Tumor Necrosis Factor-alpha Exhibits Greater Proinflammatory Activity Than Lymphotoxin In Vitro

By Christopher E. Desch, Aldo Dobrina, Bharat B. Aggarwal, and John M. Harlan

Tumor necrosis factor-alpha/cachectin (TNF-α) and lymphotoxin (LT, TNF-β) are primarily products of monocytes and lymphocytes, respectively. The proteins are 51% homologous in their primary structure, cause necrosis of Meth A sarcoma in vivo, are toxic to selected tumor cells in vitro, and bind to the same receptor on cells in vitro. However, some recent studies have indicated both qualitative and quantitative differences between recombinant human (rh) LT and rhTNF with respect to inducing human umbilical vein endothelial cell (HEC) adherence for neutrophils and release of hematopoietic growth factor and interleukin-1 (IL-1) from HEC. The rhLT used in these studies was expressed in bacteria and was consequently not glycosylated, whereas natural LT is glycosylated. Therefore, we have compared various preparations of glycosylated and nonglycosylated rhLT with two preparations of rhTNF with respect to their proinflammatory characteristics. We now report that the same LT cDNA, when expressed in mammalian cell line and appropriately glycosylated, is also markedly less potent than rhTNF on a molar basis in inducing endothelial adhesiveness for neutrophils and in directly activating neutrophil adherence to albumin-coated plastic. All recombinant proteins, however, were equipotent on a molar basis in producing cytotoxicity in L929 cells. We conclude that differences in the primary structure of rhTNF and rhLT, rather than alterations induced by prokaryote protein processing, account for the disparate proinflammatory activity in vitro.

From the Department of Medicine, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA; the Institute of General Pathology, University of Trieste, Trieste, Italy; the Cytokine Research Laboratory, Department of Clinical Immunology and Biological Therapy, University of Texas MD Anderson Cancer Center, Houston, TX; and the Division of Hematology, Department of Medicine, University of Washington, Seattle, WA. Submitted February 27, 1989; accepted January 26, 1990. Supported by Grant No. HL-03174 from the US Public Health Service.

J.M.H. is a recipient of the Established Investigatorship Award from the American Heart Association. A.D. is a recipient of a Fulbright grant and a grant from the Italian Association for Cancer Research.

Address reprint requests to John M. Harlan, MD, Division of Hematology ZA-34, Harborview Medical Center, 325 Ninth Ave, Seattle, WA 98104.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1990 by The American Society of Hematology.

this study we compare the effects of highly purified preparations of rhLT expressed in E coli (rhLT-E) and two rhLT preparations expressed in two different transfected mammalian cell lines (rhLT-M1, rhLT-M2) with two preparations of rhTNF expressed in E coli (rhTNF1, rhTNF2) on human endothelial cells, human neutrophils, and murine L929 cells. This comparison allows us to determine whether differences in the structure of LT account for the discrepancies between rhTNF and rhLT. Our studies demonstrate that both rhLT-M (glycosylated) and rhLT-E (nonglycosylated) are markedly less potent than rhTNF in stimulating HEC adhesiveness for neutrophils or neutrophil adhesion to albumin-coated plastic.

MATERIALS AND METHODS

Reagents. RhTNF, (mol wt = 17,000 d)32 and rhLT-E (mol wt = 18,000 d)32 were expressed in E coli and purified to homogeneity as previously described.3,32 RhLT-M1 was derived from the same cDNA as rhLT-E, but was expressed in a mammalian kidney cell line A293 and purified to homogeneity as described.33 This preparation consisted of a 20 Kd and a 25 Kd form. The 25 Kd form was identical to that previously described for the full length LT isolated from RPMI 1788.34 The amino-terminal sequence of the 20 Kd polypeptide was found to be truncated by 20 residues on the amino-terminal end.33 RhTNF1 and rhLT-M1 were gifts of Dr Jeffrey Browning, Biogen Inc, Cambridge, MA. The rhTNF2 preparation was expressed in E coli and purified to homogeneity as previously described.32 RhLT-M1 was purified from LT-transfected CHO cells and consisted of a major species of 20 Kd and two minor species of slightly higher molecular weight.33 The amino-terminal amino acid sequence of this preparation was found to correspond to the amino-terminus of the 25 Kd form described by Aggarwal et al.,34 the different forms probably resulting from differences in a glycosylation.

Polymyxin B and actinomycin D were purchased from Sigma Chemical Co, St Louis, MO.

Cell culture. Human endothelial cells were isolated by collagenase treatment of umbilical veins as previously described37 and grown in RPMI-1640 medium (Whittaker MA Bioproducts, Walkersville, MD) containing 10 mmol/L N'-2-hydroxyethylpiperazine-N'-ethanesulfonic acid, 20% adult bovine and newborn calf serum (1:1; HyClone Sterile Systems, Logan, UT), 20 µg/mL endothelial cell-derived growth factor prepared as described by Maciag et al.,38 and 90 µg/mL heparin as described by Thornton et al.39

Adherence assays. Neutrophil adherence to HEC monolayers was assayed as previously described.35 Human polymorphonuclear neutrophils (PMNs) were purified by Ficol-Hypaque gradient centrifugation, dextran sedimentation, and hypotonic lysis of contaminating red blood cells. The PMNs were labeled with 51Cr (as sodium chromate, 1 µCi/mL; New England Nuclear, Boston, MA) as described.40 The 51Cr-labeled PMNs were resuspended in RPMI-1640 medium at 2,000/µL. First- or second-passage HEC were plated at confluent density in 48-well plates. Growth medium was removed, and RPMI-1640 medium (250 µL) containing 2% normal calf serum, 10 µg/mL polymyxin B, and varying concentrations of polymyxin B. Medium alone or medium containing the test reagent (250 µL) was then added to each well. Test reagents were diluted in RPMI containing 2% calf serum and 10 µg/mL polymyxin B. Medium alone or medium containing the test reagent (250 µL) was then added to the wells immediately after addition of PMNs. The PMNs were allowed to adhere to BSA-coated plastic for 1 hour. The plates were then washed twice with PBS to remove nonadherent PMNs. Adherent 51Cr-labeled PMNs were harvested with NH4OH, and percent adherence was calculated as above.

L929 cytotoxicity assay. The cytotoxic activity of rhTNF, rhLT-E, and rhLT-M on murine L929 cells was determined using actinomycin D as a sensitizing agent.41 Briefly, L929 murine fibroblasts (ATCC CCL 1, American Type; Culture Collection, Rockville, MD) were grown in 75 cm2 flasks in RPMI-1640 containing 10% horse serum (Flow Laboratories, McLean, VA). The cells were harvested by trypsinization, suspended in modified minimal essential medium (Flow Laboratories) containing 10% horse serum, and plated in microtiter wells at 2.5 x 104 cells per well. At the time of the assay, actinomycin D (1 µg/mL final concentration) was added to each well followed by control medium or medium containing rhTNF, rhLT-E, or rhLT-M. After an overnight incubation, supernatant medium was harvested, and the wells were washed three times with PBS. The remaining adherent cells were fixed and stained with crystal violet in 20% methanol for 15 minutes, and then washed again four times with water to remove unbound dye. Stained cells were lysed with 0.1 mol/L sodium citrate pH 4.2/50% ethanol, and the plates were read in a photometer at an absorbance of 570 nm (A570). Control wells were incubated in medium containing actinomycin D only (A570). Maximum cytotoxicity was determined by incubation with 100 U/mL of freshly diluted rhTNF (A570 maximum). Cytotoxicity was calculated as follows:

% Cytotoxicity = 1 - (A570 Test - A570 Maximum) / (A570 Maximum - A570 Medium) x 100%

Statistics. Data were analyzed by analysis of variance treatment (GB-Stats; Dynamic Microsystems, Inc, Silver Springs, MD).

RESULTS

On a molar basis rhTNF1, rhLT-E, and rhLT-M produced equivalent cytotoxicity to murine L929 cells (Fig 1). Fifty percent cytotoxicity was demonstrated at approximately 6 pmol/L rhTNF1, 4 pmol/L rhLT-E, and 8 pmol/L rhLT-M, (means of five separate experiments). Concentrations of each recombinant protein greater than 50 pmol/L produced 100% cytotoxicity. Each recombinant protein was tested for its ability to induce HEC to express surface activity promoting PMN adherence. Treatment of HEC with rhTNF1, rhLT-E, or rhLT-M, for 4 hours resulted in a dose-dependent increase in PMN adherence (Fig 2). RhTNF1 was markedly more potent than either rhLT-E or rhLT-M, in inducing HEC adhesiveness for PMNs. PMN adherence to HEC pretreated...
Fig 1. Cytotoxic activity of rhTNF, rhLT-E, and rhLT-M.
Cytotoxicity was determined after an 18-hour incubation of actinomycin D-treated L929 cells with rhTNF (C), rhLT-E (○), and rhLT-M, (△). Values represent the means ± SEM of five separate experiments. Values for rhTNF were not significantly different from those for rhLT-E and rhLT-M, by analysis of variance.

with 250 pmol/L rhTNF, was 20% ± 1% versus 9% ± 3% with 500 pmol/L rhLT-E, and 6% ± 2% with 500 pmol/L rhLT-M, (means ± SEM of five experiments). Stimulation of HEC adhesiveness for PMNs was observed at rhTNF, concentrations as low as 15 pmol/L, whereas significant stimulation of HEC was detected only at rhLT-E or rhLT-M, concentrations greater than 60 pmol/L.

The marked disparity between rhTNF and rhLT was confirmed in a separate series of experiments with additional preparations of rhLT-M and rhTNF. RhLT-M, was derived from CHO cells and, therefore, also glycosylated. RhLT-M, was markedly less active than rhTNF, in stimulating endothelial adhesiveness for PMNs (Fig 3), although both produced equivalent cytotoxicity on L929 cells. PMN adherence to HEC pretreated with 25 pmol/L rhTNF, was 17% ± 3% versus 7% ± 3% to HEC pretreated with 50 pmol/L rhLT-M, (means ± SEM of three experiments). In the L929 assay, 50% cytotoxicity was observed with 2 pmol/L rhTNF, and 1 pmol/L rhLT-M,.

RhTNF, rhLT-E, and rhLT-M, were also tested for their capacity to stimulate directly human PMN adherence to BSA-coated plastic (Fig 4). Again, rhTNF, was significantly more potent than rhLT-E and rhLT-M, at each concentration tested. Stimulated adherence was 40% ± 5% with 10 nmol/L rhTNF, versus 28% ± 3% and 21% ± 2% with 10 nmol/L rhLT-E and rhLT-M, at 0.1 nmol/L, adherence was 24% ± 4% with rhTNF, versus 11% ± 3% and 12% ± 3% with rhLT-E and rhLT-M, respectively (means ± SE of seven experiments).

DISCUSSION
Although both rhTNF and rhLT activate HEC and PMNs, our studies demonstrate that rhTNF is markedly more potent than rhLT, regardless of whether rhLT is
PROINFLAMMATORY ACTIVITY OF rhTNF vs rhLT

derived from E coli (rhLT-E) or a mammalian cell source (rhLT-M). The disparity between rhTNF and rhLT-M is in agreement with previously published differences between rhTNF and rhLT-E.\textsuperscript{21,26} Our results with two well-characterized preparations of rhLT-M effectively eliminate the possibility that glycosylation or other differences between prokaryote and eukaryote protein processing account for the previously reported disparity between the activity of rhLT-E versus rhTNF on HEC.\textsuperscript{22,23}

Pober et al\textsuperscript{28} reported that rhTNF expressed in E coli and rhLT expressed in CHO cells were equivalent in their ability to activate HEC. Unfortunately, the amino-terminal amino acid sequence of the rhLT preparation used in their studies has not been published, and the discrepancy between their results and ours remains unexplained.

In summary, although rhTNF and rhLT are remarkably similar in a number of respects, including tumor necrosis activity in vivo, cytotoxicity for some tumor cells in vitro, and binding to the same receptor on some cell types in vitro, the proteins differ significantly in their biologic effects on human endothelial cells,\textsuperscript{21,23} human monocytes,\textsuperscript{25} human neutrophils,\textsuperscript{26} human fibroblasts,\textsuperscript{27} and several human tumor cells\textsuperscript{28} in vitro. The significant differences between the effects of rhTNF and rhLT on human cells in vitro warrant a critical comparison of the activities of the two proteins in vivo. Since rhLT-E and rhTNF produce similar effects in mice,\textsuperscript{43} it may be necessary to compare the proteins in a nonhuman primate. If rhLT is indeed less potent than rhTNF in activating monocytes, neutrophils, fibroblasts, and endothelial cells in vivo, it will likely be considerably less toxic than rhTNF. Such studies may also provide insight into whether “toxic” effects are necessary for the anti-tumor activity of the tumor necrosis factors.

ACKNOWLEDGMENT

The skilled technical support of Kathe Stanness and Penny Thompson is gratefully acknowledged. We thank Pauline Marden for her skillful word processing and Dr Nicholas Kovach for performing the statistical analysis.

REFERENCES


Tumor necrosis factor-alpha exhibits greater proinflammatory activity than lymphotoxin in vitro

CE Desch, A Dobrina, BB Aggarwal and JM Harlan