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Intratracheal Administration of Endotoxin and Cytokines

VII. The Soluble Interleukin-1 Receptor and the Soluble Tumor Necrosis Factor Receptor II (p80) Inhibit Acute Inflammation

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Intratracheal administration of endotoxin (LPS) causes acute neutrophilic inflammation via induction of pulmonary tumor necrosis factor α (TNF) and interleukin-1 (IL-1) expression. In the present study, the anti-inflammatory activity of soluble IL-1 receptor (sIL-1r) and soluble TNF receptor p80 (sTNFr-p80) in LPS-induced acute pulmonary inflammation was investigated. The sIL-1r coinjected intratracheally with LPS in rats significantly inhibits neutrophilic exudation into bronchoalveolar lavage (BAL) fluid by 47% after 6 hr compared to injection of LPS alone. TNF and IL-6 in the same BAL fluids were both lowered by approximately 50% after intratracheal coinjection of sIL-1r and LPS as compared to LPS alone. In the same model, the sTNFr-p80 inhibited acute inflammation. Paradoxically, TNF levels in BAL fluids were generally elevated after the intratracheal coinjection of LPS and monomeric sTNFr-p80 compared to injection of LPS injection alone. The combined anti-inflammatory effect of sIL-1r and sTNFr-p80 at the maximally effective individual doses is not significantly greater than the effect of either soluble receptor alone.

INTRODUCTION

The intratracheal injection of endotoxin, a lipopolysaccharide (LPS) component of the cell walls of gram-negative bacteria, upregulates interleukin-1 (IL-1) and tumor necrosis factor (TNF) expression in the lung and results in severe local acute inflammation (1). Intratracheal injection of either IL-1 or TNF also induces acute inflammation in the lung (1). The IL-1 receptor antagonist (IL-1ra) has been shown to inhibit LPS- or IL-1-induced acute pulmonary inflammation (2). Transmembrane and soluble receptors for both IL-1 and TNF have been cloned and expressed in recombinant form (3–5). Two distinct TNF receptors of molecular weights of approximately 60 kDa(p60) and 80 kDa(p80) are identified and are expressed in different anatomic portions of lymph nodes (6). Although our laboratory originally used the designation of "Type I" to refer to the p60 form (7) and "Type II" to refer to the p80 form of the receptor, other investigators have used the reverse terminology so that the designations p60 and p80 will be used in the present report to avoid confusion regarding terminology. IL-1 and TNF soluble receptors most likely compete for IL-1 and TNF *in vivo* with the corresponding membrane-bound, biologically active receptors for IL-1 and TNF. IL-1 and TNF soluble receptors therefore are likely to act as endogenous molecules to downregulate the proinflammatory actions of IL-1 and TNF. The TNF p60 soluble receptor was recently shown to inhibit LPS-induced acute inflammation in the lung (7). The purpose of the present study is to demonstrate the pharmacologic anti-inflammatory activity of the sIL-1r and the sTNFr-p80, to study the combined anti-inflammatory effect of sIL-1r and sTNFr-p80, and to investigate the effects of exogenously administered sIL-1r and sTNFr-p80 on LPS-initiated endogenous TNF and IL-6 expression *in vivo*.

MATERIALS AND METHODS

Male Lewis rats weighing approximately 225 g were anesthesized with ether and injected intratracheally with equal volume (0.5 ml) of various doses and combinations of either endotoxin (S. Typhus lipopolysaccharide, Sigma Chemical Co., St Louis, MO), recombinant murine sIL-1r (Immunex, Seattle, WA), and the monomeric or dimeric-Fc construct forms of sTNFr-p80 (Immunex, Seattle, WA). One microgram of monomeric sTNFr is the molar equivalent of 2 μg of dimeric sTNFr because the Fc portion of the dimeric sTNFr-Fc construct accounts for one-half the molecular weight of the dimeric construct. Six hours after intratracheal injection the animals were sacrificed and bronchoalveolar lavage (BAL) was performed to enumerate the absolute number of neutrophils in the intraalveolar inflammatory exudate as previously described (1). The absolute number of neutrophils in the BAL fluid specimens is expressed as the mean ± one standard deviation of the mean. TNF and IL-6 protein levels in BAL fluid specimens were determined as previously described (WEHI 164, subclone 13 bioassay for TNF, B9

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bioassay for IL-6) (8). The probability value was determined by the t test (Systat, Inc., Evanston, IL).

RESULTS

After intratracheal coinjection of 5 μg LPS and 30 μg sIL-1r, the LPS-induced exodus of neutrophils was significantly inhibited by an average of 47% in rats at 6 hr ($n = 9$), compared to rats receiving 5 μg of LPS alone ($n = 9$) (Table 1). However, the accumulation of neutrophils in BAL fluid was not significantly inhibited in rats at 6 hr after intratracheal coinjection of 5 μg LPS and 10 μg sIL-1r ($n = 12$) compared to 5 μg LPS alone ($n = 10$).

The TNF and IL-6 levels in BAL fluid from the rats receiving intratracheal coinjection of LPS (5 μg) and sIL-1r (30 μg) ($n = 9$) were lowered by 40% ($P = 0.07$, i.e., statistically not quite significant) and 50% ($P = 0.02$) (Table 2), respectively, compared to the BAL fluid of rats receiving LPS alone ($n = 9$).

Intratracheal coinjection of LPS (5 μg) and sTNFr-p80 (40 or 60 μg) significantly inhibited acute neutrophilic exodus by approximately 34 to 43% at 6 hr compared to intratracheal injection of LPS (5 μg) alone (Table 3). In contrast, intratracheal injection of LPS (5 μg) and a dimeric sTNFr-p80-Fc construct at doses of 20, 40, 80, and 120 μg did not cause a significant decrease in neutrophil numbers in BAL fluid ($n = 14$ rats treated with dimeric sTNFr-p80 at differing doses, data not shown).

The measurement of TNF levels in the BAL fluids of rats treated with LPS, LPS plus monomeric sTNFr-p80, and LPS plus dimeric sTNFr-p80-Fc construct were somewhat paradoxical (Table 4). The combination of LPS plus monomeric sTNFr-p80 that showed a significant anti-inflammatory effect did not show a decrease in TNF activity compared to rats injected with LPS alone. In fact, the coinjection of monomeric sTNFr-p80 (40 μg) with LPS (5 μg) actually increased TNF activity ($P < 0.03$). In the same experiment, on the other hand, the coinjection of the dimeric sTNFr-p80-Fc construct that did not show any anti-inflammatory activity decreased the mean TNF activity in BAL fluid compared to injection of LPS alone and significantly decreased TNF activity compared to the monomeric sTNFr-p80 ($P < 0.006$). In another set of experiments using higher doses of monomeric and dimeric sTNFr-p80 (Table 4), the dimeric receptor coinjected with LPS significantly decreased TNF activity compared to either LPS alone ($P < 0.02$) or LPS coinjected with the monomeric receptor ($P < 0.001$).

The combined effects of sIL-1r and monomeric sTNFr-p80 (Table 5) were investigated in an experiment involving four experimental groups (5 μg LPS alone, 5 μg LPS + 30 μg sIL-1r, 5 μg LPS + 40 μg sTNFr-p80, and 5 μg LPS + 30 μg sIL-1r + 40 μg sTNFr-p80). The combination of sIL-1r and sTNFr-p80 inhibited the LPS-induced inflammation by an average of 47% ($P < 0.00002$), whereas the sIL-1r or sTNFr-p80 alone caused 37% ($P < 0.007$) and 28% ($P < 0.02$) inhibition, respectively. The anti-inflammatory effect of the combination of sIL-1r and sTNFr-p80 was not statistically significantly greater than the effect of either soluble cytokine receptor alone.

TABLE 1
The sIL-1r Inhibit Intratracheal LPS-induced Acute Inflammation in Rats

Intratracheal injection	n	Neutrophils × 10^{-6}	Inhibition (%)	P value
LPS (5 μg)	10	14.3 ± 1.2		
LPS (5 μg) + sIL-1r (10 μg)	12	11.3 ± 2.8	21.0	NS
LPS (5 μg)	9	16.4 ± 2.2		
LPS (5 μg) + sIL-1r (30 μg)	9	8.7 ± 2.1	47.0	0.0001

TABLE 2
The sIL-1r Decreases LPS-Induced TNF and IL-6 Activity in BAL Fluid

Intratracheal injection	n	TNF(pg/BAL)	IL-6(pg/BAL)
LPS (5 μg)	9	11.4 ± 5.5	268.8 ± 146.1
LPS (5 μg) + sIL-1r (30 μg)	9	6.8 ± 4.5	130.3 ± 81.0

DISCUSSION

The intratracheal injection of LPS provides a simple and reproducible animal model to study the inflammatory cytokine cascade *in vivo*. An understanding of the cytokine cascade will be useful to our understanding of the pathogenesis of both infectious and noninfectious

TABLE 3
The sTNFr-p80 Inhibits Intratracheal LPS-induced Acute Inflammation in Rats

Intratracheal injection	n	Neutrophils × 10^{-6}	Inhibition (%)	P value
LPS (5 μg)	4	16.5 ± 5.4		
LPS (5 μg) + sTNFr-p80 (10 μg)	4	13.2 ± 0.8	20.0	NS
LPS (5 μg)	2	15.8 ± 1.3		
LPS (5 μg) + sTNFr-p80 (20 μg)	2	14.1 ± 1.1	11.4	NS
LPS (5 μg)	6	14.8 ± 2.5		
LPS (5 μg) + sTNFr-p80 (40 μg)	6	8.4 ± 2.2	43.3	0.001
LPS (5 μg)	9	19.1 ± 3.9		
LPS (5 μg) + sTNFr-p80 (60 μg)	9	12.6 ± 3.8	34.0	0.002

TABLE 4

Monomeric sTNFr-p80 Increases Whereas Dimeric sTNFr-p80 Decreases LPS-Induced TNF Activity in BAL Fluid

Intratracheal injection	*n*	TNF(pg/BAL)
LPS (5 μg)	6	1970 ± 1338
LPS (5 μg) + sTNFr-p80 monomer (40 μg)	6	3075 ± 1218
LPS (5 μg) + sTNFr-p80 dimer (80 μg)	6	939 ± 793
LPS (5 μg)	4	3755 ± 1510
LPS (5 μg) + sTNFr-p80 monomer (60 μg)	4	5793 ± 1140
LPS (5 μg) + sTNFr-p80 dimer (120 μg)	2	848 ± 504
LPS (5 μg) + sTNFr-p80 dimer (288 μg)	2	515 ± 357

inflammatory diseases. The intratracheal injection of LPS may be a model for the human acute respiratory disease caused by the inhalation of endotoxin-contaminated grain dust. Although LPS most likely initiates a substantial portion of the acute inflammatory response observed during gram-negative pneumonia, one must remember that the intratracheal injection of sterile LPS is not a model of septic gram-negative bacterial pneumonia.

The sIL-1r and sTNFr-p80 significantly inhibit LPS-initiated local acute neutrophilic inflammation in the lung. The anti-inflammatory activity of IL-1ra (2) and of sTNFr-p60 (7) have been previously documented by our laboratory in the same model of acute pulmonary inflammation. Of note given the different mechanisms of action of the two molecules is that both the sIL-1r, a soluble receptor, and the IL-1ra, a competitive receptor antagonist, inhibit acute inflammation. Endogenous sIL-1r, IL-1ra, and TNF soluble p60 and p80 receptors may all be postulated to play an important role in the downregulation of the IL-1- and TNF-orchestrated acute inflammatory response *in vivo*.

The observation that IL-1sr decreases both TNF and IL-6 activity in BAL fluid suggests that IL-1 may play a role in the induction of TNF and IL-6 expression. IL-1 has previously been demonstrated by many investigators to induce TNF expression in a variety of experimental systems (9). IL-1 and TNF are both also known to play a significant role in the induction of IL-6 expression, perhaps as a negative feedback mechanism, since IL-6 downregulates TNF and IL-1 expression (8). IL-6 also inhibits LPS-initiated acute pulmonary inflammation (10). Given the fact that neutrophils can synthesize both TNF (11) and IL-6 (12), the possibility must be considered that the decrease in TNF and IL-6 activity in BAL fluid represents at least in part a decrease in the number of neutrophils in BAL fluid as opposed to a decrease in IL-1 induced alveolar macrophage-derived TNF and IL-6.

TABLE 5

The Combination of Maximally Effective Doses of sIL-1r and sTNFr-p80 Does Not Cause Significantly More Inhibition of LPS-Induced Acute Inflammation Than Either Soluble Receptor Alone

Intratracheal injection	*n*	Neutrophils × 10^{-6}	Inhibition (%)	*P* value
LPS (5 μg)	8	12.8 ± 1.5		
LPS (5 μg) + sIL-1r (30 μg)	7	8.1 ± 3.2	36.8	0.007
LPS (5 μg) + sTNFr (40 μg)	5	9.2 ± 2.4	28.2	0.02
LPS + sIL-1r + sTNFr	6	6.8 ± 1.6	46.9	0.00002

The documentation that monomeric sTNFr-p80 significantly inhibits LPS-initiated acute inflammation is not unexpected since monomeric sTNFr-p60 has been previously observed to inhibit neutrophil emigration in the same model (7). Somewhat surprising, however, was the failure of the dimeric sTNFr-p80-Fc construct to block inflammation, especially since the dimeric construct was very effective in inhibiting TNF bioactivity. Of relevance in this regard is that intratracheal injection of an antiserum to TNF (13) that quite effectively blocked LPS-induced TNF activity in BAL fluid also did not inhibit inflammation (unpublished observations). The characteristics of the dimeric sTNFr-p80-Fc construct and the aforementioned TNF antiserum that appear to allow neutralization of cytotoxic TNF bioactivity while not inhibiting inflammation in our model are unclear. The failure to inhibit inflammation might be attributable to the proinflammatory potential of the two molecules' Fc regions although many investigators have convincingly demonstrated that other TNF antisera (14, 15) as well as the dimeric sTNFr-p80-Fc construct (16) are effective *in vivo* in the inhibition of endotoxic shock. The dimeric sTNFr-p80 is, in fact, much more potent than monomeric sTNFr-p80 in inhibiting endotoxic shock (16).

The paradoxical elevation of TNF activity in the BAL fluid of rats coinjected with LPS and the monomeric sTNFr-p80 was not surprising in light of our recent observation that sTNFr-p60, while inhibiting inflammation, also causes a paradoxical increase in LPS-initiated TNF activity in BAL fluid (7). A paradoxical increase in TNF in the sera of endotoxemic baboons treated with sTNFr has been previously described (17). The increase in TNF may be postulated to represent a dissociation of TNF from functionally sequestered TNF within TNF–sTNFr complexes (18). The sequestered TNF may bypass normal catabolism via internalization by target cells or digestion by neutrophil-derived proteases (19).

The observation that the combination of sIL-1r and sTNFr-p80 at maximally effective doses does not inhibit acute inflammation significantly more than either soluble receptor alone is consistent with our previous finding that the combination of sTNFr-p60 and IL-1ra does not provide significantly greater anti-inflammatory activity than either factor alone (7).

Nevertheless, the average inhibition of inflammation provided by blocking both IL-1 and TNF was slightly greater than that afforded by blocking only one cytokine. In addition, sIL-1r and sTNFr may produce synergistic effects when simultaneously administered at low (i.e., individually suboptimal) doses. Thus, the possibility remains that combination therapy with inhibitors of IL-1 and TNF may in the future prove beneficial in the treatment of inflammation.

In conclusion, recombinant sIL-1r and sTNFr-p80 are demonstrated to inhibit acute neutrophilic inflammation in the lung. The role of soluble IL-1 and TNF receptors in the endogenous downregulation of acute inflammatory diseases remains to be fully elucidated.

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