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Differences in the Bioactivity of Recombinant Human TNF, LT, and T-Cell–Derived LT-3 on Transformed Cells In Vitro and the Meth A Tumor Growing in BALB/c Mice

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Summary: Cytolytic human T cells and the continuous human T cell line YM 1.2 can secrete a lymphotoxin (LT) form, termed LT-3, when stimulated in vitro. LT-3 is immunologically related to, but functionally distinct from, both alpha LT and tumor necrosis factor (TNF). Purified, native LT-3 and recombinant human LT and TNF were tested for their ability to destroy a panel of primary and continuous cell lines in vitro and cause necrosis of 8- to 9-day cutaneous Meth A tumors growing in BALB/c mice. LT-3 is more effective than either LT or TNF in inducing destruction of continuous cell lines in vitro. LT-3 induces destruction of cell lines that are resistant to both TNF and LT, and much less LT-3 is required to destroy cells that are LT and/or TNF sensitive. In contrast, none of these mediators had any measurable negative effects when tested on primary human fibroblasts. Additional studies revealed that LT-3 is also active in vivo and can cause necrosis of cutaneous Meth A tumors when administered intraperitoneally or intratumorally on BALB/c mice. LT-3 induces more rapid, consistent, and complete tumor necrosis than equivalent doses of either LT or TNF. **Key Words:** Alpha lymphotoxin—Lymphotoxin 3—Tumor necrosis factor.

Lymphotoxins (LTs) are proteins released by activated lymphocytes that can cause growth inhibition and lysis of transformed cells in vitro and necrosis of tumors in vivo (1–3). Tumor necrosis factor (TNF) is a protein released by activated macrophages and lymphocytes that also causes growth inhibition and lysis

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of transformed cells in vitro (4,5). Human alpha LT and TNF are now well characterized and purified recombinant forms are available. Alpha LT and TNF are related molecules for they share 28% amino acid identity (6). Cytolytic human T cells and several continuous T cell lines have been shown to release other cytotoxins when stimulated in vitro. Ware and Green (6) described a cytotoxin derived from stimulated human T cells that is immunologically unrelated to either LT or TNF. Yamamoto and colleagues (7,8) demonstrated that cytolytic human T cells can release three cytotoxins that were subsequently identified as alpha LT, a TNF-like material, and a cytotoxin termed LT-3. A continuous human T cell line, termed YM 1.2, was shown to release LT-3 and this effector molecule was purified to homogeneity (7). The LT-3 protein was shown to share immunologic determinants with both TNF and LT; however, it appeared to be more lytically active on several different types of target cells in vitro. The present studies were initiated to determine if LT-3 is active in vivo and to compare the effectiveness of LT, TNF, and LT-3 both in vitro and in vivo. These studies reveal that LT-3 is more lytically active than either LT or TNF in vitro and may be a more effective antitimor agent in vivo.

MATERIALS AND METHODS

TNF, LT, and LT-3 Samples

LT-3 was purified from cell- and serum-free supernatants derived from the continuous T cell line YM 1.2 after stimulation with phorbol myristate acetate as described previously (7). Isoelectric focusing fractions were brought to 1% with purified bovine serum albumin (BSA) and then dialyzed against 1,000 vol of sterile phosphate-buffered saline [0.01 *M* phosphate (pH 7.2) in 0.15 *M* NaCl (PBS)] to remove ampholines. Samples were aliquoted, filter sterilized, and stored at −10°C. Recombinant human TNF and LT were generous gifts of Cetus Corp. (Emeryville, CA, U.S.A.) and Genentech Corp. (South San Francisco, CA, U.S.A.), respectively. Samples were suspended in RPMI-1640 + 3% fetal calf serum (RPMI-3%) for in vitro experiments and PBS + 1% BSA for in vivo studies. All samples employed for in vivo studies were tested by the QLC 100 Limulus amebocyte lysate assay method and found to contain 0–3 U of endotoxin activity. As an additional control, 1.0 ml of individual active samples was placed in sterile glass test tubes and inactivated by heating at 100°C for 20 min by immersion in a boiling water bath.

Cell Culture

Both suspension and monolayer cells were maintained in RPMI + 3% or 10% fetal bovine serum (Gibco, Grand Island, NY, U.S.A.) in an atmosphere of 95% air/5% CO_2. Adherent monolayer cells employed in these studies were as follow: continuous human—Hela (cervical carcinoma), melanoma, breast carcinoma, colon carcinoma, lung carcinoma, and fibrosarcoma; continuous murine—Meth A (weakly adherent) and B-16 melanoma; primary human—primary fibroblasts. Nonadherent continuous human cells employed in these studies were as follow: K-562

(myelogenous leukemia), Raji (B lymphoblastoid), and Molt-4 (T lymphoblastoid). Primary and continuous monolayer cells were passaged twice weekly by trypsinization and resuspension in fresh medium. Continuous cells were passaged twice weekly by dilution and resuspension in fresh medium. All cell cultures were maintained in Falcon plastic tissue culture flasks. Ascites were collected from BALB/c mice 9 days after intraperitoneal injection of 2×10^6 Meth A cells. Ascites Meth A cells were introduced and maintained in vitro in an identical fashion to nonadherent cells and were employed in these studies after 2 months of passage in culture.

In Vitro Cell Destructive Assays

A microassay described by Yamamoto et al. (9) was employed in all these studies. Briefly, 20,000 target cells in 100 μl RPMI-3% in 0.5 μg/ml of mitomycin C were added to individual wells of flat-bottomed 96-well Falcon microtiter plates. After incubation for 16 h at 37°C, the medium was discarded and the cells were treated with 25 μl of serial dilutions of individual effector molecules in RPMI-3%. After incubation for 16–48 h in a 5% CO_2/95% air mixture, the medium was discarded and the remaining cells stained with crystal violet. After thorough washing, the crystal violet was solubilized in acidified alcohol and the plate assayed in a Titertek Multiskan plate reader (Flow Laboratories, McLean, VA, U.S.A.) with a 580-nm interference filter. Viability of treated and control suspension cells was determined by the thiazolyl blue [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)] staining technique (9). In both these methods, cell destruction is proportional to the density of stain detected in each well. A unit of activity is the amount of effector molecule required to destroy 50% of 20,000 target L929 cells in 16 h in the microplate assay. This unit system is arbitrary, and all references to units in the text refer to units of activity as measured in this assay. Recombinant human LT and native LT-3 have a specific activity of 3×10^7 U/mg protein and recombinant TNF has a specific activity of 1×10^7 U/mg protein when tested in this laboratory.

In Vivo Antitumor Effects

The Meth A tumor was a generous gift from Dr. Lloyd Old (Memorial Sloan-Kettering Cancer Institute, New York, NY, U.S.A.) The tumor was maintained in the ascites form by weekly passage in normal female BALB/c mice. Cutaneous tumors were induced by intradermal injection of 5×10^5 ascites Meth A cells on the shaved backs of female BALB/c mice as described by Carswell et al. (10). Animals were employed for study 8–9 days after injection, when tumors were ~0.5 cm in diameter. All animals were preselected for tumors of similar size. Control tumor animals were either untreated or injected with the PBS + 1% BSA vehicle employed to suspend the test molecule. The percentage of necrosis was ranked by visual observation on a scale of 0–4, with 0 being no necrosis and 4 equivalent to 95–100% tumor necrosis. Tumors were visually examined every 3 h for the first 24 h then at 12- to 24-h intervals. Tumors were measured every 48–72 h and size was determined by the product of tumor length × width × thickness.

RESULTS

Comparison of In Vitro Cell Lytic Effects of Human Recombinant Alpha LT, TNF, and Purified, Native LT-3

Identical numbers of cells from different continuous or primary lines were established in individual wells of microtiter plates. Duplicate wells were then treated with serial dilutions of LT, TNF, LT-3, or vehicle control RPMI-3%. The number of lytic units required to cause destruction of 50% of each type of target cell was established as described in Materials and Methods. The results from a single experiment, which was repeated three times with essentially similar results, are shown in Table 1. In general, fewer units of TNF were required than units of LT to induce destruction of cell lines that were sensitive to both these mediators. However, TNF was effective against K-562 and Molt-4 cell lines, which were not affected by the highest levels of LT employed in these studies. In contrast, LT-3 was effective against all transformed cell lines employed, even five lines that were resistant to the highest levels of both TNF and LT. In addition, much lower levels of LT-3 were effective in causing target cell destruction than levels of either TNF or LT. None of these effectors had measurable negative effects on the primary fibroblasts employed in these studies. While the data are not shown, we found no

TABLE 1. *The effect of LT, TNF, and LT-3 on various continuous and primary cell lines in vitro*

Cell line employed	Units required to induce 50% cell lysis		
	TNF	LT	LT-3
Human continuous cells			
Hela (cervical carcinoma)	250 ± 50	300 ± 50	20 ± 5
Melanoma	350 ± 100	850 ± 100	25 ± 4
Breast carcinoma	NE	NE	28 ± 6
Lung carcinoma	NE	NE	35 ± 7
Fibrosarcoma	1,590 ± 200	NE	220 ± 20
Colon carcinoma	460 ± 80	1,050 ± 200	75 ± 12
K-562 (myelogenous leukemia)	670 ± 100	NE	100 ± 23
Raji (B lymphoid cell line)	NE	NE	85 ± 13
Molt-4 (T lymphoid cell line)	220 ± 50	NE	15 ± 4
Human primary cells			
WI-38 fibroblasts	NE	NE	NE
GM3468 fibroblasts	NE	NE	NE
Skin fibroblasts	NE	NE	NE
Murine continuous cells			
Meth A fibrosarcoma	NE	NE	125 ± 23
B-16 melanoma	NE	NE	40 ± 7

Nondividing cells (20,000) were established in duplicate wells of Falcon microtiter plates, then exposed to various dilutions of effector molecule containing up to 2,000 U of activity. The number of viable cells remaining after 24–48 h was determined for both adherent and nonadherent cells as described in Materials and Methods. The number of units causing 50% reduction of cell numbers is shown. NE, no measurable effect.

change in cell lytic activity when LT-3 was tested on various target cells in the presence of rabbit antibodies that neutralize human alpha, beta, and gamma interferons.

Induction of Necrosis of Cutaneous Meth A Tumors Growing on BALB/c Mice by LT-3 Administered Intraperitoneally

Cutaneous 8- to 9-day Meth A tumors were induced on female BALB/c mice as described in Materials and Methods. Groups of three tumor animals received either various doses of LT or vehicle control (PBS + 1% BSA) or they were not treated. The results of one experiment, which was repeated three times with similar results, are shown in Figs. 1 and 2. Central necrosis first became evident within 5–6 h after injection of ≥7,000 U. Necrosis progressed from the center to involve the entire tumor mass by 24 h as shown in Fig. 1A. While central necrosis appeared in tumors treated with lower LT-3 doses, it did not progress to involve the entire tumor mass. Doses of ≥7,000 U induced complete destruction of the tumor. Shown in Fig. 1B are tumor-bearing animals (animals A and B) injected with 15,000 U and the vehicle control animal (animal C) 32 days after treatment. The effects of this treatment on tumor growth and survival of these animals are shown in Fig. 2. Doses of LT-3 below 7,000 U caused partial necrosis and temporary inhibition of tumor growth; however, the tumor regrew and eventually killed the animals. Survival to 40 days is shown in Fig. 2; however, the animals receiving >7,000 U remained tumor-free for the 6-month duration of these studies. Untreated tumor animals and those treated with the vehicle exhibited identical growth patterns.

Induction of Necrosis of Cutaneous Meth A Tumors on BALB/c Mice by LT-3 Administered Directly into the Tumor

BALB/c mice bearing comparable 8- to 9-day cutaneous Meth A tumors were randomly divided into groups of three animals. Test animals were injected with 20 μl of various dilutions of LT directly into the tumor mass. Control tumor animals were either not treated or injected with 20 μl of the PBS + 1% BSA vehicle control. Tumors on both test and control animals were examined visually for evidence of necrosis and measured for growth as described in the previous section. A summary of the results of one study that was repeated three times is shown in Fig. 3. Central necrosis became visible within 2–3 h after injection in test animals receiving 300 U of LT-3 activity. Necrosis then progressed to involve the entire tumor mass by 24 h. Doses of LT-3 of <150 U caused some central necrosis but did not progress to involve the entire tumor mass. This dosage of LT-3 also caused temporary inhibition of tumor growth; however, tumors regrew and eventually killed the animals. Almost all animals (97%) that received ≥150 U exhibited complete tumor regression. These animals remained tumor free for the 40-day period and, while not shown, they were still tumor-free after the 6-month duration of these studies.

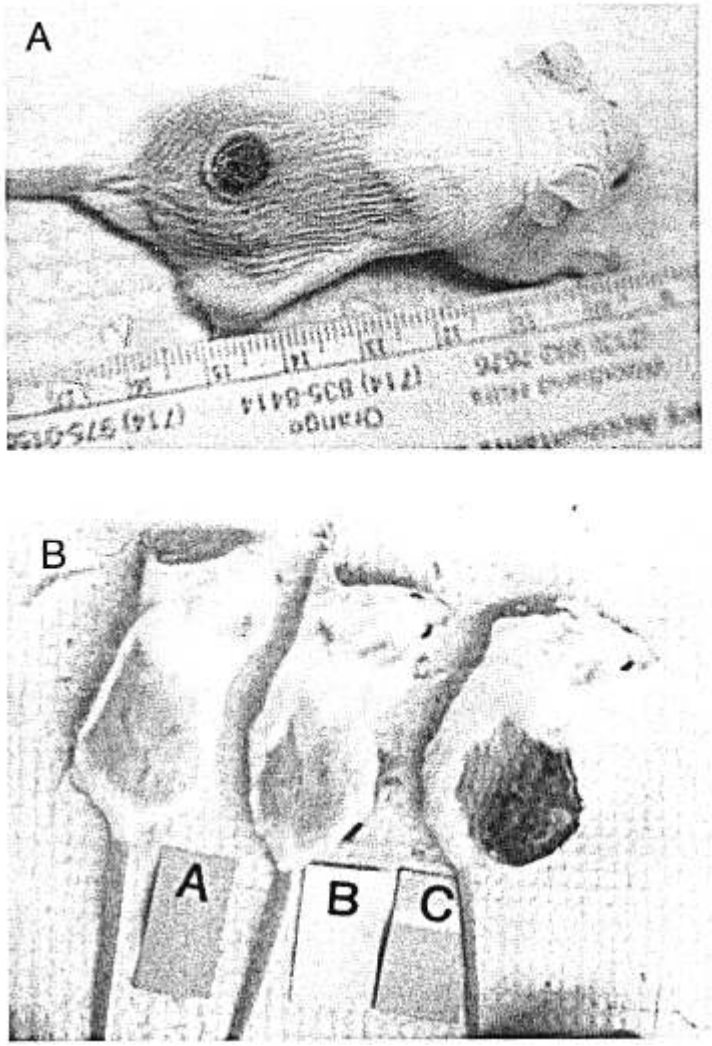

FIG. 1. The effect of human LT-3 administered intraperitoneally on 8-day cutaneous Meth A tumors growing on BALB/c mice. A: Necrosis observed 24 h after injection of 15,000 U of LT-3. B: Day 32 after tumor induction. Animals A and B received 15,000 U LT-3 and animal C received vehicle control on day 8.

The Intraperitoneal Dosage of LT, TNF, and LT-3 That Induces Negative Side-effects in Normal BALB/c Female Mice

Normal female BALB/c mice each received a single intraperitoneal injection of LT, TNF, or LT-3. Animals were visually examined every 6 h over a 3-day period. There were three animals in each dosage group. Animals treated with LT and TNF received 0.2, 0.5, and 1.0 $\times$ 10^6 U (LT animals also received 1.5 $\times$ 10^6 U). Animals treated with LT-3 received 1.0, 1.5, and 2.0 $\times$ 10^5 U. Animals

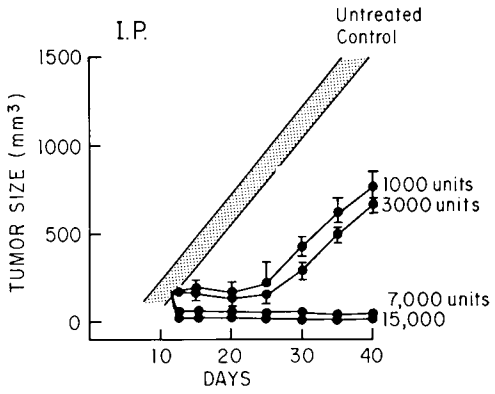

FIG. 2. Tumor-bearing animals received various amounts of LT-3 or vehicle control. The data shown represent the averages of three animals per dosage group. Tumor size was determined as described in Materials and Methods.

receiving TNF began to show signs of discomfort at 5×10^5 U. These animals stopped eating, ceased moving, and assumed a hunched position in their cages for ~24 h, then they recovered and behaved normally. Animals exhibited similar symptoms at a level of 1.5×10^6 U of LT. Animals that received LT-3 showed similar reactions at 1.5×10^5 U. In contrast, animals treated with vehicle or identical amounts of active samples that had been inactivated by heating exhibited no negative reactions.

DISCUSSION

While LT and TNF are physically related (5) and have been reported to bind to the same receptor on cells in vitro (11), we found differences in the sensitivity of individual cell lines to these two effectors. Recombinant TNF caused destruction of Molt-4 and K-562 cells; however, these cells were not affected by similar levels of recombinant LT. Weitzen et al. (12) have also reported that Molt-4 and K-562 cells are resistant to destruction induced by native human alpha LT in vitro. It has also been reported that LT and TNF differ in their ability to induce inflammation in tumor and normal tissues (13), to induce neutrophilia when injected into rats (14), and to induce interleukin 1 production by human endothelial cells in vitro

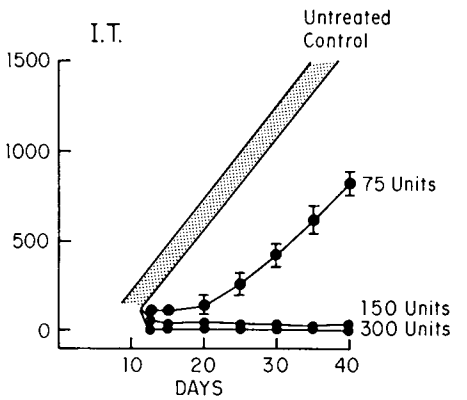

FIG. 3. The effect of human LT-3 administered directly into 8-day cutaneous Meth A tumors on BALB/c mice. Tumor-bearing animals received 20 μl of various levels of human LT-3 or vehicle control on day 8. Tumor size was determined as described in Materials and Methods. The data represent the averages of three animals per dosage group.

(15). Thus, while structurally related, these two effectors appear to have different functional capabilities both in vitro and in vivo.

When compared on a unit basis, native LT-3 is more effective than recombinant LT or TNF in causing destruction of different transformed cell lines in vitro. First, it takes less LT-3 than LT or TNF to kill target cell lines that are sensitive to these two mediators. Second, small amounts of LT-3 caused destruction of target cell lines that are resistant to the highest levels of TNF and LT we employed in these studies.

The present studies also reveal that LT-3 is active as an antitumor agent in the Meth A-BALB/c tumor-host system. LT-3 has the capacity to induce necrosis of cutaneous Meth A tumors whether administered systemically or locally into the tumor. However, direct intratumor injection is the most effective, for it requires the least amount of material to cause necrosis and complete tumor destruction. Necrosis begins rapidly after treatment of tumors with LT-3. Central necrosis was apparent 5–6 h after intraperitoneal administration and 2–3 h after intratumor injection. The central necrosis then rapidly progressed to involve the entire tumor mass by 24 h. In contrast, necrosis induced by LT and TNF in this same tumor-host model is more protracted (3,10; B. Averbook et al., submitted). Necrosis first appears 12–16 h after injection of even the highest levels of LT and TNF, and requires 48–72 h to involve the entire tumor mass.

When compared on a unit basis, LT-3 appears to be more efficient as an antitumor agent in the Meth A system than either TNF or LT. We have published studies on the effect of different dosages of LT and TNF on this tumor-host system elsewhere (3; B. Averbook et al., unpublished observations). However, we can extract and summarize certain of these data to facilitate comparison of the relative degree of effectiveness of these three interrelated mediators. The data, shown in Table 2, have been selected to allow comparison of the dose of each mediator required to induce 90–100% necrosis of 7- to 8-day cutaneous tumors and the percentage of animals that become long-term survivors. These data were assembled from different individual studies and were conducted over a 3-year

TABLE 2. *Comparison of the antitumor activity of LT, TNF, and LT-3 when administered to BALB/c mice bearing 8-day cutaneous Meth A tumors*

Effector molecule	Route of injection	Units causing 90–100% necrosis	% long-term survivors
LT	Intraperitoneal	225,000 ± 75,000	45
	Intratumor	25,000 ± 5,000	65
TNF	Intraperitoneal	240,000 ± 70,000	42
	Intratumor	28,000 ± 6,000	65
LT-3	Intraperitoneal	12,000 ± 3,000	95
	Intratumor	175 ± 25	97

BALB/c mice bearing cutaneous 8-day Meth A tumors were treated with a single injection of LT, TNF, or LT-3. Necrosis, tumor growth, and animal survival were assessed as described in Materials and Methods. The data shown are averages and are derived from individual experiments. There were ~40, 15, and 10 animals in each dosage group of LT, TNF, and LT-3 studies.

period. LT and TNF appear to be quite similar when compared on a unit basis whether injected via intraperitoneal or intratumor routes. The wide degree of variation is observed when we sum data of tumor animals treated with the same doses but derived from different experiments and is currently unexplained. In this same situation LT-3 causes 90–100% necrosis at lower doses and a greater percentage of treated animals go on to become long-term survivors. The data in Table 2 also indicate that intraperitoneal doses of TNF (5.0×10^5 U) and LT (1.5×10^6 U) that cause 90–100% necrosis are two- and fivefold below the levels that we find begin to induce negative side-effects, respectively. In contrast, levels of LT-3 (1.5×10^5 U) causing the same degree of necrosis are 10-fold below those that begin to induce negative side-effects. Negative side-effects in these studies are indicated by cessation of eating, ruffled fur, and the assuming of a hunched, unmoving position. However, these animals recovered from these negative effects after 24–36 h. Lethal dose levels were not studied. It is therefore clear that LT-3 is more toxic than either LT or TNF. LT could be ranked as superior to TNF in this tumor-host system because it is not as toxic as TNF and is effective at levels well below those that induce negative side-effects.

We have shown that LT-3 is immunologically related to both TNF and LT proteins (7,8). It will be interesting to establish the precise molecular relationship of LT-3 to TNF and LT. This will establish if LT-3 is a new LT form or a posttranslationally modified TNF or LT molecule. In either case, these data may provide important information as to why the LT-3 molecule is more effective both in vitro and in vivo. An understanding of the structure-function relationship of these three proteins could lead to the genetic engineering of molecules with maximum antitumor activities and minimal negative side-effects.

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