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Rapid Communication

Intratracheal Injection of Endotoxin and Cytokines

II. Interleukin-6 and Transforming Growth Factor Beta Inhibit Acute Inflammation

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The nature of the endogenous mediators that downregulate and curtail the exodus of neutrophils into local acute inflammatory sites is unknown. In the present report, interleukin-6 (IL-6) and transforming growth factor beta (TGFβ), members of a family of macrophage-derived proteins known as cytokines, are shown to inhibit significantly the acute neutrophilic exodus caused by an intratracheal injection of endotoxin (LPS), a proinflammatory component of the cell walls of gram-negative bacteria. Transforming growth factor beta (10 μg) and IL-6 (10 μg) coinjected intratracheally with LPS (10 μg) each inhibited the number of neutrophils in 6-hour bronchoalveolar lavage (BAL) specimens by approximately 50%. The intratracheal coinjection of IL-6, TGFβ, and LPS inhibited the LPS-induced neutrophilic inflammatory exodus by nearly 75%. Interleukin-6 also is shown to be endogenously upregulated within the lung after intratracheal challenge with endotoxin, providing evidence that IL-6 may represent an endogenous negative feedback mechanism to inhibit endotoxin-initiated cytokine-mediated acute inflammation. Interleukin-6 and TGFβ both strongly inhibited the quantity of TNF-α recovered in the BAL fluid of LPS-challenged rats, suggesting that downregulation of LPS-induced TNF-α production within the lung represents one mechanism whereby IL-6 and TGFβ exert an antiinflammatory action. Interleukin-6 and TGFβ represent novel pharmacologic and, probably, endogenous inhibitors of acute inflammation. (Am J Pathol 1991, 138:1097–1101)

Interleukin-6 (IL-6) and transforming growth factor beta (TGFβ) are multifunctional members of a family of proteins, so called cytokines, that include the proinflammatory factors interleukin-1 (IL-1) and tumor necrosis factor (TNF). Cytokines are released by inflammatory and parenchymal cells in response to endotoxin (LPS), a proinflammatory lipopolysaccharide component of the cell walls of gram-negative bacteria. Cytokines are thought to orchestrate endotoxin-initiated acute inflammation (ie, the migration of neutrophils from blood vessels into tissues). Cytokines also contribute to many of the systemic effects of endotoxemia, such as neutrophilia, lymphopenia, fever, the synthesis of acute-phase proteins by the liver, and hypotensive shock.[1–3]

Intratracheal injection of endotoxin upregulates IL-1 and TNF expression in the lung and results in severe local acute inflammation.[4] Intratracheal injection of either IL-1 or TNF also induces acute inflammation in the lung.[4] Interleukin-1 is a far more potent inducer of acute inflammation than TNF in the lung as well as in other anatomic locations.[4-6]

The role of IL-6 in local acute inflammation has not, to our knowledge, been defined. Intravenous IL-6, however, was reported by Aderka et al[7] to decrease intravenous endotoxin-induced serum TNF levels in mice. Our laboratory has confirmed that intravenous IL-6 decreases intravenous endotoxin-induced serum TNF levels in rats.[8]

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In addition, and perhaps more importantly in regard to the pathogenesis of acute inflammation, we have observed that pretreatment with IL-6 decreases IL-1 mRNA levels in splenic and hepatic whole-organ RNA extracts of endotoxemic rats.[8] Because IL-6 downregulates endotoxin-induced TNF and IL-1 expression, and because IL-1 and TNF are thought to be proximal mediators of endotoxin-induced inflammation, an experimental animal model was created to test the hypothesis that IL-6 would downregulate LPS-induced acute inflammation and to search for local LPS-induced IL-6 expression.

The role of TGFβ in acute inflammation also has not, to our knowledge, been defined. Transforming growth factor beta, similar to IL-6, has been reported to be present in a variety of cell types, including activated macrophages.[9] Transforming growth factor beta has multiple complex growth stimulatory and growth inhibitory effects on mesenchymal and epithelial cells.[10] It is also a potent immunosuppressive factor. Transforming growth factor beta is present in rheumatoid synovial fluids and can inhibit IL-1–induced lymphocyte proliferation.[9] It has been reported to suppress the production of TNFα by LPS-stimulated macrophages.[11] In light of the known immunosuppressive effects of TGFβ, its possible role as an inhibitor of acute neutrophilic inflammation was investigated. Finally, because both IL-6 and TGFβ were found to act as pharmacologic inhibitors of local LPS-induced acute inflammation, we tested the anti-inflammatory effect of combined IL-6 and TGFβ.

Materials and Methods

Rats (congenic male Lewis rats weighing approximately 225g and anesthesized with ether) were injected intratracheally with equal volumes (0.5 ml) of various doses and combinations of either endotoxin (*S. Typhus* lipopolysaccharide, Sigma Chemical Co., St. Louis, MO), recombinant human IL-6, or recombinant human $TGF\beta_1$ (gifts of Dr. Lawrence Souza, Amgen, Inc., Thousand Oaks, CA). Six hours later, the rats were killed and bronchoalveolar lavage (BAL) *via* the instillation of a tracheal catheter was performed to enumerate the absolute number of neutrophils in the intra-alveolar inflammatory exudate. The 6-hour timepoint previously has been documented by our laboratory[4] to represent the peak of the neutrophilic inflammatory exodus. Experiments were performed by randomly dividing rats into experimental groups to be injected on the same day with either LPS or with LPS plus IL-6 or TGFβ. A typical day's experiment consisted of injecting three rats intratracheally with LPS alone and three rats intratracheally with LPS plus a cytokine. The absolute number of neutrophils in the BAL specimens of the rats from each experimental group is expressed as the mean plus or minus one standard error of the mean. The probability value was determined by the two-tailed *t*-test for unpaired data. Histologic examination of Bouin's-fixed paraffin-embedded sections of the lungs after BAL was performed to evaluate the accumulation of neutrophils within pulmonary parenchyma and the adequacy of BAL. Tumor necrosis factor protein determinations in BAL fluid were determined as previously described.[12] Whole-lung RNA extraction, Northern blotting, and hybridization for IL-6 mRNA were performed as previously described.[12,13]

Results

The LPS-induced acute inflammatory exodus was inhibited by an average of 61% in rats receiving 10 μg IL-6 plus 1 μg LPS ($n = 7$), as opposed to rats receiving 1 μg LPS alone ($n = 9$) ($p < 0.0001$, Table 1). The LPS-induced neutrophilic exodus was inhibited by an average of 53% in rats receiving 10 μg IL-6 plus 10 μg LPS ($n = 18$), as opposed to rats receiving 10 μg LPS alone ($n = 18$) ($p < 0.0001$, Table 1). Intratracheal injection of IL-6 alone caused the accumulation of a very small number of neutrophils (less than 10^6 neutrophils/BAL, $n = 2$). Intratracheal injection of saline causes no or at most a very slight accumulation of neutrophils in BAL fluid.[4,14]

After having demonstrated that IL-6 can act as a pharmacologic downregulator of LPS-induced local acute inflammation, we wondered if there was evidence that host

Table 1. *IL-6 and TGFβ Inhibit Intratracheal LPS-induced Acute Inflammation*

Intratracheal injection	n	Neutrophils × 10^{-6}	Inhibition (%)	*P* value
LPS (1 μg)	9	6.36 ± 0.52	61	0.0001
LPS (1 μg) + IL-6 (10 μg)	7	2.45 ± 0.54		
LPS (10 μg)	18	20.86 ± 0.17	53	0.0001
LPS (10 μg) + IL-6 (10 μg)	18	9.84 ± 0.12		
LPS (10 μg)	6	12.67 ± 1.10	35	0.02
LPS (10 μg) + TGFβ (1 μg)	6	8.22 ± 1.20		
LPS (10 μg)	14	18.74 ± 1.06	54	0.0001
LPS (10 μg) + TGFβ (10 μg)	13	8.55 ± 0.57		

LPS, Lipopolysaccharide, TGFβ, transforming growth factor beta.

IL-6 is upregulated locally after the intratracheal injection of LPS and may therefore act as an endogenous negative feedback mechanism to stop the neutrophilic exodus that is the cellular hallmark of acute inflammation. Interleukin-6 mRNA expression was determined in whole-lung RNA extracts by Northern blotting using a rat IL-6 cDNA probe[13] at sequential timepoints after the intratracheal or intravenous injections of 100 μg LPS. Intratracheal injection of LPS caused IL-6 mRNA expression beginning at 2 hours and increasing markedly at 4 hours (Figure 1). In a further study of pulmonary IL-6 mRNA expression at later timepoints after intratracheal injection of LPS, IL-6 mRNA expression was found to remain at a very high level at 6 hours with a return to preinjection levels at 12, 24, and 48 hours (data not shown). In contrast to the kinetics of IL-6 expression after the intratracheal injection of LPS, intravenous injection of LPS caused IL-6 mRNA expression to peak at 2 hours with a substantial decrease toward baseline level by 4 hours (Figure 1).

The LPS-induced acute inflammatory exodus was inhibited by an average of 54% in rats receiving 10 μg TGFβ plus 10 μg LPS ($n = 13$), as opposed to rats receiving 10 μg LPS alone ($n = 14$) ($p < 0.0001$, Table 1). The LPS-induced accumulation of neutrophils was inhibited by 35% in rats receiving 1 μg TGFβ plus 10 μg LPS ($n = 6$), as opposed to rats receiving 10 μg LPS alone ($n = 6$) ($p < 0.02$, Table 1). Intratracheal injection of TGFβ alone did not cause a significant influx of neutrophils into the lungs (less than 0.1×10^6 neutrophils/BAL).

The combined effects of IL-6 and TGFβ on LPS-induced acute neutrophilic inflammation then were investigated. All four experimental groups (10 μg LPS alone, 10 μg LPS plus 10 μg IL-6, 10 μg LPS plus 10 μg TGFβ, and 10 μg LPS plus 10 μg IL-6 plus 10 μg TGFβ) were studied concurrently. The combination of IL-6 plus TGFβ inhibited the LPS-induced neutrophilic exodus by 74%, as opposed to 35% inhibition with IL-6 alone and 56% inhibition with TGFβ alone (Table 2). The combined inhibitory effect of IL-6 and TGFβ was significantly greater than the inhibitory effect of either cytokine alone ($p < 0.008$).

Histologic examination of the lungs in all four experimental groups ($n = 2$ rats from each group) was performed after BAL to exclude the possibility that the decrease in BAL neutrophils might be due to an increase in neutrophils remaining in the lungs of cytokine-treated rats (as might occur, for example, if IL-6 or TGFβ induced the expression of adhesion molecules that caused the neutrophils to adhere more avidly to pulmonary parenchyma). Double-blind microscopic evaluation of the lungs revealed an indistinguishable histologic appearance in the LPS-plus-cytokine–treated groups. The LPS-alone–treated group was distinguished from the other three groups by a greater number of neutrophils. One may therefore conclude that the degree of cytokine-induced inhibition of acute neutrophilic inflammation as measured by BAL is, if anything, an underestimation of the true amount of inhibition. In none of the experimental groups (not even after intratracheal injection of 10 μg LPS

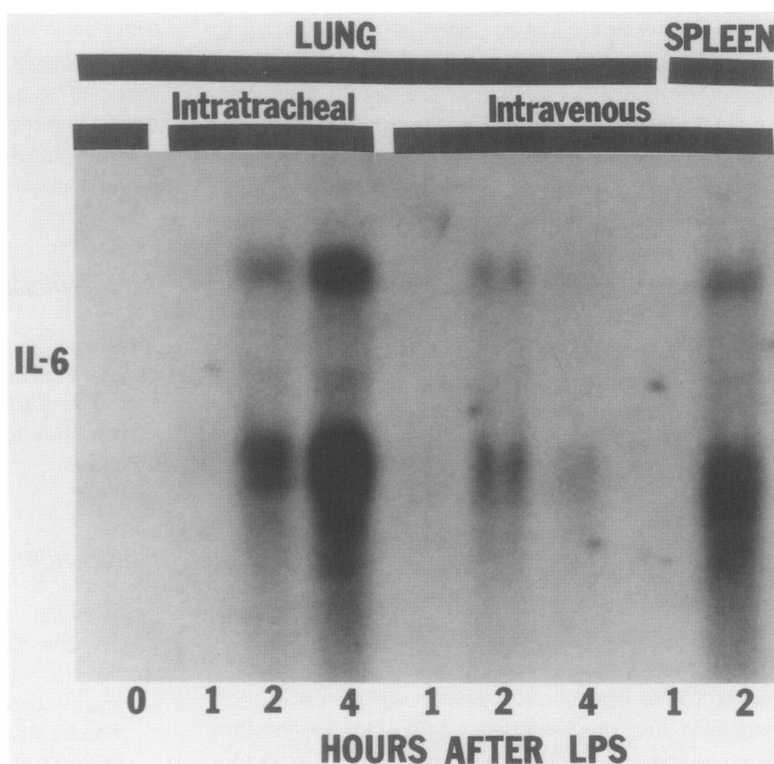

Figure 1. *IL-6 mRNA is not constitutively detected in whole-lung RNA preparations of native rats (time 0) or 1 hour after either intratracheal or intravenous injections of endotoxin. IL-6 mRNA is detectable in the lung at similar levels at 2 hours after either the intratracheal or intravenous injections of endotoxin. At 4 hours after the intratracheal injection of endotoxin, the level of pulmonary IL-6 mRNA has increased dramatically, whereas at the same timepoint after the intravenous injection of endotoxin the level of IL-6 has almost returned to preinjection level. The 4-hour timepoint marks the beginning of the neutrophilic exodus into alveoli after the intratracheal, but not the intravenous, injection of endotoxin.[4] The expression of splenic IL-6 mRNA in a whole-organ RNA preparation at 2 hours after the intravenous injection of endotoxin is shown for comparison. All lanes of the Northern blot were loaded with 25 μg whole-organ RNA and hybridized with rat IL-6 cDNA. The rat expresses two major IL-6 mRNA species that code for the same protein.[13]*

Table 2. *The Combination of IL-6 and TGFβ Inhibits Intratracheal LPS-induced Acute Inflammation More Than Either Cytokine Alone*

Intratracheal injection	n	Neutrophils × 10^{-6}	Inhibition (%)	*P* value
LPS (10 μg)	8	20.75 ± 1.42	—	—
LPS (10 μg) + IL-6 (10 μg)	8	13.44 ± 1.39	35	0.002
LPS (10 μg) + TGFβ (10 μg)	7	9.10 ± 0.88	56	0.0001
LPS (10 μg) + IL-6 (10 μg) + TGFβ (10 μg)	8	5.50 ± 0.75	74	0.0001

LPS, lipopolysaccharide; TGFβ, transforming growth factor beta.

alone) was any evidence of architectural or cellular injury to the lung observed at the light microscopic level. The LPS-induced inflammatory reaction was histologically characterized by the appearance of a modest number of neutrophils in alveolar septae, alveolar spaces, and in bronchial mucosa, as previously illustrated by our laboratory.[4] Tumor necrosis factor protein levels in the BAL fluids of the same rats were measured and were found to be decreased by 66% in both LPS-plus-IL-6– and LPS-plus-TGFβ–treated rats and to be decreased by 88% in LPS-plus-IL-6-plus-TGFβ–treated rats as compared to LPS-alone–treated rats (274 ± 30 U TNF/BAL after LPS alone, 94 ± 36 U TNF/BAL after LPS plus IL-6, 96 ± 38 U TNF/BAL after LPS plus TGFβ, and 34 ± 17 U TNF/BAL after LPS plus IL-6 plus TGFβ).

Discussion

Although much new information has emerged during the past few years regarding the acute proinflammatory effects of such cytokines as IL-1 and TNF, relatively little is known about endogenous downregulators of acute inflammation. In the present study, IL-6 and TGFβ, alone and especially in combination, are shown to inhibit strongly LPS-induced acute inflammation. Interleukin-6 and TGFβ thus represent novel antiinflammatory agents of potential therapeutic utility in clinical situations of cytokine-mediated acute inflammation.

Neutrophils first appear in BAL fluids 4 hours after intratracheal injection of endotoxin,[4] whereas neutrophils are not present in BAL fluid 4 hours after the intravenous injection of endotoxin. The marked increase in pulmonary IL-6 mRNA after intratracheal as opposed to intravenous injection of endotoxin therefore may be, in part, neutrophil derived, especially because Cicco et al[15] recently reported that neutrophils express IL-6 in response to stimulation with endotoxin *in vitro*. The concept that neutrophils may downregulate their own accumulation *via* the expression of IL-6 is attractive, but our data do not allow us to quantitate the probable increased contribution of alveolar macrophage-derived IL-6 after intratracheal administration as opposed to intravenous administration of endotoxin. Endotoxin-stimulated cells of the monocyte–macrophage lineage are known to secrete large amounts of IL-6.[16] The amounts of IL-6 and TGFβ that are released into the alveolar fluid of the lung under pathophysiologic conditions are unknown. Interleukin-6 is known, however, to attain concentrations as high as 2 μg/ml in the serum of endotoxemic rats,[8] suggesting that microgram quantities of endogenous IL-6 also might be attainable in the lung during gram-negative pneumonia.

In conclusion, pharmacologic administration of exogenous recombinant IL-6 and TGFβ inhibits endotoxin-induced local acute inflammation. The mechanism of the anti-inflammatory action of IL-6 and TGFβ may relate to the ability of these cytokines to inhibit TNFα[8,11] (and as shown by the present data) and IL-1 production[8] by macrophages. Host-derived IL-6 is upregulated locally after challenge with LPS and may act as an endogenous-negative feedback mechanism to inhibit the LPS-initiated IL-1– and TNF-mediated acute inflammatory process. Finally host macrophage-derived TGFβ may be speculated also to play a role as an endogenous inhibitor of acute inflammation. Transforming growth factor beta has been reported by Roberts et al[17] to increase collagen synthesis *in vitro* and to cause fibrosis *in vivo* after intradermal injection. Transforming growth factor beta is known to be a pleiotropic mediator and may be hypothesized to perform a dual role by acting both to resolve acute inflammation and to begin tissue repair and remodeling by initiating collagen deposition.

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