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Short Communication

Intratracheal Administration of Endotoxin and Cytokines

IV. The Soluble Tumor Necrosis Factor Receptor Type I Inhibits Acute Inflammation

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Endotoxin lipopolysaccharide (LPS) administered intratracheally to rats causes pulmonary tumor necrosis factor α (TNF) and interleukin-1 (IL-1) production and results in acute bronchoalveolar neutrophilic inflammation. In the present study, the recombinant human TNF soluble receptor type I (sTNFrI) co-injected intratracheally with LPS is shown to inhibit significantly (P < 0.0001) the number of neutrophils in bronchoalveolar lavage specimens at 6 hours as compared to intratracheal injection of LPS alone. The sTNFrI was at least as effective as the recombinant human IL-1 receptor antagonist (IL-1ra) as an inhibitor of acute inflammation. Inhibition of LPS-induced acute inflammation by the combination of sTNFrI and IL-1ra was not significantly more than the inhibition afforded by sTNFrI alone. Intratracheal co-injection of sTNFrI with LPS unexpectedly increased TNF levels in BAL specimens, perhaps by changing the normal catabolism of TNF. On the other hand, co-injection of sTNFrI and LPS decreased IL-6 levels in BAL fluid, most likely by interfering with the induction of IL-6 by TNF. The sTNFrI may prove to be an important pharmacological down-regulator of acute inflammation. (Am J Pathol 1993, 142: 1335–1338)

Lipopolysaccharide (LPS) is a constituent of the cell walls of gram-negative microorganisms that contributes to the local inflammation and systemic toxicity of gram-negative infections. LPS up-regulates the expression of tumor necrosis factor (TNF) and interleukin-1 (IL-1) *in vitro* in alveolar macrophages.[1] Intratracheal injection of LPS up-regulates TNF and IL-1 expression in the lung *in vivo* and results in severe local acute inflammation.[1,2] Intratracheal injection of either TNF or IL-1 also causes acute inflammation in the lung.[1] The IL-1 receptor antagonist (ra) has recently been shown to be pharmacologically effective in down-regulating intratracheal LPS-induced acute inflammation.[3] The soluble TNF receptor type I (sTNFrI) has been cloned and expressed in recombinant form.[4] The purpose of the present study is to demonstrate that the sTNFrI is pharmacologically effective at inhibiting acute inflammation, to compare the anti-inflammatory activity of sTNFrI with the activity of IL-1ra, to study the anti-inflammatory effect of sTNFrI combined with IL-1ra, and to study the effect of sTNFrI on endogenous TNF and IL-6 concentrations within an acute inflammatory effusion.

Materials and Methods

Intratracheal injection of Salmonella typhus LPS (Sigma Chemical Co., St. Louis, MO), sTNFrI (Synergen, Inc., Boulder, CO), and IL-1ra (Synergen) into male Lewis viral antigen-free rats was as previ-

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ously described by our laboratory.[1-3] Bronchoalveolar lavage (BAL) fluid was collected at various time points (2 to 12 hours) after intratracheal injection, and BAL neutrophils were enumerated also as previously described.[1-3] The number of neutrophils per BAL in each experimental group is expressed as the mean ± one standard deviation. TNF and IL-6 protein levels in BAL fluids were assayed as previously described (WEHI 164, subclone 13 bioassay for TNF, B9 bioassay for IL-6) and are expressed as the mean ± one standard error.[5] Statistical analysis was performed by the *t*-test for unpaired data.

Results

Intratracheal co-injection of LPS (5 μg) and sTNFrI (10 μg or 100 μg) significantly ($P < 0.0001$) inhibits acute neutrophilic inflammation by approximately 50% after 6 hours as compared to the intratracheal injection of LPS (5 μg) alone (Table 1). During each day's experiment, the two experimental groups (LPS plus sTNFrI, usually $n = 3$ rats, versus LPS alone, usually $n = 3$ rats) were run contemporaneously. The experiments were repeated on 4 different days at the dose of 10 μg sTNFrI and on eight different days at the dose of 100 μg sTNFrI. Essentially no neutrophils are present in rats receiving intratracheal injections of vehicle alone (Table 1). TNF and IL-6 protein levels in BAL fluids from a representative day's experiment (Table 2) show an approximately 90-fold higher level in TNF ($P < 0.05$) and a 50% lower level in IL-6 ($P < 0.05$).

A comparison of the anti-inflammatory activity of sTNFrI to the activity of IL-1ra at the maximally effective doses of each agent was undertaken. During the same experiments, the anti-inflammatory activity of the combination of sTNFrI and IL-1ra was determined. The sTNFrI ($P < 0.003$, $n = 8$), the IL-1ra ($P < 0.03$, $n = 8$), and the combination of the sTNFrI and IL-1ra ($P = 0.0015$, $n = 8$) all significantly inhibited the exodus of neutrophils as compared to LPS alone ($n = 8$) in experiments repeated on four different days in which two rats were contemporaneously studied in each experimental group on each of the four days. The sTNFrI (63% inhibition of neutrophil accumulation) was on average more effective than IL-1ra (46% inhibition of neutrophil accumulation) in inhibiting acute inflammation (Table 3), although the difference did not reach statistical significance ($P = 0.12$). The combination of sTNFrI and IL-1ra was not significantly more effective than sTNFrI alone (Table 3). TNF and IL-6 protein levels in the BAL fluids from a representative day's experiment show that the presence of sTNFrI either alone or in combination with IL-1ra causes a greater than 30-fold increase in TNF in the BAL fluid and a decrease in IL-6 levels. IL-1ra does not substantially affect either TNF or IL-6 levels in BAL fluid (Table 4).

A kinetic study of the anti-inflammatory effect of sTNFrI was undertaken (Figure 1) at 1, 2, 4, 6, and 12 hours after the intratracheal injection of LPS with and without sTNFrI. Inhibition of neutrophil influx afforded by sTNFrI was again documented at 6 hours, but was no longer apparent at 12 hours after the injection of LPS. Also in concurrence with the results of our previous experiments, TNF bioactivity in BAL fluid was paradoxically elevated at 6 and 12 hours after co-injection of LPS plus sTNFrI as compared to TNF activity after injection of LPS alone (Figure 2). IL-6 bioactivity was again diminished at 6 hours after co-injection of sTNFrI with LPS (Figure 2).

Table 2. *Intratracheal Co-Injection of sTNFrI and LPS Increases TNF and Decreases IL-6 Levels in BAL Fluid as Compared to Intratracheal Injection of LPS Alone*

Intratracheal injection	n	TNF (U/BAL)	IL-6 (ng/BAL)
LPS	3	4 ± 1	61 ± 3
LPS+sTNFrI	4	362 ± 98	30 ± 9

Discussion

The sTNFrI is demonstrated to very significantly inhibit an LPS-induced local acute inflammatory reaction. The observation that the sTNFrI inhibits acute inflammation to the same extent as the combination of

Table 1. *sTNFrI Inhibits Acute Neutrophilic Inflammation after Intratracheal Injection of LPS*

Experimental group	*n*	PMNs/BAL	*P* value
LPS (5 μg)	12	$17.8 \pm 3.9 \times 10^6$	0.0001
LPS (5 μg) + sTNFrI (10 μg)	13	$8.5 \pm 3.2 \times 10^6$	
LPS (5 μg)	24	$16.3 \pm 5.1 \times 10^6$	0.0001
LPS (5 μg) + sTNFrI (100 μg)	24	$8.3 \pm 3.4 \times 10^6$	
Vehicle	5	$0.021 \pm 0.01 \times 10^6$	

PMNs = neutrophils.

Table 3. *sTNFrI+IL-1ra Is No More Effective than sTNFrI at Inhibiting Intratracheal LPS-Induced Acute Inflammation*

Experimental group	*n*	PMNs/BAL	% Inhibition
LPS (5 μg)	8	$21.1 \pm 10.0 \times 10^6$	
LPS (5 μg) + IL-1ra(100 μg)	8	$11.3 \pm 5.3 \times 10^6$	46
LPS (5 μg) + sTNFrI (10 μg)	8	$7.7 \pm 3.1 \times 10^6$	63
LPS (5 μg) + IL-1ra (100 μg) + sTNFrI (10 μg)	8	$6.8 \pm 2.1 \times 10^6$	68

PMNs, neutrophils.

Table 4. *TNF and IL-6 Levels in BAL Fluids after Intratracheal Co-Injection of sTNFrI and IL-1ra*

Experimental group	*n*	TNF (U/BAL)	IL-6 (ng/BAL)
LPS	2	34 ± 11	65 ± 1
LPS + IL-1ra	2	20 ± 1	82 ± 2
LPS + sTNFrI	2	1450 ± 125	48 ± 9
LPS + IL-1ra + sTNFrI	2	1140 ± 528	23 ± 4

sTNFrI and IL-1ra at near maximally effective doses of each individual molecule suggests that IL-1 release is mediated by TNF. If binding of LPS-induced TNF by the sTNFrI prevents the expression of IL-1, then the IL-1ra would not be expected to confer any additional anti-inflammatory activity. The hypothesis that TNF is responsible for the expression of IL-1 is consistent with the work of Fong and colleagues, who showed that an antiserum to TNF inhibited the appearance of serum IL-1 in endotoxemic baboons.[6] The suggestion in our experiments that the sTNFrI is a slightly more effective inhibitor of acute inflammation than IL-1ra would be consistent with the concept that TNF is a more proximal member of the proinflammatory cytokine cascade, and that TNF causes the expression of proinflammatory cytokines (for example, IL-8) in addition to IL-1.

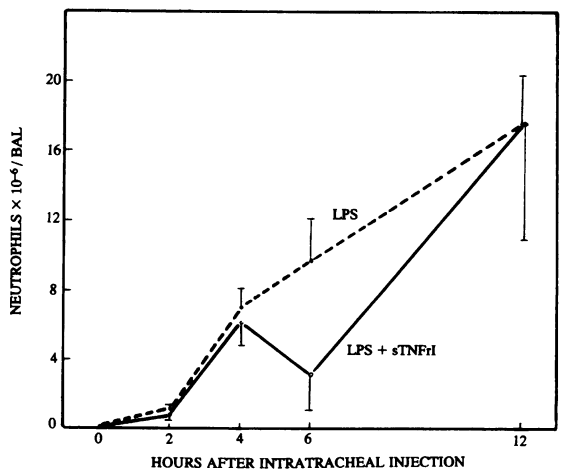

Figure 1. *Intratracheal co-injection of LPS plus sTNFrI significantly inhibits bronchoalveolar neutrophil influx at 6 hours but not at prior or later time points as compared to intratracheal injection of LPS alone.*

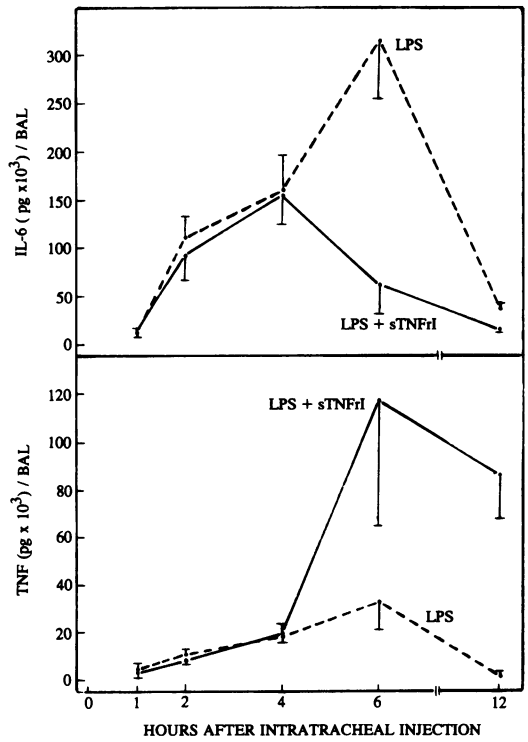

Figure 2. *The BAL fluid after intratracheal coinjection of LPS plus sTNFrI (same rats as shown in Figure 1) shows an increase in TNF activity at 6 and 12 hours and a decrease in IL-6 activity at 6 hours as compared to BAL fluid from rats injected with LPS alone (n = 6 in each group at 1, 2, and 4 hours, n = 3 in each group at 6 hours, and n = 7 in each group at 12 hours).*

A marked reduction in neutrophil influx was noted at 6 hours after co-injection of LPS and sTNFrI, despite the finding that TNF activity in BAL fluids was substantially increased in rats receiving co-injections of LPS and sTNFrI. A possible explanation for this paradoxical observation is that TNF may be buffered by sTNFrI and then gradually released. In this regard, Van Zee et al[7] have demonstrated that the early rise in TNF in septic baboons is abolished by sTNFrI, followed by a delayed appearance of circulating TNF. sTNFrI prevented hemodynamic collapse in septic baboons, and the late increase in circulating TNF was not accompanied by any deterioration in hemodynamic function. Aderka et al[8] have shown that TNF soluble receptors can initially dampen, but then prolong, TNF activity *in vitro*.

Additionally, TNF is usually catabolized via internalization by target cells or via digestion by neutrophil-derived proteases[9] after LPS challenge, processes that would not proceed at the usual rate due to the binding and functional sequestration of TNF by sTNFrI and to the decreased number of neutrophils present in sTNFrI-treated animals. Measurement of IL-6 showed that co-injection of sTNFrI and LPS decreases IL-6 in BAL fluid, a finding that is consistent with the concept that TNF plays a significant role in the induction of IL-6 expression[5] and also supports the conclusion that sTNFrI inhibits TNF activity *in vivo* despite the measurement of high levels of TNF activity in BAL fluid. The increase in TNF in the BAL fluid of rats receiving intratracheal co-injections of LPS and sTNFrI is not likely to be due to an increase in vascular permeability, because 1) vascular permeability is linked to neutrophil emigration and less neutrophils were present in the BAL fluid of rats receiving sTNFrI; 2) TNF levels are much higher in the alveolar space than in the circulation, and any increase in edema fluid in the lung would therefore be unlikely to increase the TNF concentration in BAL fluid; and 3) the IL-6 concentration was decreased in the BAL fluid of rats receiving sTNFrI.

The rapid and relatively transient expression of TNF that occurs after exposure to LPS[6,10] as well as the danger of impairing host defenses by inhibiting TNF expression[11–15] may limit the usefulness of the sTNFrI during clinical gram-negative infections. On the other hand, the kinetics of expression of TNF activity in patients with gram-negative infections and bacteremia may well differ from TNF expression after experimental bolus injections of endotoxin, and the clinical usefulness of the sTNFrI in sepsis therefore remains to be determined. sTNFrI may prove to be of substantial therapeutic utility in non-infectious inflammatory diseases such as autoimmune diseases, transplant rejection, or graft versus host disease in which TNF can be hypothesized to play an important pathogenetic role. The observation that TNF is elevated in a local inflammatory effusion after administration of sTNFrI suggests that the *in vivo* fate or catabolism of TNF/sTNFrI complexes will be important to determine in future study.

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