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RAPID COMMUNICATION

Recombinant Human Alpha Lymphotoxin (Tumor Necrosis Factor-Beta) Induces Peripheral Neutrophilia and Lymphopenia in the Rat

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Recombinant human alpha lymphotoxin (rLT) administered intravenously to Lewis rats induces peripheral neutrophilia and lymphopenia in a dose-response dependent fashion. A dose of 30,000 units of rLT induced a neutrophilia (1589 ± 326 to 5554 ± 1050 neutrophils/cu mm) and lymphopenia (10,368 ± 992 to 4636 ± 878 lymphocytes/cu mm) at 2 hours after injection that was highly significant ($P < 0.001$ and $P < 0.001$, respectively) in comparison with vehicle controls. The kinetics of the neutrophilia that peaked at 2 hours as well as of the lymphopenia were highly reminiscent of the neutrophilia and lymphopenia following intravenous administration of either recombinant human interleukin-1 (IL-1) α or β to rats. The peripheral neutrophilia was accompanied by a significant depletion of bone marrow neutrophils ($P < 0.001$), as is also known to occur after administration of IL-1. Systemic blood pressure was not affected by rLT, which suggested that the changes in circulating leukocyte subsets were not attributable to hemodynamic changes nor to the hemodynamic-change-related release of adrenal hormones. Adrenalectomy did not alter the rLT-induced neutrophilia or lymphopenia, which suggested that rLT does not mediate its hematologic effects on peripheral blood leukocytes via the release of adrenal hormones. Pretreatment of rats with dexamethasone, indomethacin, or aspirin also did not alter rLT-induced neutrophilia or lymphopenia, which suggested that rLT-induced hematologic effects were not mediated via arachidonic acid metabolites, in stark contrast to IL-1 induced neutrophilia, which is inhibited by both dexamethasone and indomethacin. (Am J Pathol 1987, 128:5–12)

LYMPHOTOXINS are a family of proteins that are released by stimulated lymphocytes *in vitro* and that are functionally defined by their ability to exert either cell lytic or growth inhibitory effects.[1,2] The ability of lymphotoxins to lyse tumor cells *in vitro* as well as their tumorilytic effect on experimental tumors *in vivo* is reminiscent of the effects of macrophage-derived tumor necrosis factor and has led some investigators to rename alpha lymphotoxin as tumor necrosis factor-beta.[3,4] Human alpha lymphotoxin is now available in recombinant form as an 18.6-kilodalton peptide that assembles into a 40–60-kilodalton molecule.[3–5] Recombinant lymphotoxin (rLT) has recently been observed to induce acute neutrophilic inflammatory reactions after injection into mice and rabbit skin.[6] The purpose of the present study is to investigate the effect of intravenously administered rLT on circulating white blood cell subsets. Recombinant LT was found to induce a marked neutrophilia and lymphopenia in a dose-response-dependent fashion and with kinetics similar to those observed after intravenous injection of interleukin-1 (IL-1). The peripheral neutrophilia observed after administration of rLT was accompanied by a depletion of bone marrow neutrophils, which suggested that the source of the increase was, at least in part, from the bone marrow, although an additional contribution from the marginal pool of peripheral neutrophils cannot be excluded. The rLT-induced peripheral hematologic changes were independent of the release of endogenous adrenal hormones and were not inhibited by

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dexamethasone, indomethacin, or aspirin (all inhibitors of arachidonic acid metabolism).

Materials and Methods

Lewis rats, male, weighing 200–250 g were obtained from Harlan–Sprague–Dawley (Indianapolis, Ind). Recombinant LT was the gift of Genentech, Inc. (San Francisco, Calif) and has a molecular weight of 18.6 kilodaltons. The dosages of rLT are expressed in units of lytic activity defined as the reciprocal of the dilution required to lyse 7500 murine L929 cells as previously described in a microplate assay.[7] The specific activity of rLT was 2.9×10^7 U/mg. Recombinant LT was diluted in 1% normal Lewis rat serum (1% NRS) in sterile phosphate-buffered saline. Dose-response and kinetic experiments were performed by administering various doses of rLT (10, 100, 500, 1500, 3000, 30,000, 50,000 or 75,000 units) intravenously in a volume of 0.5 ml injected into the dorsal vein of the penis. Injection time was approximately 30 seconds. Blood for the quantitation of circulating leukocytes and for blood smears was obtained by tail bleeding under ether anesthesia immediately before the intravenous injection as well as 0.5, 1, 1.5, 2, 4, 6, and 24 hours after injection. Three vehicle control injections (0.5 ml 1% NRS) were performed concurrently with the dose-response/kinetic experiments. The absolute number of circulating leukocytes/cu mm was quantitated with a Coulter counter (Hialeah, Fla). White blood cell differentials were performed by counting 100 white blood cells on modified Wright's-stained smears (Diff-Quik Stain Set; American Scientific Products, McGaw Park, Ill).

Statistical analysis of the rLT-induced changes in circulating white blood cell subsets and in bone marrow cells was performed by studying rats given injections of either 30,000 units rLT (n = 6) or 1% NRS (n = 6) and sacrificed at 2 hours. Peripheral blood was obtained by tail bleeding immediately before injection and at the time of sacrifice. Bone marrow hematopoietic cell subsets were quantitated by the method of Chervenick et al.[8] Briefly, at the time of sacrifice, one humerus was immediately dissected free of soft tissue and washed, and the ends of both epiphyses were cut off with a scalpel. The bone marrow was eluted from the humerus by washing the marrow with 10 ml of Isoton II buffer (Coulter) injected through a 21-gauge needle together with heparin and a red blood cell lysing agent (Zapoglobin, Coulter). The absolute number of cells per humerus was determined with the Coulter counter. The contralateral humerus was used to prepare bone marrow smears. Bone marrow smears were stained by the same modified Wright's method used to stain peripheral blood smears, and differential counts were performed on 500 cells per smear according to standard morphologic criteria for the rat as reported by Hulse.[9]

Experiments designed to explore the role of arachidonic acid metabolism in rLT-induced peripheral hematologic effects were performed by pretreating rats with dexamethasone, indomethacin, or aspirin before administration of rLT. Indomethacin (Merck, Sharp and Dohme, West Point, Pa), 5 mg/kg, and dexamethasone (Organon, West Orange, NJ), 50 mg/kg, were administered intravenously 1 minute before the intravenous injection of rLT via the dorsal vein of the penis. Aspirin (Gendex, Inc., Jersey City, NJ), 100 mg/kg, in a volume of 0.5 ml saline, was administered via gavage 30 minutes before the intravenous injection of rLT. Peripheral blood for the purpose of performing blood smears and quantitating the absolute numbers of circulating leukocytes was obtained by tail bleeding 0, 2, and 6 hours after injection.

Adrenalectomy was performed in the designated experimental groups to probe a possible role for endogenous adrenal hormone release in the rLT-induced effects on circulating leukocyte numbers. Adrenalectomy was performed through bilateral dorsal incisions under ether anesthesia with blunt removal by forceps of the entire suprarenal fat pad, and either rLT (n = 5) or vehicle control (n = 5) was administered 3–4 days after adrenalectomy.

Blood pressure was measured 1 hour after the intravenous administration of either 30,000 U rLT (n = 3) or vehicle control (n = 3). Blood pressure was measured with the Harvard rat tail blood pressure system (Harvard Instruments, Cambridge, Mass) in triplicate and averaged. Hematocrit was measured by the standard capillary tube technique at time 0 and 2 hours in the rats receiving 30,000 units rLT (n = 6) or vehicle control (n = 6).

Endotoxin contamination of the rLT was ruled out as a cause of neutrophilia by boiling rLT for 10 minutes, injecting 3000 units boiled rLT/rat in two animals, and checking for any changes in circulating white blood cell subsets. Additionally, rLT was assayed for endotoxin by the QCL-1000 chromogenic limulus amebocyte lysate method (M. A. Bioproducts, Walkersville, Maryland).

Statistics were performed by the paired or unpaired *t* test as appropriate, and a *P* value of <0.05 was considered to be significant. Arithmetic averages are expressed as $\pm$ 1 standard deviation (SD).

Results

Recombinant LT induced peripheral neutrophilia in a dose-response-dependent fashion (Figure 1). As

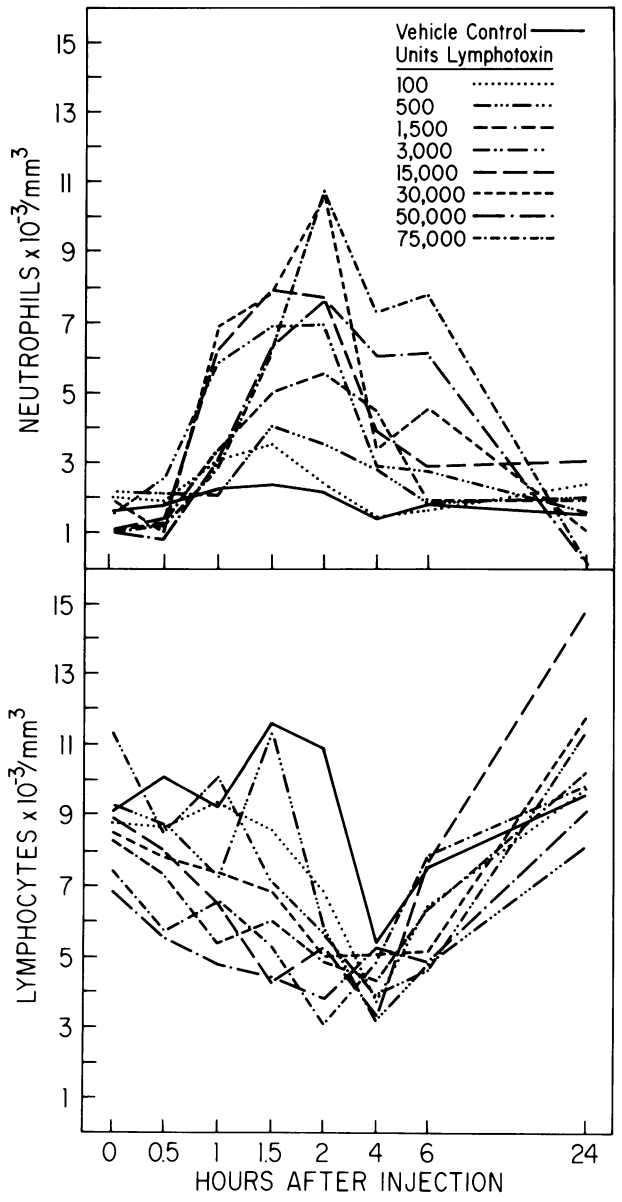

Figure 1—Dose-response and kinetic study of the effect of rLT on absolute numbers of circulating neutrophils and lymphocytes demonstrates a dose-response-dependent neutrophilia and lymphopenia (n = 1 at each dose). The neutrophilia attains a maximum 1.5–2 hours after intravenous injection. Rats given the vehicle control (n = 3) do not demonstrate changes in numbers of circulating neutrophils, but do show lymphopenia at 4 hours after injection.

little as 100 units rLT caused neutrophilia, and the magnitude of neutrophilia progressed increasingly with increasing doses of 100, 500, 1500, 3000, and 15,000 units rLT. Increasing the dose between 15,000 and 75,000 U/rat caused a variable further increase in the absolute number of circulating neutrophils. The dose of 10 units rLT did not cause neutrophilia. Neutrophilia attained a maximum 1.5–2 hours after injection, regardless of the dose, and declined by 4 hours. Of possible interest is the observation that at the three highest doses of rLT there was a slight increase of neutrophils between 4 and 6 hours after injection, and at the two highest doses of rLT a marked neutropenia was observed 24 hours after injection.

Recombinant LT also induced peripheral lymphopenia in a generally dose-response-dependent fashion (Figure 1). The magnitude and kinetics of lymphopenia in rats were somewhat more difficult to interpret than the magnitude and kinetics of neutrophilia because rats given the vehicle control alone invariably showed a lymphopenia at 4 hours after injection. Nevertheless, the lymphopenic effects of rLT could be demonstrated as early as 0.5 hours after injection, and the lymphopenia generally continued progressively until 4 hours. Rats receiving vehicle control (n = 3) did not demonstrate any evidence at all of lymphopenia within the first 2 hours after injection, an observation that is consistent with previous observations by our laboratory in a large number of rats. The dose of 10 units rLT also did not cause lymphopenia within the first 2 hours after injection.

An intermediate dose of rLT (30,000 U/rat) was chosen for study of the effects of rLT on more sizable groups of rLT-injected (n = 6) and vehicle control (n = 6) rats. The dose of 30,000 U/rat corresponds to 1.04 μg/rat or 5.8×10^{-11} mol/rat. Recombinant LT induced a very reproducible neutrophilia and lymphopenia (Figure 2) with an increase from 1589 ± 326 to 5554 ± 1050 neutrophils/cu mm ($P < 0.001$) and a decrease of 10,368 ± 992 to 4636 ± 878 lymphocytes/mm³ ($P < 0.001$) (Table 1). Recombinant LT caused the appearance of a very small number of immature myeloid forms in the circulation (Table 1). No significant changes in numbers of circulating monocytes or eosinophils were observed when compared with vehicle controls (Table 1). The possibility that rLT might have induced a transient neutropenia at a time before 30 minutes was examined by performing peripheral blood smears at 1,5, and 10 minutes after intravenous injection of 30,000 U rLT (n = 2) or vehicle control (n = 2). No neutropenia was observed at these early time points in either rLT or vehicle control-treated rats. Examination of the bone marrow demonstrated that the peripheral neutrophilia occurring at 2 hours was accompanied by a marked depletion of bone marrow neutrophils ($P < 0.001$) (Table 2). No other significant changes in absolute numbers of hematopoietic cells were observed in the bone marrow.

Rats receiving 30,000 units rLT were pretreated with dexamethasone, indomethacin, or aspirin for investigation of the possible role of arachidonic acid

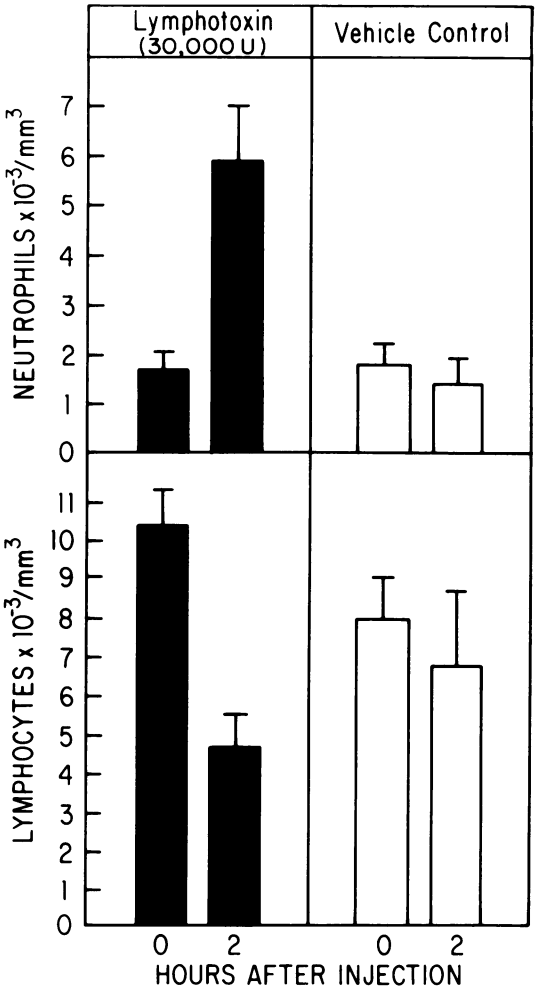

Figure 2—Lymphotoxin (n = 6) induces a marked neutrophilia ($P < 0.001$) and lymphopenia ($P < 0.001$) 2 hours after injection, whereas vehicle control (n = 6) does not induce any significant changes in circulating leukocyte subsets.

metabolites on rLT-induced peripheral hematologic effects. Lymphotoxin (positive control group, n = 6) induced neutrophilia and lymphopenia that peaked at 2 hours after injection. Vehicle control (negative control group, n = 6) did not cause any changes in the numbers of circulating neutrophils or lymphocytes at either 2 or 6 hours after injection. Indomethacin, dexamethasone, and aspirin (n = 6 in each experimental group) did not inhibit the lymphotoxin-induced neutrophilia or lymphopenia. Indomethacin and aspirin alone (n = 6 in each negative control group) did not affect the numbers of circulating leukocytes at either 2 or 6 hours. Dexamethasone alone (n = 6) did not cause any change in the number of circulating neutrophils, but did cause the expected progressive lymphopenia at 2 and 6 hours (Figure 3 and Table 3).

Adrenalectomized rats given rLT (n = 5) developed neutrophilia and lymphopenia that were comparable in magnitude to those noted in normal rats. Vehicle control (n = 5) did not cause a significant neutrophilia or lymphopenia in adrenalectomized rats (Figure 4 and Table 4).

Blood pressure 1 hour after administration of 30,000 units rLT was 111 ± 3 mmHg and 1 hour after administration of vehicle control was 111 ± 0.5 mmHg. No significant differences in hematocrit were noted in rLT- as compared with vehicle control-treated rats.

Heat inactivation of rLT by boiling completely abrogated its neutrophilia- and lymphopenia-inducing activity. The rLT contained less than 3 units of endotoxin as measured by the limulus lysate assay.

Discussion

Recombinant human alpha lymphotoxin is an extremely potent inducer of peripheral neutrophilia and lymphopenia. The source of the circulating neutrophils is at least partly via the release of bone marrow neutrophils. The mechanism of the lymphopenia remains to be elucidated but might be hypothesized to include increased margination of circulating lymphocytes along endothelium. Tumor necrosis factor α (TNFα), a molecule that is similar in several aspects to LT, induces leukocyte adhesion molecules *in*

Table 1—Recombinant Human Lymphotoxin Induces Neutrophilia and Lymphopenia in Peripheral Blood

	Lymphotoxin (n = 6) (cells/cu mm [%])		Vehicle (n = 6) (cells/cu mm [%])	
White blood cell differential	0 minutes	120 minutes	0 minutes	120 minutes
Immature neutrophils	0 (0)	230 ± 73 (2.1 ± 0.75)	0 (0)	0 (0)
Mature neutrophils	1,589 ± 326 (12.7 ± 2.3)	5,554 ± 1,050 (51.6 ± 3.5)	1,760 ± 459 (17.5 ± 1.9)	1,462 ± 508 (17.1 ± 4.6)
Lymphocytes	10,368 ± 992 (82.5 ± 2.0)	4,636 ± 878 (43.0 ± 4.0)	7,947 ± 1,100 (79.1 ± 2.7)	6,731 ± 1,831 (78.8 ± 6.3)
Eosinophils	108 ± 53 (0.8 ± 0.4)	49 ± 85 (0.5 ± 0.8)	116 ± 94 (1.1 ± 0.9)	56 ± 70 (0.6 ± 0.8)
Monocytes	521 ± 260 (4.0 ± 1.6)	305 ± 109 (2.8 ± 0.9)	244 ± 142 (2.3 ± 1.2)	311 ± 146 (3.5 ± 1.2)
Total white blood cells	12,586 ± 1,408	10,774 ± 1,772	10,067 ± 1,623	8,560 ± 2,230

Table 2—Recombinant Human Lymphotoxin Induces Release of Neutrophils From the Bone Marrow

White blood cell differential	Cells × 10^{-6} (%)/humerus	
	Lymphotoxin (n = 6)	Vehicle (n = 6)
Erythroid		
Pronormoblasts	0.60 ± 0.3 (1.5 ± 0.5)	0.65 ± 0.2 (1.0 ± 0.3)
Early Normoblasts	1.44 ± 0.5 (2.4 ± 0.9)	1.36 ± 0.4 (2.1 ± 0.3)
Intermediate Normoblasts	6.98 ± 0.9 (11.6 ± 1.2)	6.72 ± 1.1 (10.5 ± 0.7)
Late Normoblasts	10.49 ± 1.6 (17.4 ± 2.0)	10.54 ± 2.2 (16.4 ± 0.5)
Myeloid		
Myeloblasts	1.46 ± 0.2 (2.4 ± 0.2)	1.40 ± 0.2 (2.2 ± 0.4)
Promyeleocytes	1.25 ± 0.3 (2.0 ± 0.4)	1.28 ± 0.9 (1.9 ± 0.8)
Myelocytes	5.52 ± 0.9 (9.1 ± 0.7)	5.44 ± 2.4 (8.2 ± 1.9)
Metamyelocytes	3.10 ± 0.5 (5.1 ± 0.4)	2.37 ± 0.6 (3.7 ± 0.5)
Band Cells	2.15 ± 0.7 (3.6 ± 1.3)	3.18 ± 0.9 (4.9 ± 0.9)
Segmented Neutrophils	3.78 ± 1.3 (6.1 ± 1.8)	10.55 ± 1.7 (16.6 ± 1.8)
Eosinophils	2.68 ± 0.4 (4.4 ± 0.5)	2.53 ± 0.8 (3.9 ± 1.0)
Basophils	0.44 ± 0.3 (0.7 ± 0.4)	0.49 ± 0.2 (0.7 ± 0.3)
Monocytes	2.07 ± 0.6 (3.4 ± 0.7)	1.66 ± 0.5 (2.6 ± 0.7)
Mast Cells	1.65 ± 0.3 (2.7 ± 0.3)	1.44 ± 0.5 (2.3 ± 0.9)
Histiocytes	1.98 ± 0.8 (3.3 ± 1.2)	2.13 ± 0.8 (3.4 ± 1.5)
Lymphoid		
Lymphocytes	11.57 ± 3.0 (19.0 ± 3.3)	10.22 ± 0.1 (16.1 ± 2.2)
Plasma cells	1.79 ± 0.5 (3.0 ± 0.9)	1.00 ± 0.5 (1.6 ± 0.9)
Megakaryocytes	1.39 ± 0.8 (2.3 ± 1.6)	1.06 ± 0.8 (1.9 ± 0.9)
Total nucleated cells/humerus	60.34 ± 8.5	64.02 ± 12.9

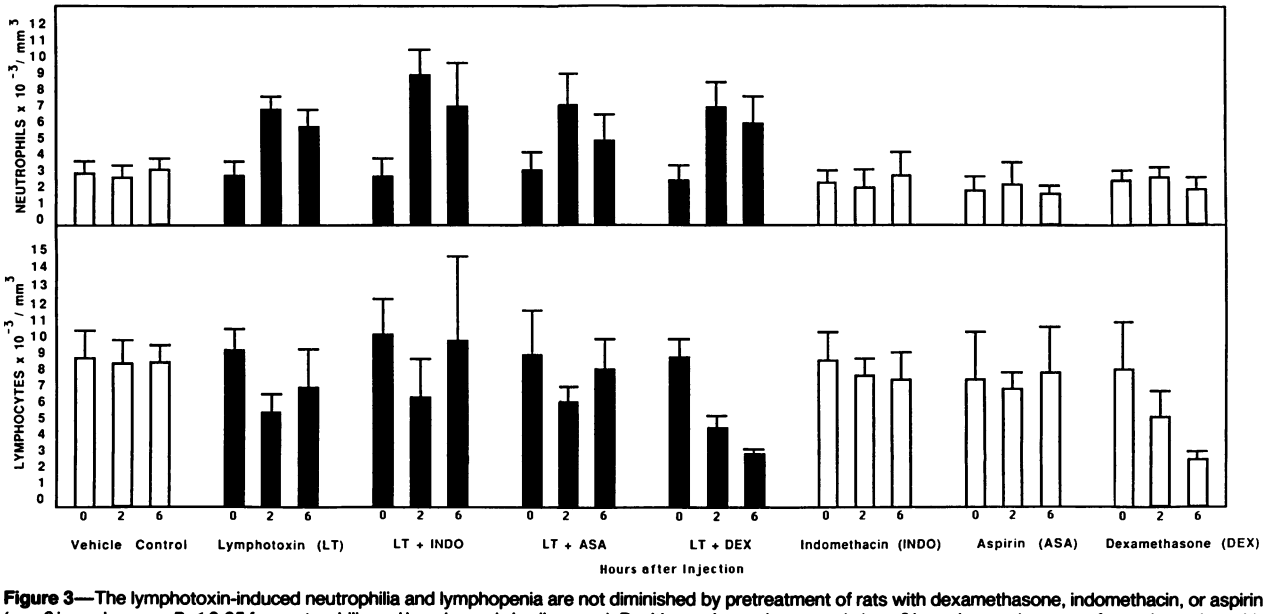

Figure 3—The lymphotoxin-induced neutrophilia and lymphopenia are not diminished by pretreatment of rats with dexamethasone, indomethacin, or aspirin (n = 6 in each group; $P < 0.05$ for neutrophilia and lymphopenia in all groups). Positive and negative controls (n = 6 in each group) were performed together with the inhibition experiments. The only effect on circulating leukocytes noted in the negative control groups was the expected dexamethasone-induced lymphopenia.

Table 3—Recombinant Lymphotoxin-Induced Neutrophilia and Lymphopenia Are Not Diminished by Indomethacin, Aspirin, or Dexamethasone

	Hours after injection	Vehicle control (n = 6)	Lymphotoxin (n = 6)	Lymphotoxin + indomethacin (n = 6)	Lymphotoxin + aspirin (n = 6)	Lymphotoxin + dexamethasone (n = 6)	Indomethacin (n = 6)	Aspirin (n = 6)	Dexamethasone (n = 6)
Neutrophils	0	2653 ± 776	2562 ± 984	2461 ± 1104	2555 ± 1384	2177 ± 839	2026 ± 764	1690 ± 663	2112 ± 507
$\times 10^{-3}/mm^3$	2	2424 ± 808	6558 ± 935	8881 ± 1418	6951 ± 1949	6763 ± 1333	1993 ± 889	2081 ± 1031	2382 ± 578
	6	2774 ± 801	5561 ± 1072	6904 ± 2498	4698 ± 1468	5735 ± 1546	2782 ± 1178	1521 ± 404	1667 ± 638
	0	8584 ± 1682	9124 ± 1216	10024 ± 2004	8866 ± 2480	8512 ± 1262	8179 ± 1870	7198 ± 2891	7970 ± 2643
Lymphocytes	2	8110 ± 1539	5223 ± 1061	6118 ± 2519	5848 ± 751	4164 ± 669	7258 ± 1130	6674 ± 887	5002 ± 1278
$\times 10^{-3}/mm^3$	6	8193 ± 1284	6914 ± 2115	9808 ± 4746	7940 ± 1670	2585 ± 344	7125 ± 1634	7890 ± 2426	2183 ± 629

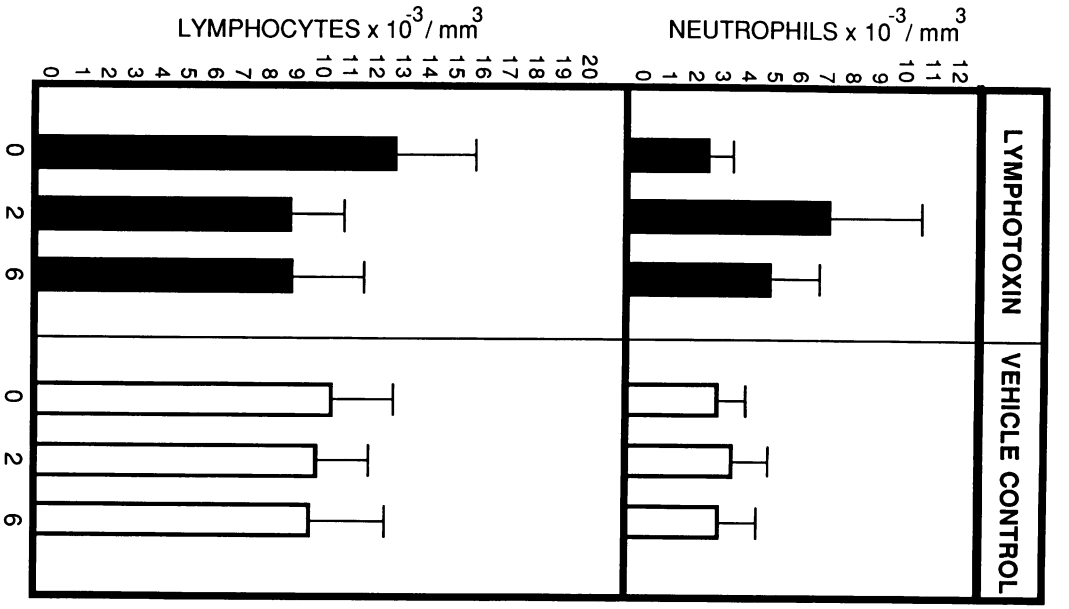

Figure 4—Adrenalectomy did not abrogate either the lymphotoxin-induced neutrophilia ($P < 0.05$) or lymphopenia ($P < 0.01$). No significant changes in circulating leukocytes were noted in vehicle-control-treated adrenalectomized rats (n = 5 in each group).

Table 4—Recombinant Lymphotoxin-Induced Neutrophilia and Lymphopenia Occur in Adrenalectomized Rats

	Hours after injection	Lymphotoxin (n = 5)	Vehicle control (n = 5)
Neutrophils	0	2500 ± 799	2864 ± 1058
$\times 10^{-3}$/cu mm	2	7057 ± 3496	3257 ± 1527
	6	4756 ± 1945	2904 ± 1243
Lymphocytes	0	12790 ± 2840	10295 ± 2271
$\times 10^{-3}$/cu mm	2	8650 ± 2173	9866 ± 1762
	6	8829 ± 2688	9606 ± 2574

vitro,[10] and kinetic studies *in vitro*[11] have demonstrated that preincubation of endothelial cells with TNFα for 4 hours results in maximum lymphocyte adhesion, a time course that would not be inconsistent with our *in vivo* observations. Lymphotoxin was demonstrated not to indirectly induce changes in circulating leukocyte subsets by lowering blood pressure and thereby causing the release of adrenal hormones. A direct adrenal hormone-releasing effect of rLT as a cause of the neutrophilia and lymphopenia was ruled out by experiments with adrenalectomized rats. The neutrophilia-inducing effects of rLT could not be explained by endotoxin contamination because boiling abrogated the activity, no endotoxin was detectable by the limulus amebocyte lysate assay, and, finally, the kinetics of the changes in circulating neutrophils did not include the inital neutropenia that is observed in endotoxin-treated rats.

The kinetics of rLT-induced neutrophilia are strikingly similar to IL-1-induced neutrophilia in the rat (data submitted for publication). The rLT-induced neutrophilia was not inhibited by the inhibitors of arachidonic acid metabolism, dexamethasone, indomethacin, or aspirin. Recombinant IL-1β-induced neutrophilia has been shown in our laboratory to be completely inhibited in the rat by pretreatment with dexamethasone or indomethacin (submitted for publication). Thus, rLT and rIL-1β induce neutrophilia via two distinct mechanisms, one which appears independent and one dependent on arachidonic acid metabolism.

Lymphotoxin prepared by stimulation of leukocytes with phytohemagglutinin[12] and a lymphotoxin-containing preparation derived from the human lymphoid cell line RPMI · 1788[13] have been reported to result in granulocytosis and lymphopenia peaking at 2–4 hours after intravenous injection into a small number of cancer patients with advanced disease. The discussion that followed both of those previous reports[12,13] raised questions regarding the possible additional presence of endotoxin and/or IL-1 in the lymphotoxin-containing supernatants. Our present observations employing rLT demonstrate that neither endotoxin nor IL-1 accounts for the changes in circulating white blood cell subsets and support the contention that the hematologic effects of LT in humans and rats may be similar.

Recently, our laboratories reported that rLT induces acute neutrophilic inflammatory infiltrates after intradermal injection into mice and rabbits.[6] The observation that a lymphocyte product can mediate peripheral neutrophilia as well as neutrophilic inflammatory infiltrates is of note because the presence of neutrophils in immunologically mediated inflammation is most often taken to represent the result of humorally rather than cell-mediated immune events. Shalaby and colleagues have reported that rLT augments the phagocytic and cytotoxic activities of neutrophils,[14] and recently Hemmi and colleagues have reported that lymphotoxin is a differentiation factor for myeloid cells.[15] The apparent multiple roles of lymphotoxin as an activator of neutrophils,[14] a neutrophil-releasing factor and a myeloid-differentiation factor[15] are teleologically consistent with one another. The *in vivo* role of LT is unlikely to be restricted to tumor rejection, and LT may prove to be a central endogenous mediator of the acute inflammatory response.

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