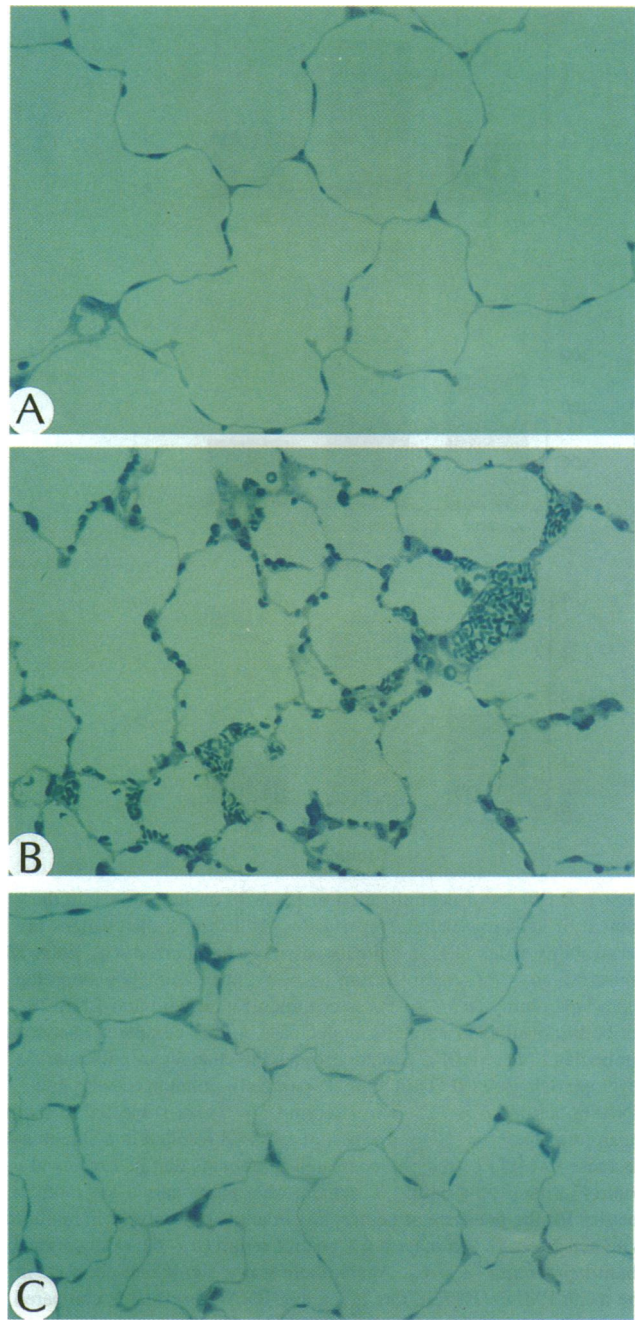


**Figure 4.** Lung injury and neutrophil sequestration after hepatic ischemia and 12 h of reperfusion. **A** illustrates pulmonary permeability index (PI) as measured by extravasation of Evans blue. Animals undergoing hepatic ischemia/reperfusion ( $n = 7$ ) had a significantly increased PI as compared to sham operated control animals ( $n = 7$ ,  $*P < 0.005$ ). **B** demonstrates pulmonary myeloperoxidase (MPO) levels in animals subjected to hepatic ischemia/reperfusion as compared to sham-operated control animals ( $n = 5$  in both groups,  $*P < 0.05$ ). **C** demonstrates pulmonary tissue morphometrics for the presence of neutrophils from animals subjected to hepatic ischemia/reperfusion versus sham laparotomy ( $*P < 0.001$ ).

multiple organ system failure syndrome, regardless of the precipitating event (5, 24–26). Activated neutrophils release a variety of inflammatory mediators, including proteolytic enzymes, arachidonic acid metabolites, reactive oxygen species, and cytokines; these mediators can impact directly on the pulmonary microvasculature, leading to increased pulmonary microvascu-

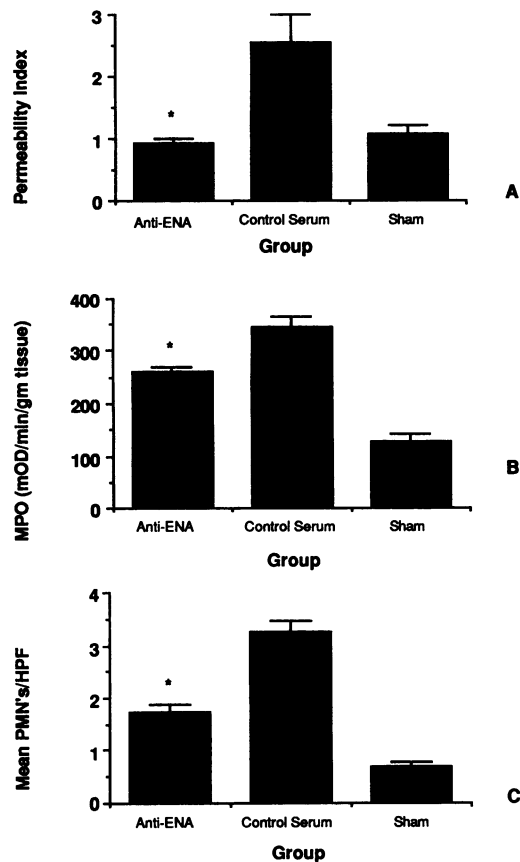


**Figure 5.** The effect of neutralization of TNF on pulmonary ENA-78 levels after 6 h of hepatic reperfusion. Pulmonary ENA-78 levels were significantly attenuated in animals passively immunized with rabbit anti-TNF serum before the initiation of hepatic ischemia/reperfusion ( $n = 3$ ) as compared to animals receiving control serum ( $n = 3$ ,  $*P < 0.005$ ). “Sham” ( $n = 3$ ) is the sham-operated animals that did not receive either the control or neutralizing rabbit anti-TNF sera.



**Figure 6.** Photomicrograph of the effects of neutralization of ENA-78 on hepatic ischemia/reperfusion-induced lung injury. **A**, **B**, and **C** show the histopathologic changes in the lungs of sham-operated controls, hepatic ischemia/reperfusion animals who had received control serum, and hepatic ischemia/reperfusion animals who were passively immunized with neutralizing antibodies to ENA-78, respectively ( $1 \mu\text{m}$  sections stained with toluidine blue,  $\times 400$ ).

lar permeability, pulmonary hemorrhage, and increased leukocyte migration into the pulmonary interstitium (5, 24, 26–28). Our laboratory has previously used a rat model of lobar hepatic ischemia/reperfusion and has identified pathophysiologic events in the lung that were associated with the release of hepatic-derived TNF (2, 3). These findings were unassociated with translocation of bacterial or circulating portal venous endotoxin (2). While these investigations were important in estab-



**Figure 7.** Effects of neutralization of ENA-78 on lung injury in the context of hepatic ischemia/reperfusion. **A** illustrates the changes in permeability index (PI) in animals passively immunized with ENA-78 antiserum ( $n = 6$ ), control serum ( $n = 7$ ), or in animals undergoing sham laparotomy ( $n = 6$ ). Passive immunization with anti-ENA-78 antibodies resulted in a significant decrease in PI as compared to control antibodies ( $*P < 0.01$ ). **B** demonstrates the changes in pulmonary myeloperoxidase (MPO) in animals passively immunized with anti-ENA-78 serum ( $n = 7$ ), control serum ( $n = 7$ ), or subjected to sham laparotomy ( $n = 6$ ). Neutralization of ENA-78 resulted in a significant decrease in MPO activity as compared to animals that had received control serum ( $*P < 0.005$ ). **C** demonstrates pulmonary tissue morphometrics for the presence of neutrophils in animals passively immunized with anti-ENA-78 serum ( $n = 6$ ), control serum ( $n = 6$ ), or undergoing sham laparotomy ( $n = 6$ ). Again, there was a significant decrease in the mean PMN's/HPF in rats given anti-ENA-78 serum as compared to rats given control serum ( $P < 0.001$ ).

lishing the relationship of hepatic ischemia/reperfusion injury and development of lung injury, perhaps the most intriguing result was the finding that TNF is a major proximal mediator in this event (2, 3). The early elevation in plasma TNF correlated with the magnitude of lung MPO activity and microvascular permeability (2, 3).

Although TNF was initially reported to be a neutrophil chemotactic/activating factor (4), recent studies have failed to find a direct neutrophil chemotactic effect of TNF (5, 6). This observation would suggest that potential cytokine networks are operative *in vivo* and are dependent upon the initial generation of TNF, followed by the production of additional cytokines that may influence neutrophil chemotaxis/activation. This hypothesis has been substantiated with the discovery of a superfamily

of small proteins with specific neutrophil chemotactic properties (7).

These chemotactic cytokines, or chemokines, share significant homology with a family of polypeptides that possess four conserved cysteine amino acid residues, the first pair of cysteines separated by one amino acid residue (C-X-C) (7). In general, this family has potent neutrophil chemotactic/activating properties, such as the induction of the respiratory burst, exocytosis, generation of arachidonic acid metabolites, shedding of L-selectin, and expression of  $\beta_2$ -integrins (CD11b/CD18 complex) (7). The chemokines with specific neutrophil activating properties include NAP-2, GRO-alpha, GRO-beta, GRO-gamma, IL-8, and ENA-78 (8–11). This latter chemokine *in vitro* has been found to be produced by a number of immune and nonimmune cells, including endothelial cells, neutrophils, monocytes, pulmonary epithelial cells, and pulmonary fibroblasts in response to TNF or IL-1 (8, 12). These previous findings support the concept that both immune and nonimmune cells of the lung have the capacity to produce chemokines that contribute to the pathogenesis of pulmonary injury via the elicitation and activation of neutrophils.

In our current investigation, we have extended our previous studies of hepatic ischemia/reperfusion-induced lung injury and determined whether an interrelationship of TNF and ENA-78 exists that could account for the development of lung injury. We found a time-dependent production of pulmonary-derived ENA-78, in the context of hepatic ischemia/reperfusion injury, that was significantly expressed during significant pulmonary microvascular permeability and neutrophil sequestration. Immunolocalization of ENA-78 within the lung was associated with a staining pattern that included cells of the entire ACM. This finding was intriguing and suggested that all of the cellular constituents of the ACM could express ENA-78. However, this pattern of immunolocalization could also have been explained by the presence of ENA-78 bound to the extracellular matrix of the ACM. ENA-78, similar to other members of the C-X-C chemokine family can bind to heparin and, *in vivo*, may have been bound to the glycosaminoglycans within the extracellular matrix (7, 8, 12). In addition, immunolocalization within the lung was specific for ENA-78 since our antibodies do not cross-react with other C-X-C or C-C chemokine family members and preabsorption of these antibodies to human ENA-78 before the performance of immunohistochemical staining completely abrogated their ability to immunolocalize ENA-78 in rat lung.

Previously, we found that neutralization of TNF resulted in significant attenuation of lung injury in association with hepatic ischemia/reperfusion injury (2, 3). In this study, we found that neutralizing TNF was efficacious in inhibiting pulmonary ENA-78 expression. This finding supported the notion that a TNF-dependent induction of pulmonary ENA-78 may be a mechanism that contributes to the pathogenesis of pulmonary injury. To test this premise, *in vivo* neutralization of ENA-78 by passive immunization resulted in significant attenuation of both pulmonary microvascular permeability and neutrophil infiltration in the lung. These findings were comparable to our previous studies (2, 3), suggesting that ENA-78, and perhaps other members of the C-X-C chemokine family, may represent more distal mediator(s) in a series of events initiated by TNF.

One can speculate, in the context of hepatic ischemia/reperfusion injury, that the mechanisms involved in neutrophil elicitation, activation, and ensuing neutrophil-mediated microvascular injury in the lung, are dependent upon a cascade of events.

Hepatic ischemia/reperfusion injury leads to early TNF production and the release of this potent mediator into the pulmonary microvasculature, causing pulmonary endothelial activation with the expression of adhesion molecules. Subsequently, TNF can simultaneously induce the local generation of ENA-78 or potentially other members of the C-X-C chemokine family, resulting in neutrophil activation and upregulation of the neutrophil-derived  $\beta_2$ -integrin (CD11b/CD18). The interaction of CD11b/CD18 complex with its receptor/ligand, intracellular adhesion molecule-1, on the pulmonary endothelium results in firm adhesion of neutrophils to the endothelium (7, 29, 30). The subsequent steps leading to neutrophil transendothelial migration and extravasation into the interstitium of the lung may be dependent upon both the continued expression of  $\beta_2$ -integrins and neutrophil migration along an established ENA-78 concentration gradient. Finally, activation and influx of neutrophils into the pulmonary interstitium by ENA-78 or potentially other members of the C-X-C chemokine family can result in a neutrophil-mediated pulmonary microvascular injury. Thus, our findings demonstrate the importance of a C-X-C chemokine (ENA-78) in mediating a TNF-dependent pulmonary injury in the context of hepatic ischemia/reperfusion-induced pulmonary injury in the rat.

## Acknowledgments

This research was supported in part by National Institutes of Health grants HL-50057, HL-46487, HL-02401, HL-31693, HL-35276, and HL-03072-01A1 and the American Heart Association grant 94016190.

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