UC Irvine
UC Irvine Previously Published Works

Title

Purified native and recombinant human alpha lymphotoxin [tumor necrosis factor (TNF)-beta] induces inflammatory reactions in normal skin

Permalink

https://escholarship.org/uc/item/9wj5867d

Journal

Journal of Clinical Immunology, 7(4)

ISSN

0271-9142

Authors

Averbook, Bruce J
Yamamoto, Robert S
Ulich, Thomas R
et al.

Publication Date

1987-07-01

DOI

10.1007/bf00915556

Copyright Information

Peer reviewed

Purified Native and Recombinant Human Alpha Lymphotoxin [Tumor Necrosis Factor (TNF)-beta] Induces Inflammatory Reactions in Normal Skin

BRUCE J. AVERBOOK,[1,2] ROBERT S. YAMAMOTO,[1] THOMAS R. ULICH,[1,3] E. W. B. JEFFES,[1,4] IRENE MASUNAKA,[1] and GALE A. GRANGER[1]

These studies report findings that demonstrate that human alpha lymphotoxin (LT) induces local, visible, and microscopic inflammatory reactions in normal skin. Skin sites in rabbits, when inoculated with a single injection of native or recombinant human alpha lymphotoxin, demonstrated erythema, swelling, and warmth within 5 hr. Erythema peaked between 24 and 48 hr and resolved by 72 hr. Histologic studies of skin sites injected with native LT revealed polymorphonuclear neutrophil (PMN) infiltration and edema beginning as early as 3 hr posttreatment. Individual skin sites that received three daily injections of native LT exhibited persistent erythema and swelling. Palpable induration was evident 24 hr after the second injection in the series. Histologic examination revealed the presence of many PMNs with associated focal dermal destruction, in the form of microabscesses, and scattered mononuclear cells. In contrast, control materials and recombinant human tumor necrosis factor (TNF-alpha) did not induce visible skin reactions in the rabbit. Several additional controls excluded endotoxin as being the agent responsible for the inflammatory skin reactions observed. The ability of LT to induce inflammation may have a role in its antitumor activity and it may be an important endogenous mediator in other immunologic reactions.

KEY WORDS: Lymphotoxin; tumor necrosis factor; inflammation; skin reactive factor.

[1]To whom correspondence should be addressed at Department of Molecular Biology and Biochemistry, University of California, Irvine, Irvine, California 92717.
[2]Department of Surgery, University of California, Irvine, Irvine, California 92717.
[3]Department of Pathology, University of California, Irvine, Irvine, California 92717.
[4]Department of Dermatology, University of California, Irvine, Irvine, California 92717.

INTRODUCTION

Lymphotoxins (LT) are an inducible family of cell lytic and growth inhibitory proteins that are released by stimulated human lymphocytes *in vitro* (1, 2). One of the human LT forms, termed alpha, is available in purified form from native or recombinant sources. The recombinant form is an 18,600 MW peptide that assembles into a 40,000–60,000 MW molecule and the native form is a 27,000 MW peptide that is glycosylated and assembles into a 90,000–100,000 MW molecule (3–5). The peptide chain in each form is similar. Both native and recombinant LT forms will cause necrosis of experimental tumors growing in mice and considerable interest has been generated for their potential use as antitumor agents in human cancer patients (3–5). This finding has led one group to rename alpha LT tumor necrosis factor beta (TNF-beta) (3, 4).

Several investigators have reported on supernatant factors derived from antigen- or mitogen-stimulated human lymphocytes which cause inflammation and reactions when injected into normal skin (6–13). Materials with this activity have been termed skin reactive factors and lymph node permeability factors. While studying the histology of Meth-A tumors in BALB/c mice treated with purified human LT, we noted marked infiltration by inflammatory cells (14). We therefore proceeded to discern whether lymphotoxin could be responsible for the inflammatory reactions in these tumors. The present report indicates that native and recombinant LT can induce inflammation and skin reactions and that chronic administration results in destruction of normal tissues.

MATERIALS AND METHODS

Human Alpha Lymphotoxin Production and Purification

Cell-line maintenance, LT production, and LT purification have been described in detail elsewhere (15). A unit of lytic activity is defined as the reciprocal of the dilution required to lyse 7500 murine L929 cells in an *in vitro* microplate assay.

Recombinant Human Alpha Lymphotoxin and Tumor Necrosis Factor

Recombinant human LT (TNF-beta) and tumor necrosis factor (TNF-alpha) were provided by Genentech, Inc. (San Francisco, CA), and Cetus (Emeryville, CA), respectively. When tested in our own laboratory on L929 cells in the microplate assay, the specific activity of native LT, recombinant LT, and recombinant TNF was 6×10^6 to 3×10^7 U/mg. All preparations, including the purified materials, contained less than 0–3 U of endotoxin as measured by the QCL-1000 chromogenic limulus amebocyte lysate test method (M.A. Bioproducts, Walkersville, MD). All samples were suspended in sterile phosphate-buffered saline (PBS) at a pH of 7.2 containing 1% bovine serum albumin (BSA).

Histology of LT-Inoculated, Normal Mouse Skin

The hind limbs of six female BALB/c mice (Newport Pharmaceuticals Int., Newport Beach, CA) were shaved. Right legs were injected intradermally (ID) with 20 μl of native LT, equivalent to 1800 U of lytic activity, and left legs were injected intradermally with 20 μl of PBS. Two animals were sacrificed at 24, 48, and 72 hr. The areas injected were harvested along with underlying muscle and fixed in 10% neutral buffered formalin. Tissues were then embedded in paraffin, sectioned, and prepared as hematoxylin and eosin-stained slides. Slides were evaluated by a pathologist who was not aware of the treatment paradigm. The area of greatest density of neutrophils (PMNs) was counted per 40× high-power field on each slide.

Effects of Human LT on Normal Rabbit Skin

Female, New Zealand white rabbits (ages 8–16 weeks) (Simunek Breeders, Vista, CA) were used in the following experiments. Each rabbit was pre-

tested and shown to demonstrate a skin reaction in response to LT. An adequate skin response test was defined as erythema greater than or equal to 0.8 cm from a 1000-U intradermal inoculation.

Dose Response of Skin Reaction. Serial dilution of LT in PBS were made into 20-μl samples corresponding to 10,000, 1000, 100, and 10 U. These samples were injected at individual sites id in the back of a rabbit, as was a 20-μl sample of PBS as a control. These sites were observed over a 4-day period. This experiment was repeated twice.

Visible Skin Reaction in Response to a Single Injection. A single dose of 10 μl of native LT (equivalent to 4200 U of activity) was injected id into the right shaved back of a rabbit, then observed over a 4-day period. A single control injection was used, with 10 μl of PBS injected into the left shaved back of the same animal. This was repeated two more times on different animals. Three identical experiments were performed on separate rabbits with recombinant LT.

Histology of Skin Reaction from Single and Multiple Injected Sites

Skin biopsies for histologic evaluation were obtained from rabbits at different times after a single or multiple id injections of PBS or native LT using 4200 U in 20 μl. A rabbit was inoculated daily with native LT using 4200 U in 20 μl at different sites along with an equivalent volume of PBS placed 4 cm away from the LT-treated sites. One area received repeated daily injections of LT and a control site was similarly treated with PBS. The animal was sacrificed with a 6-ml intracardiac injection of 6 M KCl. Biopsies were taken from sites corresponding to 3, 6, 24, 48, and 72 hr after single injections, as were sites chronically injected with LT or PBS. Sharp dissection was used to harvest skin samples with a portion of underlying subcutaneous tissue. All biopsies were placed in 10% neutral buffered formalin prior to embedding, sectioning, and H&E staining. This experiment was repeated twice. All slides were evaluated by a pathologist who was not aware of the treatment paradigm. The area of greatest neutrophil density on each slide was counted per 40× high-power field.

Treatment of Rabbit Skin with Recombinant TNF (TNF-Alpha)

Fifty microliters of TNF-alpha containing from 50,000 to 60,000 U was also injected into four

different rabbits who had been pretested and shown to have skin reactions in response to LT. These animals were observed over a 4-day period.

Correlation of LT and TNF in Vitro Cell Lytic Activity with Skin Reactivity After Native Polyacrylamide Gel Electrophoresis (PAGE)

Twenty microliters of native LT (4.2 × 10^5 U/ml) and 20 μl of recombinant TNF (1 × 10^6 U/ml) (Cetus Corp.) were each run separately on native (0.5 × 6.0-cm) polyacrylamide tube gels as described previously (16). Each gel was sliced into 1-mm sections. Five abutting sections were placed in 150 μl of 1% BSA (1 mg BSA/ml PBS), macerated, and allowed to elute overnight. Eluates were assayed for lytic activity using the microplate assay and injected intradermally, as 50-μl samples, into rabbit skin. The animals were observed over 4 days. This experiment was repeated two times.

Heat-Inactivated LT and Skin Reactions

Twenty microliters of native or recombinant LT (4200 U) was placed in plastic bullet tubes and heated to 100°C in a boiling-water bath for 20 min. Treated samples were injected into skin of different rabbits along with 20 μl (4200 U) of unheated native or recombinant LT at separate sites, respectively. Two rabbits received native LT as a control and two received recombinant LT (rLT) as a control. Animals were observed over 48 hr for signs of skin reactivity.

In addition, 15,000 U rLT in 50 μl PBS was heat inactivated as described above and injected into three rabbits along with positive and negative controls. Six-millimeter Baker punch biopsies were taken of each injection site after local anesthesia with 1% lidocaine. Tissues were then prepared and examined as described previously. The above experiment was duplicated using 50,000 U rLT in 50 μl PBS in a single rabbit.

Two rabbits received repeated intradermal injections of 50,000 U of heat-inactivated rLT in 50 μl of PBS in the same sites along with positive and negative controls for three consecutive days. Six-millimeter punch biopsies were obtained 24 hr after the last injection and prepared and examined as described previously.

RESULTS

Mouse Skin Reaction

Tissues harvested at 24, 48, and 72 hr from BALB/c mice injected id on the right hind limb with 1800 U of native LT and on the left hind limb with PBS revealed no grossly visible changes between control and test sites. Microscopic examination, however, revealed the presence of significant numbers of inflammatory cells at LT-treated but not at control sites (Figs. 1A and B and 2). Polymorphonuclear neutrophils (PMNs) were observed in the dermis, subcutaneous tissues, and superficial muscle layers in 24-hr specimens from LT-treated sites. Samples at both 48 and 72 hr showed progressively diminishing numbers of PMNs with a few mononuclear cells. Control sites revealed no significant inflammatory cell infiltrates at any time assessed.

Dose Response of Rabbit Skin Reaction

Individual animals were injected id at multiple sites with serial dilutions of native and recombinant LT. The diameter of erythema was inversely proportional to the dilution and the duration of the skin reaction lasted only 16–24 hr at higher dilutions. Skin reactions were observed with as little as 10 U; however, all erythema faded by 24 hr at dilutions containing LT at concentrations of 100 U or less. We also found that two of five randomly chosen rabbits showed no visible response to LT inoculation when screened with 1000-U id injections. However, responder animals remained so even after repeated challenges.

Visible Skin Reaction in Response to a Single id Injection of LT in Rabbits

Three female New Zealand white rabbits were injected id on the right shaved back with 4200 U (10 μl) of native LT and id on the left shaved back with 10 μl of PBS. This was repeated in three additional rabbits using recombinant LT. LT-treated sites showed erythema and edema which began after 5 hr, peaked between 24 and 48 hr (up to 2 cm), and then returned to normal by 72 hr. Figure 3 illustrates the visible reaction observed in rabbit skin at 5 hr. Two animals were also treated daily with 4200 U of native LT at the same site for 3 days. Individual skin sites that received three daily injections of native LT exhibited persistent erythema and swell-
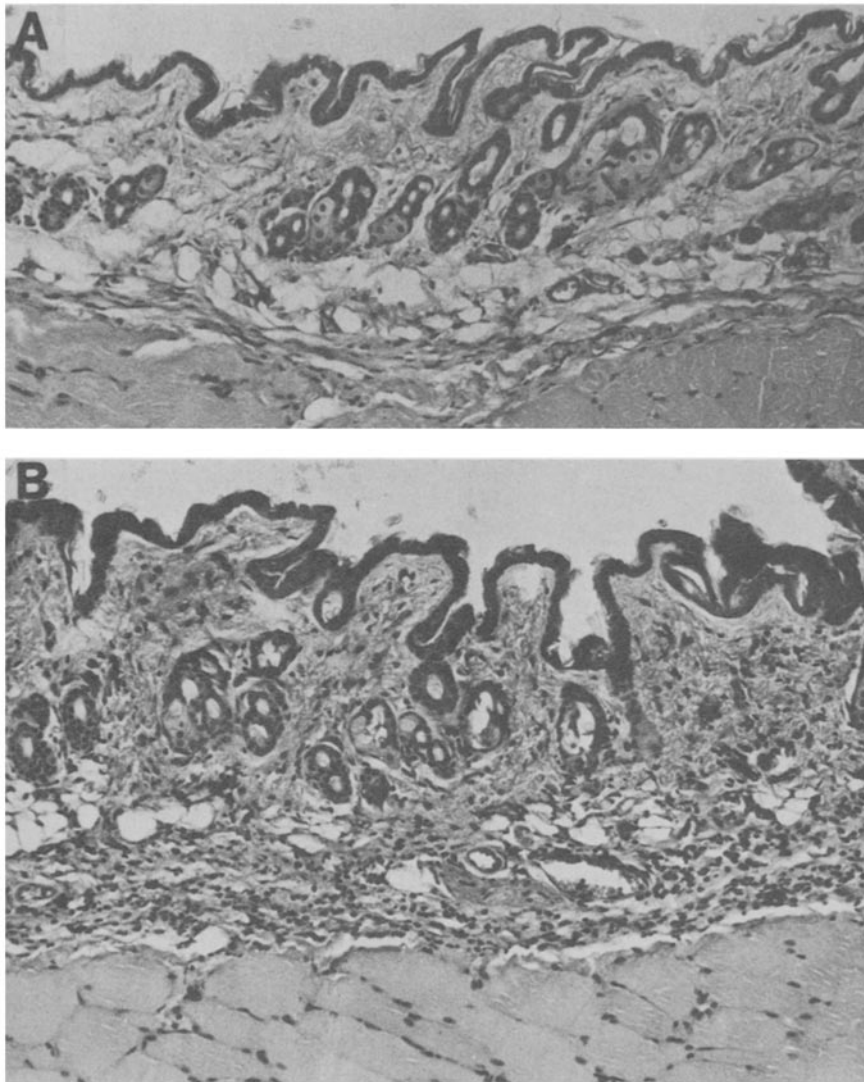
Fig. 1. Hematoxylin and eosin-stained skin from BALB/c mice 24 hr after intradermal injection with (A) PBS (20 µl) and (B) 1800 U (20 µl) of native LT. Note the edema and intense inflammatory cell infiltrate, predominantly PMN, in the dermis of the test site (B). 100×.

ing. Palpable induration was detectable 24 hr after the second injection in the series. Erythema up to 2 cm with palpable induration of up to 1 cm was present on the fourth day of examination.

Histology of Rabbit Skin Reactions from Single and Multiple Injected Sites

Skin samples for histologic evaluation were obtained from rabbits at different times after a single or multiple id injection of native LT. A high-power view of a control skin site at 6 hr is shown in Fig. 4A. There were no grossly visible changes at any control sites. Microscopic examination revealed scattered, generally superficial dermal, perivascular mononuclear infiltrates in some controls irrespective of the time course after inoculation. Histologic examination of skin sites receiving a single LT injection revealed PMN margination in blood vessels and increased numbers of PMNs in the dermis beginning as early as 3 hr posttreatment. Increasing numbers of PMNs were seen at 6 hr (Fig. 4B). There was PMN cytoclasis as evidenced by nuclear and cytoplasmic debris during the first 24 hr. The number of PMNs in the dermis peaked by 24 hr and began to decrease thereafter (Fig. 5). Seventy-two
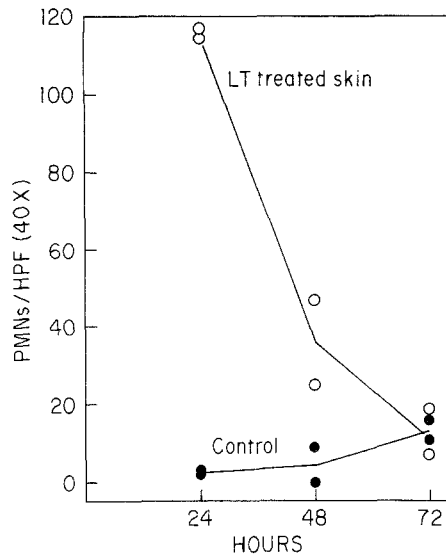
Fig. 2. Scatter plot over time of the densest area of PMN infiltration in mouse skin after treatment with 1800 U of purified native LT.

hours postinoculation specimens showed minimal changes compared to control. Animals receiving three daily LT injections at the same site were examined 24 hr after the final injection. Histologic examination revealed the presence of many PMNs with associated focal dermal destruction, in the form of microabscesses made up of PMNs, cellular debris, and karyorrhectic material (Fig. 4C). Scattered mononuclear cells were apparent at a slightly increased number in the dermis of chronically dosed skin.

Treatment of Rabbit Skin with Recombinant TNF (TNF-Alpha)

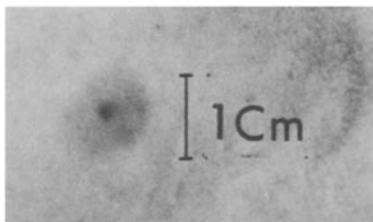While the data are not shown, animals treated with TNF-alpha showed no visible evidence of

Fig. 3. Visible skin reaction in NZW rabbit 5 hr after intradermal inoculation with 4200 U of native LT (10 μl). Indelible ink was used to mark the site of injection and is seen at the center of the skin reaction. The skin reaction at this time exhibits erythema, edema, and warmth.

inflammatory reaction at all concentrations tested up to 60,000 U.

Correlation of LT and TNF in vitro Cell Lytic Activity with Skin Reactivity After Native Polyacrylamide Gel Electrophoresis

Purified native LT and TNF-alpha were subjected to electrophoresis on native PAGE tube gels as described in Materials and Methods. While the data are not shown, only those PAGE fractions which contained lytically active LT induced a skin reaction. The same experiment conducted with recombinant TNF showed no skin reaction from either lytic or nonlytic fractions.

Heat-Inactivated LT Does Not Cause Skin Reactions

Boiling either native or recombinant LT at all levels tested for 20 min completely destroyed the ability of the sample to cause visible or histologic inflammatory skin reactions whether given in single or repeated injections. In contrast, positive controls of unheated LT, native or recombinant, were fully active in causing a skin reaction.

DISCUSSION

Recombinant and native human LT can induce inflammatory reactions in normal skin. Grossly visible evidence of inflammation was not present in the mice but rabbits responded with erythema and edema 5–6 hr after LT inoculation which peaked between 24 and 48 hr and, with single injections, resolved without tissue destruction. While the data are not shown, we have found similar reactions to single 1000-U LT injections in skin of guinea pigs. The cellular infiltrate in rabbits and mice was primarily PMN, which was characteristic of acute inflammation. The number of PMNs found in the skin gradually diminished by 48 and 74 hr to reveal a background of scattered mononuclear cells. Although not shown here, biopsies of recombinant LT-treated skin sites were identical to that of the purified molecule. There were differences in responsiveness between individual rabbits to LT injections and good responders continued to give strong reactions even after several months and multiple skin tests.

Chronic id administration of LT caused greater visible inflammation than single injections. Grossly,
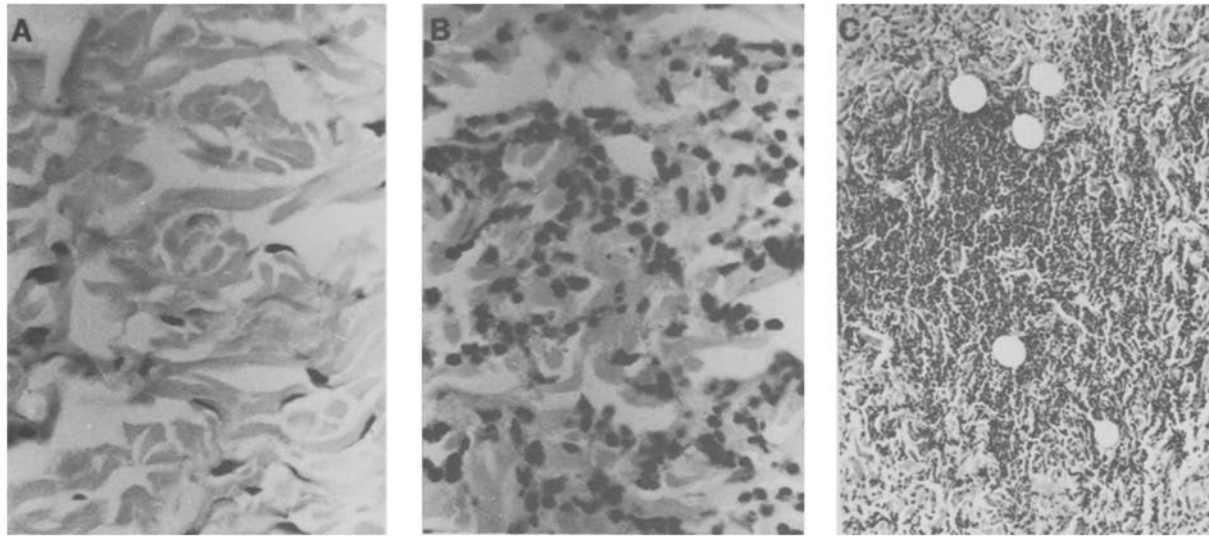
Fig. 4. Hematoxylin and eosin-stained skin sites from a NZW rabbit 6 hr after intradermal injection with (A) PBS (20 μl) and (B) 4200 U (20 μl) of native LT. (C) NZW rabbit skin 24 hr after the last of three daily, consecutive injections of native LT (4200 U). B shows a massive influx of PMNs infiltrating dermal tissue. Note the microabscess formation in C. (A, B) 400× and (C) 100×; reduced 70% for reproduction.

sites treated over 72 hr with three injections exhibited persistent edema, erythema, and palpable induration. Histological examination of these sites revealed heavy infiltration with PMNs and smaller numbers of mononuclear cells. Moreover, there were areas of normal tissue destruction in the form of microabscesses. Since recombinant LT will activate PMNs *in vitro* (17), it is possible that native LT

is capable of activating inflammatory cells that then cause tissue destruction. Further study will be required to ascertain whether LT can directly, independent of inflammatory cells, destroy normal tissue *in vivo*.

The delay in visible inflammatory responses may indicate the induction of additional mediators which produce the visible response seen. Of note, however, is that histologic changes occur prior to visible signs of inflammation. Although the data are not shown, we found that LT injections into rabbits classified as poor visible responders still showed microscopic PMN infiltration into skin sites when examined at 24 hr. More studies are needed to characterize the components of LT-induced inflammation.

Endotoxin as a cause for the visible inflammatory reaction attributed to LT was eliminated by heat inactivation of LT, PAGE, and control samples. Had endotoxin been the inciting agent for the inflammatory reactions observed, other samples off the PAGE gel would have elicited visible skin reactions. On the contrary, skin reactivity migrated solely with lytic activity. In addition, sites biopsied after single or repeated injection with heat-inactivated LT revealed no PMN infiltration. Although the data are not shown, levels of polymixin B capable of neutralizing 0.5 ng of lipopolysaccharide when mixed with 50,000 U of rLT in 50 μl of PBS did not neutralize inflammatory skin reactions.
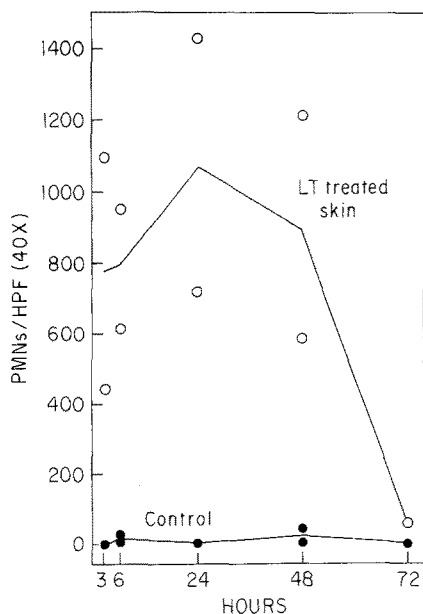

Fig. 5. Scatter plot over time of the densest area of PMN infiltration in rabbit skin after treatment with 4200 U of purified native LT.

Our results indicate that recombinant TNF (TNF-alpha) does not give rise to visible skin reactions in rabbits compared to comparable doses of LT in animals that are known responders to LT-induced inflammation. This is surprising, for LT and TNF-alpha share 28% amino acid sequence homology (18, 19) and appear to bind to the same receptor site on target cells *in vitro* (20). These results indicate that LT and TNF-alpha may exert *in vivo* antitumor effects via different mechanisms. Finally, they may also have different roles in various aspects of cell-mediated reactions *in vivo*. Histologic studies of TNF-treated skin sites are under way.

This is the first evidence demonstrating a lymphotoxin to be an inflammatory agent in tissue. Polymorphonuclear neutrophils not only are activated by LT *in vitro*, but will migrate *in vivo* into tissues inoculated with LT. *In vitro* studies indicate that cytolytic lymphocytes of all classes can release the alpha LT molecule (21). Indeed, even B lymphocytes can release the alpha protein when induced by the proper stimulus *in vitro* (22). These results raise the possibility that LT may have a role in a wide variety of endogenous inflammatory and immunologic reactions such as allograft rejection and autoimmune diseases. The presence of large numbers of neutrophils seen in examinations of immunologically mediated inflammatory sites (e.g., renal allograft rejection) is generally thought to suggest a humorally mediated immune response. Our studies, however, suggest that the presence of large numbers of neutrophils in such sites may also be a result of cell-mediated immune events. The ability of LT to induce inflammation may have a role in its antitumor activity and is currently under investigation. Now that purified materials and monospecific antibodies are available, the role of these molecules in various types of immunologic reactions can be established.

ACKNOWLEDGMENTS

REFERENCES

1. Devlin JJ, Klostergard J, Yamamoto RS, Granger GA: Lymphotoxins: After fifteen years of research. *In* Lympho-kines: A Forum for Immunoregulatory Cell Products, E Pick (ed). San Diego, Academic Press, 1982, pp 313–336

2. Granger GA, Williams TW: Lymphocyte cytotoxicity *in vitro:* Activation and release of a cytotoxic factor. Nature 218:1253–1254, 1968

3. Gray PW, Aggarwal B, Benton C, Bringman T, Henzel W, Jarrett J, Lung D, Moffat B, Ng P, Sevedersky L, Palladino M, Nedwin G: Cloning and expression of cDNA for human lymphotoxin, a lymphokine with tumour necrosis activity. Nature 312:721–724, 1984

4. Wang A, Creasey A, Ladner M, Lin L, Strickler J, Van Aresdell J, Yamamoto R, Mark D: Molecular cloning of the complementary DNA for human tumor necrosis factor. Science 228:149–154, 1985

5. Orr SL, Plunkett M, Masunaka I, Granger GA: Purification and peptide characterization of human alpha-lymphotoxin obtained from the continuous human lymphoblastoid cell line IR 3.4. *In* Cellular and Molecular Biology of Lymphokines, C Sorg, A Schimpl (eds). Orlando, FL, Academic Press, 1985, pp 555–559

6. Willoughby DA, Boughton B, Schild HO: A factor capable of increasing vascular permeability present in lymph node cells. Immunology 6:484–498, 1963

7. Bennett B, Bloom R: Reactions *in vivo* and *in vitro* produced by a soluble substance associated with delayed-type hypersensitivity. Proc Natl Acad Sci USA 59:756–762, 1968

8. Willoughby DA, Walters MNI, Spector WG: Lymph node permeability factor in dinitrochlorobenzene skin hypersensitivity reaction in guinea-pigs. Immunology 8:578–584, 1965

9. Schwartz HJ, Leon MA, Pelley RP: Concanavalin A-induced release of skin-reactive factor from lymphoid cells. J Immunol 101(1):265–268, 1970

10. Pick E, Krejci J, Turk JL: Release of skin reactive factor from guinea-pig lymphocytes by mitogens. Nature 225:236–238, 1970

11. Houck JC, Barrantes D, Irausquin H: Skin reactive factor and lymph node permeability factor. Agents Actions 3(5):278–283, 1973

12. Spirer Z, Rudich A, Assif E, Zakut V, Bogair N: Release of skin reactive factor by human lymphocytes. Int Arch Allergy Appl Immunol 46:331–338, 1974

13. Houck J, Hellman KB, Chang CM: The biochemistry of lymphocyte-derived mediators of immunological inflammation. Agents Actions 8(1–2):73–79, 1978

14. Averbook B, Yamamoto R, Masunaka I, Orr S, Granger G: Human alpha lymphotoxin: pharmacokinetics and effects on Meth A tumors in BALB/c mice. Fed Proc 45(3):271 (abstr 682), 1986

15. Yamamoto RS, Kobayashi M, Plunkett JM, Masunaka IK, Orr SL, Granger G: Production and detection of lymphotoxin *in vitro:* micro-assay for lymphotoxin. *In* Practical Methods in Clinical Immunology, T Yoshida (ed). New York, Churchill Livingstone, 1986, pp 126–133

16. Klostergard J, Granger GA: Human lymphotoxins: Purification to electrophoretic homogeneity of the alpha heavy receptor bearing class. Mol Immunol 18:455–458, 1981

17. Shalaby MR, *et al.:* Activation of human polymorphonuclear neutrophil functions by interferon-gamma and tumor necrosis factors. J Immunol 135(3):2069–2073, 1985

18. Pennica D, *et al.:* Human tumor necrosis factor: Precursor structure, expression and homology to lymphotoxin. Nature 312:724–728, 1984

19. Aggarwal BB, Henzel WJ, Moffat B, Kohr WJ, Harkins RN: Primary structure of human lymphotoxin derived from 1788 lymphoblastoid cell line. J Biol Chem 260(4):2334–2344, 1985

20. Aggarwal BB, Eessalu TE, Hass PE: Characterization of receptors for human tumour necrosis factor and their regulation by gamma interferon. Nature 318(19):665–667, 1985

21. Yamamoto RS, Ware CF, Granger GA: The human LT system. XI. Identification of LT and TNF-like LT forms from stimulated natural killers, specific and nonspecific cytotoxic human T cells in vitro. J Immunol 137(6):1878–1884, 1986

22. Yamamoto RS, Johnson D, Masunaka I, Granger GA: Phorbol myristate acetate induction of lymphotoxins from continuous human B lymphoid cell lines in vitro. J Biol Response Modif 3:76–87, 1983