INFECTION AND IMMUNITY, Mar. 1996, p. 870–877
0019-9567/96/$04.00+0

Vol. 64, No. 3

Streptococcal Cysteine Protease Augments Lung Injury Induced by Products of Group A Streptococci

THOMAS P. SHANLEY,[1]* DENNIS SCHRIER,[2] VIVEK KAPUR,[3] MICHAEL KEHOE,[4] JAMES M. MUSSER,[3] AND PETER A. WARD[1]

Department of Pathology, University of Michigan Medical School, Ann Arbor, Michigan 48109-0602[1]; Department of Immunopathology, Parke-Davis, Ann Arbor, Michigan[2]; Department of Microbiology, University of Newcastle-on-Tyne, Newcastle, United Kingdom[4]; and Section of Molecular Pathobiology, Department of Pathology, Baylor College of Medicine, Houston, Texas 77030[3]

Received 29 August 1995/Returned for modification 25 October 1995/Accepted 22 December 1995

Streptococcus pyogenes **infections in humans may be associated with severe clinical manifestations, including adult respiratory distress syndrome and a toxic shock-like syndrome. These observations have led to the investigation of products of group A streptococci that may contribute to increased virulence. Streptococcal pyrogenic exotoxin B is a highly conserved precursor of an extracellular cysteine protease that is secreted by *S. pyogenes*. We investigated the ability of this streptococcal cysteine protease (SCP) to act synergistically with either streptococcal cell wall antigen (SCW) or streptolysin-O (SLO) to augment lung injury in rats. Intratracheal administration of either SCW or SLO alone caused lung injury, as measured by pulmonary vascular leak. Bronchoalveolar lavage (BAL) fluid analysis showed that SCW induced neutrophil accumulation and appearance of interleukin-1β and tumor necrosis factor alpha. In contrast, SLO induced neither neutrophil influx nor significant cytokine elevations in BAL fluids. Intratracheal administration of SCP with either SCW or SLO resulted in synergistic augmentation of lung vascular permeability and accumulation of BAL neutrophils. The synergy was reduced when SCP was either heat inactivated or coinstilled with a peptide inhibitor of the protease. SCP in the presence of SCW resulted in a significant increase in BAL fluid tumor necrosis factor alpha content but not in immunoreactive interleukin-1β. Moreover, the copresence of SCP with SCW resulted in increased BAL fluid nitrite-nitrate levels, indicative of nitric oxide production. These data demonstrate that SCP acts synergistically with other *S. pyogenes* products (SCW or SLO) to increase tissue injury and provide additional evidence that SCP may function as an important virulence factor in group A streptococcal infections.**

In recent years, attention has focused on severe infections in humans caused by *Streptococcus pyogenes* (group A streptococci). These infections are characterized by fulminant and often fatal septic episodes and have led to the description of a streptococcal toxic shock syndrome (4, 11). Because of the increased severity of group A streptococcal infections, there has been renewed interest in the study of putative virulence factors, including three extracellular protein exotoxins: streptococcal pyrogenic exotoxins A (SPE A), B (SPE B), and C (SPE C) (21, 34, 35). SPE B is a highly conserved precursor of an extracellular cysteine protease expressed by virtually all isolates of *S. pyogenes* (22). SPE B is expressed as a 40-kDa protein that is autocatalytically truncated to a 28-kDa molecule which when reduced functions as an active cysteine protease. Several lines of evidence suggest that this streptococcal cysteine protease (SCP) may be a critical virulence factor in streptococcal infections. Patients with low acute-phase levels in serum of antibody to SCP are more likely to succumb to invasive group A streptococcal infections than are individuals with high serum antibody levels (12). This result is supported by the demonstration that active immunization with purified SCP protects mice from lethal challenge with group A streptococci (15). In vitro, SCP cleaves biologically inactive interleukin-1β (IL-1β) precursor to produce mature, active IL-1β (16). Additionally, SCP cleaves fibronectin and degrades vitronectin in vitro (17). These observations led us to assess the ability of SCP to increase tissue injury in vivo.

In the present study, purified, activated SCP coinstilled intratracheally with either group A *Streptococcus* cell wall antigen (SCW) or streptolysin-O (SLO) in rat lungs resulted in a synergistic augmentation of lung injury as measured by increased lung vascular permeability and accumulation of bronchoalveolar lavage (BAL) neutrophils. Intratracheal SCW administration resulted in increased BAL fluid content of IL-1β and tumor necrosis factor alpha (TNF-α). Although no significant increase in immunoreactive IL-1β was observed following coinstillation of SCP with SCW, a substantial increase in TNF-α was found, suggesting increased production of TNF-α as a mechanism responsible for SCP-mediated augmentation in lung injury. Furthermore, a significant increase in BAL fluid nitrite-nitrate content was found following coinstillation of SCW with SCP, suggesting that nitric oxide production may contribute to the synergistic augmentation of injury observed. Despite in vitro data demonstrating that SLO can stimulate production of TNF-α and IL-1β from macrophages (10), neither TNF-α nor IL-1β was detected in BAL fluids from SLO-challenged rats in vivo. The data suggest that additional mechanisms are responsible for the observed synergistic effects between SCP and SLO, perhaps related to the ability of SCP to cleave extracellular matrix proteins (17).

Our results demonstrate that SCP acts synergistically with other group A streptococcal products to augment injury in an in vivo model of acute lung injury and provide additional evidence that this protease is an important virulence factor in group A *Streptococcus* infections.

* Corresponding author. Mailing address: Department of Pathology, MSRB1 Rm. 7526, The University of Michigan Medical School, 1301 Catherine St., Box 0602, Ann Arbor, MI 48109-0602. Phone: (313) 747-2929. Fax: (313) 764-4308.

MATERIALS AND METHODS

Reagents. All reagents were purchased from Sigma Chemical Corp. (St. Louis, Mo.) except where noted.

Purification and activation of SCP and inhibitor. SCP was purified to apparent homogeneity from culture supernatants as previously described (12). In the course of these experiments, two preparations of SCP were used, one for the SCW studies and one for the SLO studies. Differences in the bioactivity of these preparations explain subtle differences among the SCP-only-treated animals in these studies. The bioactivity of the SCP is estimated by determining its proteolytic ability against bovine casein (16). A nonspecific, tripeptide inhibitor of SCP, *N-t*-butoxycarbonyl-leucyl-valyl-glycyl diazomethyl ketone (Boc-LVG-CHN_2), was purchased from Enzyme System Products (Dublin, Calif.) (3).

SCW preparation. The SCW preparation was purchased from Lee Laboratories (Grayson, Ga.). This preparation, designated 10S, is a highly immunogenic peptidoglycan polysaccharide preparation resulting from sonication and subsequent centrifugation of group A *Streptococcus* bacteria. This preparation possessed no hemolytic activity as determined by a hemolysis assay (see below). No endotoxin content was detected by *Limulus* lysate assay.

SLO. SLO was expressed and purified to homogeneity in the laboratory of M.K. and was activated by reduction with 10 mM dithiothreitol. Activity was quantitated by the hemolysis assay described below. No endotoxin was detected by *Limulus* lysate assay at the highest dilution used for in vivo studies (1:500).

Erythrocyte (RBC) hemolysis assay. Hemolytic activity of SLO was determined by a procedure modified from the work of Bhakdi et al. (2). Briefly, 8 to 10 ml of whole blood was obtained from rats anesthetized with ketamine. Whole blood was diluted to 50 ml with phosphate-buffered saline, pH 7.4 (PBS), and spun at 2,500 × *g* for 10 min, and the supernatant was discarded. RBCs were washed three times with PBS in a similar manner. A 4% RBC solution was made by adding 4 ml of rat RBCs to 96 ml of PBS. SLO was activated with 10 mM dithiothreitol for 5 min at room temperature. Serially diluted, activated SLO in 50 μl of PBS was added to duplicate wells of a 96-well microtiter plate. A total of 50 μl of 4% RBC solution was added to each well and incubated at 37°C for 30 min. Hemolytic dilutions were determined by visual reading. For all in vivo studies employing SLO, the highest dilution that yielded 100% hemolysis was used (usually 1:500).

Rat model of SCW- and SLO-induced lung injury. Adult male (275 to 300 g) Long-Evans rats (specific pathogen free) (Harlan Sprague-Dawley, Indianapolis, Ind.) were used for all studies. Intraperitoneal ketamine (15 to 30 mg/100 g of body weight) was given for sedation and anesthesia. The trachea was exposed via a midline, ventral neck incision, and a 25-gauge needle was inserted into the trachea. All intratracheal injections were given during inspiration in a volume of 300 μl of PBS. Either SCW (250 μg) or SLO (1:500 dilution) was given intratracheally to initiate injury. Negative control animals received 300 μl of PBS intratracheally. Immediately thereafter, trace amounts (1 μCi) of ^{125}I-labeled bovine serum albumin were injected intravenously to measure pulmonary vascular permeability, as described elsewhere (20). Rats were sacrificed at 6 h, and the pulmonary circulation was flushed by placing a 21-gauge needle through the right ventricle into the pulmonary artery and infusing 10 ml of saline. Lung injury was quantitated by determining vascular permeability index as follows. For calculation of the permeability index, the amount of radioactivity remaining in the saline-perfused lungs was determined by gamma counting. This value, as counts per minute, was divided by the amount of radioactivity present in 1.0 ml of blood collected from the inferior vena cava also at the time of sacrifice to obtain the permeability index. Ten micrograms of SCP was coinstilled with either SCW or SLO where indicated. Inhibition of the exotoxin was performed by boiling (100°C) for 30 min or by the coinstillation of 10 μg of the inhibitor, Boc-LVG-CHN_2, described above.

BAL. At the time of sacrifice, 8 ml of PBS was instilled and withdrawn three times from the lungs via an intratracheal cannula. Lavage fluids were retrieved, and total cell counts were performed with a Coulter counter (Hialeah, Fla.). BAL fluid samples were subjected to Cytospin centrifugation (700 × *g* for 7 min) and stained with Diff-Quik products (Baxter, Miami, Fla.) for determination of leukocyte differentials. Cells were separated from BAL fluid by centrifugation at 1,500 × *g* for 10 min.

BAL fluid cytokine determination. The BAL fluid was analyzed for immunoreactive IL-1β content via a murine IL-1β enzyme-linked immunosorbent assay (ELISA) (Biosource, Camarillo, Calif.). This ELISA cannot differentiate between inactive (precursor) IL-1β and active murine IL-1β. It has demonstrated cross-reactivity with rat IL-1β.

TNF-α bioactivity content in BAL fluids was determined by a cytotoxicity assay employing a fibrosarcoma cell line (WEHI 164 clone 13) as previously described (6).

Anti-murine IL-1β antibody. Goats were immunized with 20 μg of murine IL-1β (R & D Systems) in complete Freund's adjuvant and then serially boosted with 10 μg of murine IL-1β in incomplete Freund's adjuvant until serum bleeds from the goat reached a titer of $>10^{-6}$ against murine IL-1β as determined by indirect ELISA. Where indicated, anti-murine IL-1β was administered at a dose of 0.5 ml intravenously and 1.0 ml intraperitoneally at the commencement of injury. Lung vascular permeability was determined as described above.

Measurement of BAL fluids content of NO_2^--NO_3^-. Nitrite in BAL fluids was measured with the Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride, 25% hydrochloric acid) forming a chromophore absorbing at 543 nm (33). Nitrate was reduced to nitrite with nitrate reductase (EC 1.6.2.2) from *Aspergillus* sp., and 2.5 nM NADP (reduced form) was added. Absorbance was then measured as NO_2^- (the combination of nitrite and reduced nitrate was designated NO_2^--NO_3^-). Data from BAL fluids were presented in nanomoles per milliliter.

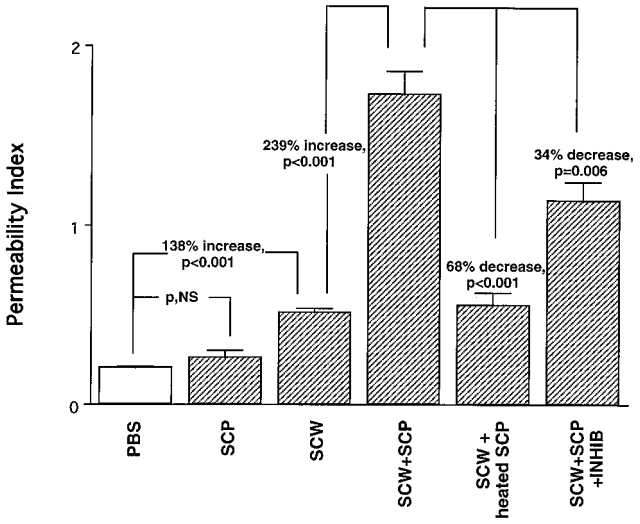

FIG. 1. Permeability index in SCW-induced lung injury. Shown is the effect of SCP in SCW-induced lung injury in the rat as assessed by permeability (leakage of ^{125}I-bovine serum albumin) 6 h after commencement of injury as described in Materials and Methods. Either heat inactivation of SCP or coadministration of SCP with its inhibitor diminished the augmentation of lung injury attributed to SCP. NS, not significant in relation to a P value of ≤0.05. All values are expressed as means ± SEM with $n = 6$ for all intervention groups.

Morphological analysis. Rats undergoing SCW-induced lung injury as described above were sacrificed 6 h after commencement of injury. Whole lungs were inflated with 10% buffered formalin via a trachea cannula and removed en bloc. Lungs were stained with hematoxylin and eosin and were examined by light microscopy.

Statistical analysis. Data sets were examined with two-way analysis of variance, and mean values were then compared between groups by a Tukey's procedure with statistical significance defined at $P < 0.05$. All values were expressed as means ± standard errors of the mean (SEM).

RESULTS

Effect of SCP on SCW-induced lung injury. Intratracheal administration of SCW (250 μg) resulted in a significant increase in permeability index (to 0.51 ± 0.03) compared with negative controls (PBS alone; 0.20 ± 0.02, $P < 0.001$) (Fig. 1). Because SCP cleaves fibronectin and degrades vitronectin in vitro (10), SCP alone was given at a dose of 10 μg ($n = 4$). Compared with the effects of PBS alone (negative control), no significant change in permeability index was observed in these animals (0.26 ± 0.04) (Fig. 1). Coadministration of SCP (10 μg) with SCW resulted in a striking increase (239%) of the permeability index (to 1.73 ± 0.13, $P < 0.001$). Heat inactivation of SCP prior to coinstillation with SCW (0.55 ± 0.08) resulted in a total loss of any increment in the permeability index caused by addition of SCP to SCW (Fig. 1). Coaddition of an inhibitor of SCP, Boc-LVG-CHN_2, with SCW and SCP resulted in a 34% decrease in permeability (1.14 ± 0.10, $P < 0.01$ [Fig. 1]). That the tripeptide protease inhibitor did not completely abolish the synergistic effect of SCP was probably due to the dose used (10 μg) being at a molar concentration significantly lower than that for SCP. These data indicate that the combination of SCW with SCP results in the maximal amount of lung injury as reflected by the permeability index.

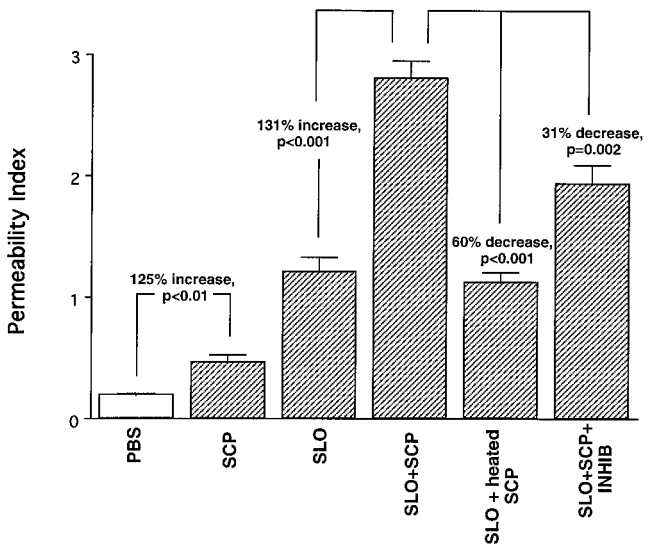

FIG. 2. Permeability index in SLO-induced lung injury. Shown is the effect of SCP in SLO-induced lung injury in the rat as assessed by permeability (leakage of ^{125}I-bovine serum albumin) 6 h after commencement of injury as described in Materials and Methods. Either heat inactivation of SCP or coadministration of SCP with its inhibitor diminished the augmentation of lung injury attributed to SCP. All values are expressed as means ± SEM with $n = 6$ for all intervention groups.

Effect of SCP on SLO-induced lung injury. In this series of studies, intratracheal instillation of SCP (10 μg) caused a permeability index of 0.46 ± 0.06, a significant increase ($P = 0.037$) compared with the effect of PBS alone (0.20 ± 0.01) (Fig. 2). The titer of SLO was determined for hemolytic activity as described above. The lowest SLO dilution, which gave 100% hemolysis of rat RBCs, was 1:500. This dilution was used for in vivo studies. SLO caused a significant increase in permeability (1.21 ± 0.12, $P < 0.001$ [Fig. 2]) compared with negative control animals. Coinstillation of SCP with SLO resulted in a 131% increase in permeability (to 2.80 ± 0.15, $P < 0.001$) compared with the effect of SLO alone (Fig. 2). Heating (100°C, 30 min) SCP also resulted in its loss of ability to cause synergistic effects with SLO (Fig. 2). The addition of the tripeptide inhibitor to SCP in the copresence of SCP and SLO resulted in a partial decrease (31%, $P = 0.002$) in lung injury as reflected by the permeability index (Fig. 2).

Effects of SCP with or without SCW or SLO on lung neutrophil accumulation. The effects of SCP on BAL fluid cell content from rats treated with SCW or SLO were assessed. SCW administration induced a large influx of neutrophils into lungs compared with PBS-treated animals (Table 1). Coinstillation of SCP with SCW resulted in a 190% increase in BAL neutrophils compared with instillation of SCW alone (Table 1). Inhibition of SCP with Boc-LVG-CHN_2 in the copresence of SCW and SCP resulted in a 44% decrease in BAL neutrophils compared with rats given SCW plus SCP (Table 1). Heat inactivation of SCP diminished the increase in BAL neutrophils by 58% to a level not statistically different from that with SCW alone (Table 1). Therefore, the augmentation of lung injury mediated by SCP as measured by permeability changes was concurrent with a significant increase in BAL fluid neutrophils.

Somewhat similar findings were observed in SLO-induced lung injury. The increase in BAL inflammatory cell content following SLO challenge was composed almost entirely of mononuclear cells (Table 2). However, the addition of SCP to SLO-induced injury resulted in a substantial accumulation of neutrophils recovered from BAL fluids (Table 2). Addition of the tripeptide inhibitor to SLO with SCP resulted in a partial decrease (62%) in BAL neutrophils (Table 2). Again, the increased lung permeability observed as a result of SCP coinstillation with SLO was associated with a large increase in BAL neutrophils.

BAL content of IL-1β following SCP administration. BAL fluids were examined for cytokine content. As SCP has demonstrated IL-1β convertase activity in vitro (10), BAL fluids were analyzed for IL-1β content by ELISA. Instillation of PBS (negative control animals) resulted in very little detectable IL-1β in BAL fluids (11.0 ± 1.9 pg/ml) (Fig. 3). SCW alone resulted in a significant increase (to 49.4 ± 6.8 pg/ml, $P < 0.001$) in IL-1β content in BAL fluids (Fig. 3). The addition of SCP to SCW-challenged rats resulted in BAL fluid IL-1β levels that were not significantly different from those of SCW-positive controls (40.8 ± 6.2 pg/ml [Fig. 3]). BAL fluid IL-1β levels in rats treated either with SCW plus heat-inactivated SCP (29.2 ± 10.0 pg/ml) or with the inhibitor in the copresence of SCW and SCP (38.4 ± 2.8 pg/ml) did not differ statistically from positive control animals (Fig. 3).

Because the ELISA employed does not discriminate between precursor and active IL-1β, we attempted to indirectly determine if part of the mechanism by which SCP synergizes with SCW to augment injury is via increased activation of IL-1β by coadministering anti-IL-1β at the commencement of injury. SCP (10 μg) alone resulted in a permeability value of 0.24 ± 0.06 not significantly different from that of negative control animals receiving PBS alone (0.16 ± 0.04 [Fig. 4]). The permeability index in response to SCP was unchanged by coadministration of anti-IL-1β (0.24 ± 0.04 [Fig. 4]). As shown in Fig. 4, the administration of anti-IL-1β in the presence of SCW (0.43 ± 0.04) resulted in only a modest decrease (10%) in lung vascular permeability compared with SCW alone (0.48 ± 0.07), though this result did not reach statistical significance. The coadministration of SCP with SCW resulted in a significant

TABLE 1. Cell content in BAL fluids from SCW-challenged rats

Cells	Material instilled intratracheally[a]					
	PBS	SCP	SCW	SCW + SCP	S + S + I*[b]	SCW + heated SCP
Mononuclear cells	1.21 ± 0.06	0.34 ± 0.08	2.46 ± 0.71	2.75 ± 0.62	1.72 ± 0.15	1.05 ± 0.29
Neutrophils	0.02 ± 0.01	0.99 ± 0.16	14.29 ± 1.93	41.55 ± 6.68[c]	23.30 ± 3.73[d]	17.42 ± 5.03[e]

[a] Numbers represent means ± SEM (×10^6); $n = 5$ for each group.
[b] S + S + I = SCW (250 μg) + SCP (10 μg) + inhibitor (10 μg).
[c] Versus SCW alone, $P < 0.001$.
[d] Versus SCW + SCP, $P = 0.038$.
[e] Versus SCW + SCP, $P = 0.013$.

TABLE 2. Cell content in BAL fluids from SLO-challenged rats

Cells	Material instilled intratracheally[a]				
	PBS	SCP	SLO	SCP + SLO	SLO + SCP + inhibitor
Mononuclear cells	0.91 ± 0.15	0.93 ± 0.18	2.97 ± 0.38	2.80 ± 0.38	1.47 ± 0.23
Neutrophils	<0.020	0.22 ± 0.11	0.08 ± 0.02	3.69 ± 0.29[b]	1.41 ± 0.28[c]

[a] Numbers represent means ± SEM ($\times 10^6$); $n = 6$ for each group.
[b] Versus SCP alone or SLO alone, $P < 0.001$.
[c] Versus SCP + SLO, $P < 0.01$.

increase (204%) in lung permeability compared with the SCW-alone group (1.46 ± 0.14, $P < 0.01$ [Fig. 4]). Under these conditions, anti-IL-1β in the presence of both SCW and SCP resulted in a significant decrease (48%) in lung permeability compared with the SCW-plus-SCP group (0.76 ± 0.13, $P < 0.05$ [Fig. 4]). These findings suggest that IL-1β significantly contributes to the synergism observed between SCW and SCP. However, the data do not conclusively demonstrate an increased conversion of inactive IL-1β precursor to its mature, biologically active form.

Despite the observation that SLO has been shown to induce IL-1β production in vitro, no increase in BAL fluid IL-1β content was found in SLO-challenged rats by the above-described ELISA (data not shown).

Effect of SCP on BAL fluid TNF-α content. Although no statistically significant difference in BAL fluid immunoreactive IL-1β content was found in animals receiving SCW with SCP (compared with SCW alone), a significant difference in TNF-α content was found. BAL fluids from negative control animals (PBS alone) contained little detectable TNF-α (47.1 ± 11.0 pg/ml [Fig. 5]). SCP alone increased BAL fluid TNF-α (to 231.5 ± 33.7 pg/ml, $P < 0.001$) as did SCW (708.3 ± 299.4 pg/ml, $P = 0.018$ [Fig. 5]) compared with negative control animals (PBS alone). However, coinstillation of SCP with SCW resulted in a substantial increase in TNF-α content (to 21,040.8 ± 2,424.5 pg/ml, $P < 0.001$, compared with SCW alone) (Fig. 5). This increase was diminished either by heat inactivating the protease (with the TNF-α content falling to 1,528 ± 511.0 pg/ml; 92% decrease, $P < 0.001$) or by the addition of the protease inhibitor with SCW and SCP (with the TNF-α content falling to 3,713 ± 1,078.0 pg/ml; 82% decrease, $P < 0.001$) (Fig. 5). These data suggest that a potential mechanism by which SCP augments injury in the presence of SCW is by increased intrapulmonary production of TNF-α.

As SLO has also been shown to cause increased levels of TNF-α production in vitro, BAL fluids from SLO-challenged animals were also tested for TNF-α content. No TNF-α was detectable in the BAL fluids of animals challenged with SLO alone or in the presence of SCP (data not shown). Therefore, while the synergistic effects of SCW and SCP can be linked to TNF-α content in BAL fluids, no such association was found with SCP and SLO.

Effect of SCP on BAL fluid content of nitrite-nitrate. Because it has been suggested that nitric oxide may collaborate with streptococcal products (streptolysin-S) and oxidants to cause enhanced killing of endothelial cells in vitro (7), nitrite-nitrate levels in BAL fluids from rats treated as described above were determined. BAL fluids from negative control animals (PBS alone) contained very little NO_2^--NO_3^- (8.2 ± 2.8 nmol/ml [Fig. 6]). Intratracheal administration of SCP alone led to no significant change in this level (5.6 ± 1.7 nmol/ml). However, following intratracheal challenge with SCW, a significant increase in BAL fluid NO_2^--NO_3^- content (14.8 ± 1.4 nmol/ml) was observed in comparison with negative control animals ($P < 0.01$) (Fig. 6). Coinstillation of SCP with SCW

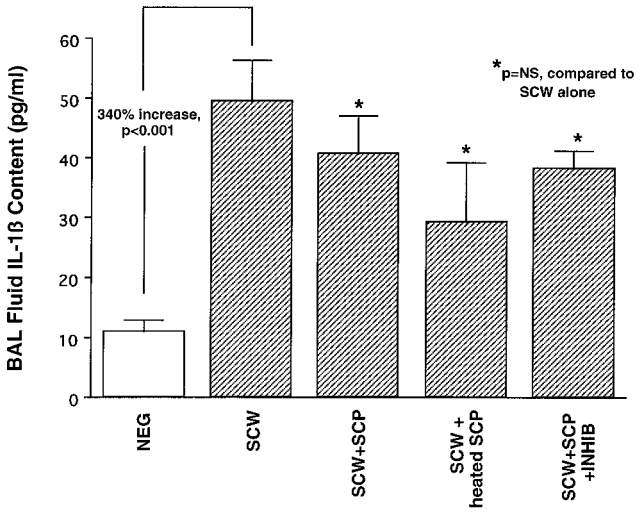

FIG. 3. Effect of SCP on BAL fluid content of IL-1β 6 h after commencement of SCW-induced lung injury as determined by ELISA. Intervention groups were compared by single-factor analysis of variance ($P = 0.113$) with $n = 6$ in all groups. NS, not significant.

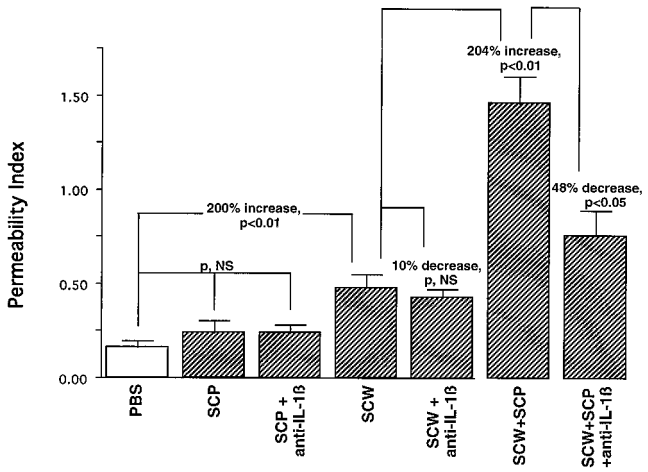

FIG. 4. Effect of anti-IL-1β on SCP-, SCW-, and SCW-plus-SCP-induced lung injury in the rat as assessed by permeability (leakage of ^{125}I-bovine serum albumin) 6 h after commencement of injury as described in Materials and Methods. While anti-IL-1β had no significant effect on permeability changes induced by either SCP or SCW alone, the antibody in the copresence of SCP plus SCW resulted in a significant decrease in lung injury. All values are expressed as means ± SEM with $n = 6$ for all intervention groups. NS, not significant.

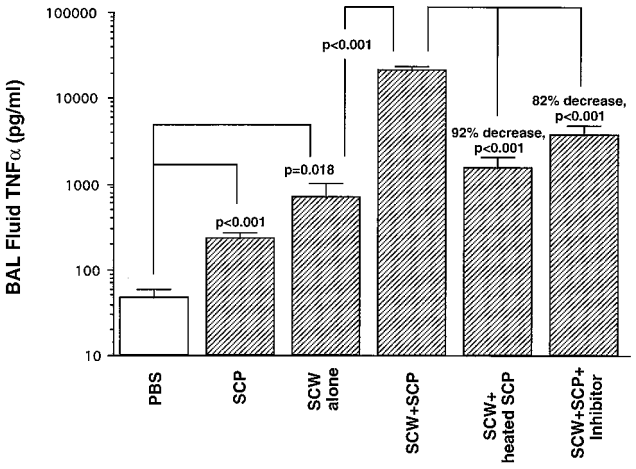

FIG. 5. Effect of SCP on BAL TNF-α 6 h after SCW-induced lung injury as determined by WEHI cytotoxicity assay. All values are expressed in picograms per milliliter with $n = 6$ in all groups. Note that the y axis is on a logarithmic scale.

led to a synergistic increase in BAL fluid NO_2^--NO_3^- content compared with that of positive control animals (37.3 ± 7.2 nmol/ml, $P = 0.025$). Heat inactivation of SCP resulted in a significant decrease in this value (18.9 ± 4.8 nmol/ml, $P = 0.041$ [Fig. 6]), while the protease inhibitor in the copresence of SCW and SCP also led to a partial (35%), though nonsignificant, reduction in the measured NO_2^--NO_3^- content (24.1 ± 2.6 nmol/ml, $P = 0.051$). These results suggest that products of the inducible nitric oxide synthase pathway are present following challenge with SCW and that augmentation of injury in the copresence of SCW and SCP may be due to increased production of NO, as determined by NO_2^--NO_3^- measurement in BAL fluids. At least in rats, this increased production of NO_2^--NO_3^- may be reflective of the great increase in BAL neutrophils (Table 1), which are known to be able to generate NO.

Histological analysis of lungs treated with SCW with or without SCP. No morphological changes were seen in animals receiving only PBS intratracheally (Fig. 7A). Lungs of rats treated with SCW alone (Fig. 7B) showed intra-alveolar neutrophils and a mild degree of hemorrhage and fibrin deposition in the intra-alveolar component. Lungs from rats treated with SCW together with SCP (Fig. 7C and D) had dramatically increased accumulation of neutrophils in addition to extensive intra-alveolar hemorrhage and fibrin deposition. The intense inflammatory cellular response caused by SCP was very much diminished by prior heat inactivation (Fig. 7E).

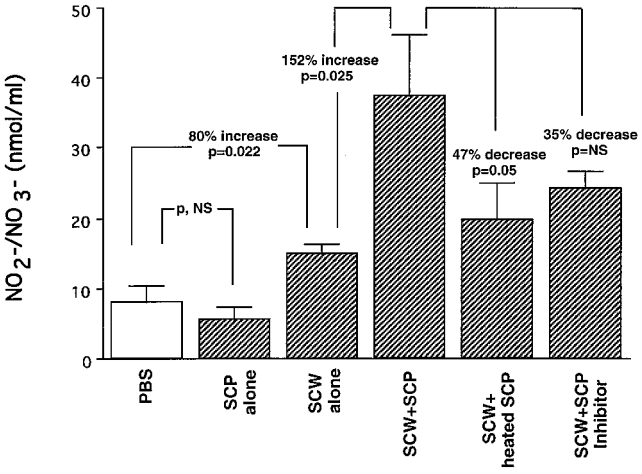

FIG. 6. Effect of SCP on production of NO_2^--NO_3^- in BAL fluids 6 h after SCW-induced lung injury as described in Materials and Methods. Values of NO_2^--NO_3^- represent means ± SEM with $n = 6$ in all groups. NS, not significant.

DISCUSSION

By the use of lung injury induced in rats, we have evaluated the potential role of products of *S. pyogenes* in the development of inflammatory injury and examined the potential role of SCP in synergistically augmenting this injury. Intratracheal administration of SCW resulted in lung injury commensurate with increased neutrophils and presence of cytokines (TNF-α and IL-1β) in BAL fluids. Interestingly, SLO induced lung injury that was not associated with either significant neutrophil influx into the lungs or TNF-α or IL-1β production. Instillation of SCP with either SCW or SLO resulted in significant, synergistic increases in both pulmonary vascular leakage and BAL neutrophils. The augmented injury observed with SCP plus SCW was associated with significant increases in BAL fluid content of TNF-α and NO_2^--NO_3^-. This is the first demonstration in vivo that products of group A streptococci can act synergistically to increase tissue injury.

The mechanism of lung injury induced by SCW is similar to that observed for other neutrophil-dependent pulmonary models of inflammation such as immunoglobulin G immune complex-mediated (31) or lipopolysaccharide (LPS)-mediated (28, 29) lung injury. In these models, lung injury was associated with increased BAL fluid neutrophils and increased levels of TNF-α and IL-1β. It was important to determine BAL fluid content of IL-1β since it is known that SCP displays IL-1β convertase activity in vitro. No increase in immunoreactive IL-1β was detected following instillation of SCP with SCW (Fig. 3). However, in the absence of a rat IL-1β bioactivity assay or an ELISA system that could discriminate between the pro- and mature forms of rat IL-1β, we cannot exclude the possibility of increased bioactive, mature IL-1β following SCP administration. An indirect method for determining if IL-1β contributes to the observed synergism in this model was via the administration of anti-IL-1β. While anti-IL-1β did not significantly decrease lung injury (as quantitated by permeability) resulting from SCW administration, the antibody did provide a significant degree of protection in the group receiving SCW plus SCP. This result indicates that IL-1β is an important mediator of the observed synergism. While it suggests that the addition of SCP to SCW may result in increased conversion of IL-1β to its active form, this conclusion cannot be confirmed without the determination of the level of bioactive IL-1β under these experimental conditions.

A significant finding in these studies was the substantial increase in BAL TNF-α following coinstillation of SCP with SCW (Fig. 5). The intrapulmonary production of TNF-α following SCW challenge may be related to direct stimulation of lung macrophages by SCW. The cell wall of group A streptococci contains a variety of immunogenic molecules, such as peptidoglycans and lipoteichoic acid, that have demonstrated in vitro cytotoxicity (5, 18). Peptidoglycan from another gram-positive organism, *Staphylococcus epidermidis*, has the ability to stimulate TNF-α production from human monocytes in a manner that is greatly potentiated in the presence of serum (19). Under such conditions, complement products were considered

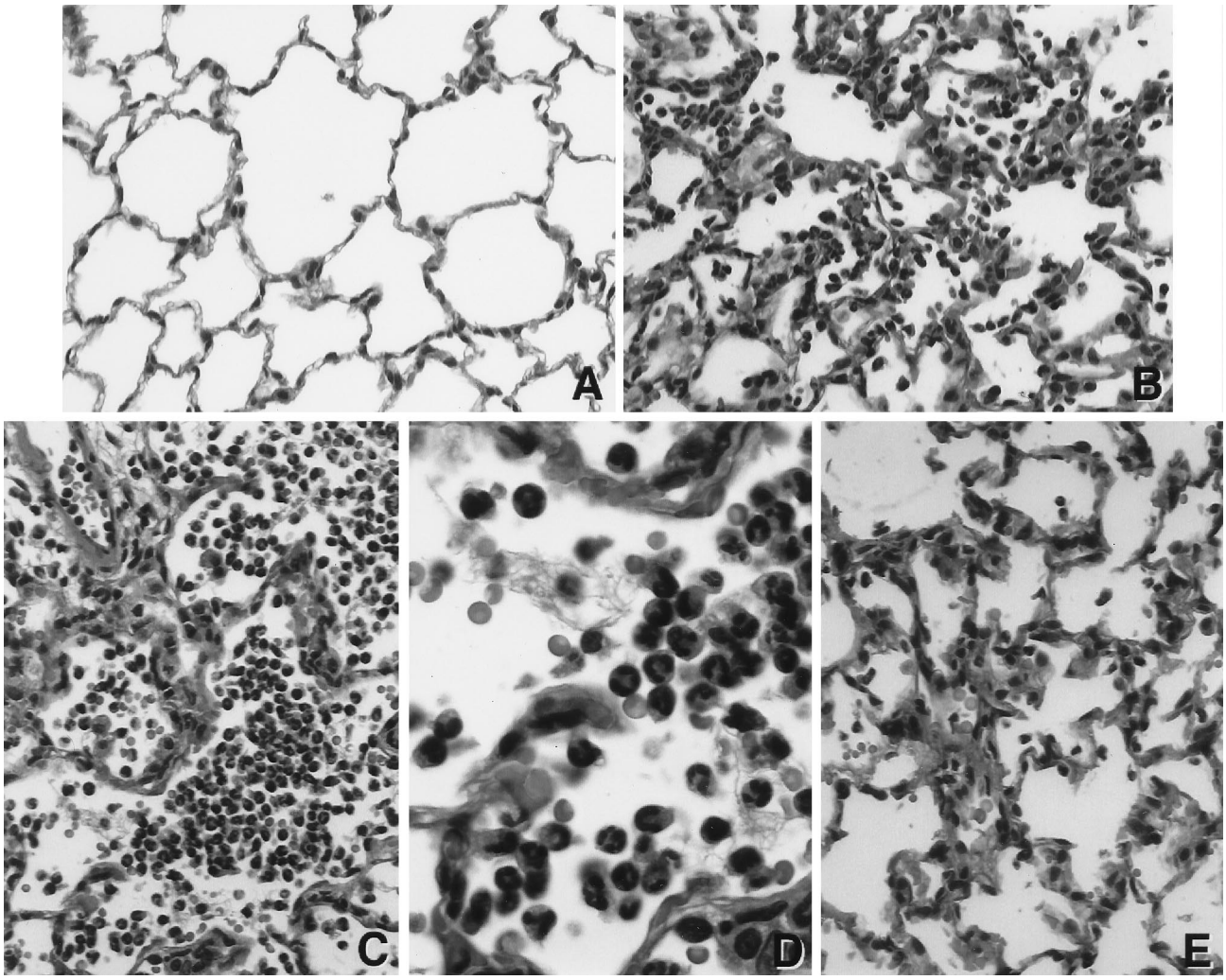

FIG. 7. Histological analysis of rat lung sections at 6 h after commencement of injury. Shown at top are normal architecture in negative control (PBS) rats (A) (magnification, ×40) and SCW treatment (B) (magnification, ×40), indicating neutrophil infiltration, mild alveolar hemorrhage, and fibrin deposition. When SCP was coinstilled with SCW (C and D) (magnification, ×40 and ×100, respectively), neutrophil content was dramatically increased in the alveolar compartment together with alveolar hemorrhage and fibrin deposition. Under conditions of heat inactivation of the SCP (E) (magnification, ×40), the neutrophil accumulation and hemorrhage were greatly reduced. Results are representative of two animals in each group.

to play a key role in potentiating the release of TNF-α from monocytes (19). Therefore, a potential mechanism by which SCP augments production of TNF-α may be by increased activation of complement. It is also possible that SCP may possess TNF-α convertase activity, although this hypothesis remains to be tested.

Because TNF-α can induce inducible nitric oxide synthase expression both in vitro and in vivo (32), it is possible that the increase in BAL NO_2^--NO_3^- content may be related to increased production of TNF-α. In vitro studies have demonstrated that the combination of sublethal doses of nitroprusside (a generator of nitric oxide), a porforin (streptolysin-S), and H_2O_2 is highly cytotoxic (7), suggesting that even modest increases in nitric oxide production may be highly toxic in the milieu of multiple inflammatory mediators and bacterial products. An additional explanation for the finding of increased BAL NO_2^--NO_3^- content may be that the presence of SCP causes increased conversion of precursor IL-1β to active IL-1β, which acts as a potent inducer of inducible nitric oxide synthase activity. In cultures of rat aortic smooth muscle cells, SCP in the presence of precursor IL-1β resulted in a nearly 60-fold increase in nitrite accumulation (16). It is of interest to note that a second exotoxin of *S. pyogenes*, SPE A, has been shown to induce TNF-α production from macrophages in vitro (10). To what extent SPE A may also contribute to the pathogenesis of tissue injury associated with streptococcal organisms remains to be determined.

Results obtained from in vivo studies employing SLO as an inducer of lung injury are consistent with previous findings that demonstrated the synergistic effects by streptococcal products in a tissue culture model of epithelial and endothelial cell killing (7–9). These in vitro studies have demonstrated that SLO is cytolytic and results in production of porin-type lesions in plasma membranes (7). These holes may be large enough to account for the albumin leakage quantitated in our in vivo system. In addition, the presence of such pores may provide SCP access to matrix proteins such as fibronectin and vitronectin which can be cleaved in vitro by SCP (17). Additionally, SLO-induced holes in the plasma membrane might allow radicals ready access to intracellular targets, resulting in cell injury

and death. Of note, the coinstillation of SCW (250 μg) with SLO (1:500 dilution) and SCP (10 μg) was uniformly fatal to rats (data not shown), suggesting that synergism may extend to all three streptococcal products. The observation of synergism between SCP and other products of *S. pyogenes* is noteworthy because similar observations have been made for products of gram-negative organisms. For instance, LPS priming has been shown to synergize with hemolysin A of *Escherichia coli*, causing pulmonary vascular abnormalities (30).

This study represents the first description of the ability of SCP to augment SCW- and SLO-induced injury in an in vivo model of lung injury. These results may have important implications in defining the pathogenesis of severe streptococcal infections. There continue to be reports of severe invasive infections, streptococcal toxic shock-like syndrome, and death in patients infected with group A streptococci. Such reports include a series of deaths among California children who acquired group A streptococcal superinfections during varicella-zoster virus infection (14). Often, these infections have been characterized by signs and symptoms that are consistent with the presence of elevated systemic TNF-α. Focal tissue infections have been associated with an intense febrile response. Severe, invasive infections with *S. pyogenes* are usually characterized by neutropenia, fever, hypotension, shock, and multiple organ failure, responses that can be seen with increased systemic levels of TNF-α (1, 23, 25). A common occurrence in these patients is the development of acute lung injury (adult respiratory distress syndrome) for which TNF-α has also been considered a potential mediator (13, 24, 26, 27). The current studies add to data that SCP is an important virulence factor in group A *Streptococcus* infection, acting to potentiate injury via a mechanism at least in part due to increased TNF-α production.

ACKNOWLEDGMENTS

We thank L.-L. Li for technical assistance in the preparation of SCP.

This work was supported in part by National Institutes of Health grants HL-31963, GM-2950, HD-00850, and AI-33119; American Heart Association grant-in-aid 006640; and funds from the Mosun Foundation and the Texas Advanced Technology Program. J.M.M. is an Established Investigator of the American Heart Association. T.P.S. is a National Institutes of Health Fellow of the Pediatric Scientist Development Program.

REFERENCES

1. **Beutler, B.** 1989. Cachectin in tissue injury, shock and related states. Crit. Care Clin. **5:**353–367.
2. **Bhakdi, S., M. Roth, A. Sziegoleit, and J. Tranum-Jensen.** 1984. Isolation and identification of two hemolytic forms of streptolysin-O. Infect. Immun. **46:**394–400.
3. **Bjorck, L., P. Akesson, M. Bohus, J. Trojnar, M. Abrahamson, I. Olafsson, and A. Grubb.** 1989. Bacterial growth blocked by a synthetic peptide based on the structure of a human proteinase inhibitor. Nature (London) **337:**385–386.
4. **Cone, L. A., D. R. Woodard, P. M. Schlievert, and G. S. Tomory.** 1987. Clinical and bacteriologic observations of a toxic shock-like syndrome due to *Streptococcus pyogenes*. N. Engl. J. Med. **317:**146–149.
5. **DeVuono, J., and C. Panos.** 1978. Effect of L-form *Streptococcus pyogenes* and of lipoteichoic acid on human cells in tissue culture. Infect. Immun. **22:**255–265.
6. **Espevik, T., and J. Nissen-Meyer.** 1986. A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor/tumor necrosis factor from human monocytes. J. Immunol. Methods **95:**99–105.
7. **Ginsburg, I., D. F. Gibbs, L. Schuger, K. J. Johnson, U. S. Ryan, P. A. Ward, and J. Varani.** 1989. Vascular endothelial cell killing by combinations of membrane-active agents and hydrogen peroxide. Free Radical Biol. Med. **7:**369–376.
8. **Ginsburg, I., and R. Kohen.** 1995. Synergistic effects among oxidants, membrane-damaging agents, fatty acids, proteinases and xenobiotics: killing of epithelial cells and release of arachidonic acid. Inflammation **19:**101–118.
9. **Ginsburg, I., R. Misgav, A. Pinson, J. Varani, P. A. Ward, and R. Kohen.** 1992. Synergism among oxidants, proteinases, phospholipases, microbial hemolysins, cationic proteins, and cytokines: a possible major cause of cell and tissue destruction in inflammation (a working hypothesis). Inflammation **16:**519–537.
10. **Hackett, S., and D. L. Stevens.** 1992. Streptococcal toxic shock syndrome: synthesis of tumor necrosis factor and interleukin-1 by monocytes stimulated with pyrogenic exotoxin A and streptolysin O. J. Infect. Dis. **165:**879–885.
11. **Hoge, C. W., B. Schwartz, D. F. Talkington, R. F. Breiman, E. M. MacNeill, and S. J. Englender.** 1993. The challenging epidemiology of invasive group A streptococcal infections and the emergence of streptococcal toxic shock-like syndrome. A retrospective population-based study. JAMA **269:**384–389.
12. **Holm, S. E., A. Norrby, A.-M. Bergholm, and M. Norgren.** 1992. Aspects of pathogenesis of serious group A streptococcal infections in Sweden. J. Infect. Dis. **166:**31–37.
13. **Hyers, T. M., S. M. Tricomi, P. A. Dettenmeier, and A. A. Fowler.** 1991. Tumor necrosis factor levels in serum and bronchoalveolar lavage fluid of patients with adult respiratory distress syndrome. Am. Rev. Respir. Dis. **144:**268–271.
14. **Jones, S. P.** 1994. California counties investigate five chickenpox-related deaths. Infect. Dis. Child. **7**(5)**:**20, 37.
15. **Kapur, V., J. T. Maffei, R. S. Greer, L.-L. Li, G. J. Adams, and J. M. Musser.** 1994. Vaccination with streptococcal extracellular cysteine protease (interleukin-1β convertase) protects mice against challenge with heterologous group A streptococci. Microb. Pathog. **16:**443–450.
16. **Kapur, V., M. W. Majesky, L.-L. Li, R. A. Black, and J. M. Musser.** 1993. Cleavage of interleukin 1β (IL-1β) precursor to produce active IL-1β by a conserved extracellular cysteine protease from *Streptococcus pyogenes*. Proc. Natl. Acad. Sci. USA **90:**7676–7680.
17. **Kapur, V., S. Topouzis, M. W. Majesky, L.-L. Li, M. R. Hamrick, R. J. Hamill, J. M. Patti, and J. M. Musser.** 1993. A conserved *Streptococcus pyogenes* extracellular protease cleaves human fibronectin and degrades vitronectin. Microb. Pathog. **15:**327–346.
18. **Leon, O., and C. Panos.** 1983. Cytotoxicity and inhibition of normal collagen synthesis in mouse fibroblasts by lipoteichoic acid from *Streptococcus pyogenes* type 12. Infect. Immun. **40:**785–794.
19. **Mattsson, E., J. Rollof, J. Verhoef, H. Van Dijk, and A. Fleer.** 1994. Serum-induced potentiation of tumor necrosis factor alpha production by human monocytes in response to staphylococcal peptidoglycan: involvement of different serum factors. Infect. Immun. **62:**3837–3843.
20. **Mulligan, M. S., J. M. Hevel, M. A. Marletta, and P. A. Ward.** 1991. Tissue injury caused by deposition of immune complexes is L-arginine dependent. Proc. Natl. Acad. Sci. USA **88:**6338–6342.
21. **Musser, J. M.** 1992. Clinical relevance of streptococcal pyrogenic exotoxins in streptococcal toxic shock-like syndrome and other severe invasive infections. Ped. Ann. **21:**821–828.
22. **Musser, J. M., A. R. Hauser, M. Kim, P. M. Schlievert, K. Nelson, and R. K. Selander.** 1991. *Streptococcus pyogenes* causing toxic-shock-like syndrome and other invasive diseases: clonal diversity and pyrogenic exotoxin expression. Proc. Natl. Acad. Sci. USA **88:**2668–2672.
23. **Schirmer, W. J., J. M. Schirmer, and D. E. Fry.** 1989. Recombinant human tumor necrosis factor produces hemodynamic changes characteristic of sepsis and endotoxemia. Arch. Surg. **124:**445–448.
24. **Suter, P. M., S. Suter, E. Girardin, P. Roux-Lombard, G. E. Grau, and J.-M. Dayer.** 1991. High bronchoalveolar levels of tumor necrosis factor and its inhibitors, interleukin-1, interferon and elastase, in patients with adult respiratory distress syndrome after trauma, shock, or sepsis. Am. Rev. Respir. Dis. **145:**1016–1022.
25. **Tracey, K. J., B. Beutler, S. F. Lowry, J. Merryweather, S. Wolpe, I. W. Milsark, R. J. Hariri, T. J. Fahey III, A. Zentella, J. D. Albert, G. T. Shires, and A. Cerami.** 1986. Shock and tissue injury induced by recombinant human cachectin. Science **234:**470–474.
26. **Tracey, K. J., S. F. Lowry, and A. Cerami.** 1988. Cachectin/TNF-alpha in septic shock and septic adult respiratory distress syndrome. Am. Rev. Respir. Dis. **138:**1377–1379.
27. **Tran Van Nhieu, J., B. Misset, F. Lebargy, J. Carlet, and J. F. Bernaudin.** 1993. Expression of tumor necrosis factor-alpha gene in alveolar macrophages from patients with adult respiratory distress syndrome. Am. Rev. Respir. Dis. **147:**1585–1589.
28. **Ulich, T. R., K. Guo, S. Yin, J. del Castillo, E. S. Yi, R. C. Thompson, and S. P. Eisenberg.** 1992. Endotoxin-induced cytokine gene expression *in vivo*. Am. J. Pathol. **141:**61–68.
29. **Ulich, T. R., L. R. Watson, S. Yin, K. Guo, P. Wang, H. Thang, and J. del Castillo.** 1991. The intratracheal administration of endotoxin and cytokines. Am. J. Pathol. **138:**1485–1496.
30. **Walmrath, D., H. A. Ghofrani, S. Rosseau, H. Schutte, A. Cramer, W. Kaddus, F. Grimminger, S. Bhakdi, and W. Seeger.** 1994. Endotoxin "priming" potentiates lung vascular abnormalities in response to *Escherichia coli* hemolysin: an example of synergism between endo- and exotoxin. J. Exp. Med. **180:**1437–1443.
31. **Ward, P. A., and M. S. Mulligan.** 1993. Molecular mechanisms in acute lung injury. Adv. Pharmacol. **24:**275–292.
32. **Warner, R. L., R. Paine, P. J. Christensen, M. A. Marletta, M. K. Richards,**

S. E. Wilcoxen, and P. A. Ward. 1995. Lung sources and cytokine requirements for *in vivo* expression of inducible nitric oxide synthase. Am. J. Respir. Cell Mol. Biol. **12:**649–661.

33. **Werner-Felmayer, G., E. R. Werner, D. Fuchs, A. Hansen, G. Reibnegger, and H. Wachter.** 1990. Tetrahydrobiopterin-dependent formation of nitrite and nitrate in murine fibroblasts. J. Exp. Med. **172:**1599–1607.
34. **Yu, C.-E., and J. J. Ferretti.** 1989. Molecular epidemiologic analysis of the type A streptococcal exotoxin (erythrogenic toxin) gene (*speA*) in clinical *Streptococcus pyogenes* strains. Infect. Immun. **57:**3715–3719.
35. **Yu, C.-E., and J. J. Ferretti.** 1991. Frequency of the erythrogenic toxin B and C genes (*speB* and *speC*) among clinical isolates of group A streptococci. Infect. Immun. **59:**211–215.

Editor: V. A. Fischetti