Lymphotoxin Is an Autocrine Growth Factor for Epstein-Barr Virus–infected B Cell Lines

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Summary

Because human lymphotoxin (LT) was originally isolated from a lymphoblastoid cell line, we investigated the role of this molecule in three newly established Epstein-Barr virus (EBV)–infected human B cell lines. These lines were derived from acute lymphoblastic leukemia (Z-6), myelodysplastic syndrome (Z-43), and acute myelogenous leukemia (Z-55) patients who had a prior EBV infection. Each lymphoblastoid cell line had a karyotype that was different from that of the original parent leukemic cells, and all expressed B cell, but not T cell or myeloid surface markers. In all three lines, rearranged immunoglobulin heavy chain joining region (J_H) bands were found, and the presence of EBV DNA was confirmed by Southern blotting. Z-6, Z-43, and Z-55 cell lines constitutively produced 192, 48, and 78 U/ml LT, respectively, as assessed by a cytotoxicity assay and antibody neutralization. Levels of tumor necrosis factor (TNF) were undetectable. Scatchard analysis revealed that all the cell lines expressed high-affinity TNF/LT receptors with receptor densities of 4197, 1258, and 1209 sites/cell on Z-6, Z-43, and Z-55, respectively. Furthermore, labeled TNF binding could be reversed by both unlabeled TNF, as well as by LT. Studies with p60 and p80 receptor-specific antibodies revealed that the three lines expressed primarily the p80 form of the TNF receptor. When studied in a clonogenic assay, exogenous LT stimulated proliferation of all three cell lines in a dose-dependent fashion at concentrations ranging from 25 to 500 U/ml. Similar results were obtained with [^{3}H]TdR incorporation. Monoclonal anti-LT neutralizing antibodies at concentrations of 25–500 U/ml inhibited cellular multiplication in a dose-dependent manner. It is interesting that in spite of a common receptor, TNF (1,000 U/ml) had no direct effect on Z-55 cell growth, whereas it partially reversed the stimulatory effect of exogenous LT. In addition, TNF inhibited Z-6 and Z-43 cell proliferation, and its suppressive effect was reversed by exogenous LT. Both p80 and p60 forms of soluble TNF receptors suppressed the lymphoblastoid cell line proliferation and their inhibitory effect was partially reversed by LT. Our data suggest that (*a*) LT is an autocrine growth factor for EBV-transformed lymphoblastoid B cell lines; and (*b*) anti-LT antibodies, soluble TNF/LT receptors, and TNF itself can suppress the growth of lymphoblastoid cells, probably by modulating or competing with LT. It is therefore possible that soluble TNF/LT receptors, TNF, and anti-LT antibodies may be exploitable for clinical trials in disorders in which EBV has been implicated as an etiologic factor.

EBV can infect and immortalize human B lymphocytes and, as a result, permanent lymphoblastoid cell lines are established (1). EBV-carrying B cell lines may evolve from cells transformed in vitro (1) or from individuals who had a prior EBV infection (2, 3). Although the mechanism by which these cells are immortalized is largely unknown, it has been shown that the cells contain most, if not all, the herpesvirus genome as a multicopy episome (4), and that EBV, through expression of the full set of light virus-coded "latent" proteins, may protect human B cells from programmed cell death (apoptosis) (5).

Over the last several years it has become clear that a critical event in the process of B cell immortalization by EBV is the establishment of an autocrine loop where the cells produce a growth factor that supports their own proliferation (6–9). Several molecules may contribute to this activity. A protein with IL-1 bioactivity has been reported to stimulate EBV-transformed B cell proliferation in an autocrine fashion (10–12). Similarly, affinity-purified soluble CD23, a B cell activation antigen expressed at high levels in EBV-immortalized cells, which also functions as a low-affinity receptor for IgE (13, 14), was reported to promote growth in EBV-immortalized B cells by some investigators (15) but not by others (16, 17). IL-6 has been found to contribute to up to 30% of the autocrine growth–stimulating activity in many EBV lymphoblastoid cell lines (18). Other investigators reported that normal human EBV-transformed B cells constitutively produce IL-5 (19), and that IL-5 probably stimulates EBV-transformed B cell proliferation in an autocrine fashion (20). Recently, Pike et al. (21) have identified lactic acid as a soluble factor that promotes growth of EBV-immortalized B cells in serum-free conditions.

Previously, we have reported the isolation of human lymphotoxin (LT)[1] from a lymphoblastoid cell line (22, 23). Subsequently, we screened over 200 EBV-transformed human B cell lines and found that all lines without exception, produce LT constitutively (Aggarwal B. B., unpublished data). The role of LT in these cells is not understood. It is known that recombinant LT enhances the proliferation of activated B cells and augments B cell proliferation induced by IL-2 (24). Furthermore, Seregina et al. (25) have recently reported that conditioned medium from a human lymphoblastoid B cell line RPMI-6410t that stimulated its own growth, contained a high concentration of LT. Although this study suggested that LT was an autocrine growth factor for EBV-immortalized B cells (25), another study showed that the EBV-transformed DUL lymphoblastoid cells were not affected by LT (26). We therefore analyzed LT expression and activity in three different lymphoblastoid cell lines recently established in our laboratory. We found that all three lines constitutively produce and secrete LT and express high-affinity LT receptors. In addition, their proliferation is stimulated by LT and inhibited by anti-LT neutralizing antibodies, soluble TNF/LT receptors, and TNF.

Materials and Methods

Cell Lines. The EBV B cell lines we studied arose spontaneously from suspension cultures of bone marrow (BM) cells from three patients who had previously been infected with EBV. Z-6 line was developed from BM cells of a 26-yr-old acute lymphoblastic leukemia patient whose BM cells did not show any cytogenetic abnormality; Z-43 cell line was established from marrow cells obtained from an 84-yr-old patient who had refractory anemia with excess of blasts in transformation (RAEB-t) and a karyotype of 46, XX, t(3q;5q); and Z-55 is a cell line derived from BM cells of an 80-yr-old patient with acute myelogenous leukemia whose blasts were diploid. This study was performed with informed consent and was approved by the Human Ethics Committee of our institution.

Heparinized BM cells obtained from the three patients at diagnosis were layered over Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) and centrifuged (400 g, 4°C) for 20 min to remove neutrophils and RBCs. Low-density BM cells (10^6/ml) were cultured in RPMI-1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS (Flow Laboratories, Inc., McLean, VA). Cultures were maintained in 25- or 75-cm^2 tissue culture flasks (Becton Dickinson & Co., Oxnard, CA) and were fed by replacement of 80% of the medium with an equal volume of fresh complete medium every 3 d for several months. Cells from the established lines were maintained in logarithmic growth before testing. Cell density was decreased to 2–5 × 10^5 cells/ml and culture medium was exchanged every 24 h. Viability was assessed using the trypan blue exclusion test. Cytospins were stained with May-Grunwald Giemsa for morphological analysis.

LT, TNF, Anti-LT Neutralizing Antibodies, Anti-TNF Receptor Antibodies, and Soluble TNF Receptors. Recombinant human (rh) LT (sp act 5 × 10^7 U/mg) and monoclonal anti-LT neutralizing antibodies were generously provided by Genentech, Inc. (South San Francisco, CA). rhTNF-α (sp act 2 × 10^7 U/mg) was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Antibodies against the p60 and p80 forms of the TNF receptor were developed in a rabbit injected with purified recombinant forms of these proteins (27). The antibodies were purified by affinity chromatography. Soluble TNF receptors (p60 and p80) produced in *Escherichia coli* were kindly provided by Dr. T. Kohno (Synergen Corp., Boulder, CO).

Immunophenotype Analysis. The mAbs used to determine immunophenotype were anti-CD2, CD3, CD7, CD9, CD10, CD13, CD14, CD19, CD20, CD21, CD22, and CD33, all of which were obtained from the Becton Dickinson monoclonal center (Mountain View, CA), except anti-CD9 mAb 50N19 which was kindly provided by B. M. Longsnecker (University of Alberta, Edmonton, Alberta, Canada). Staining of the leukemia cells (5 × 10^6 cells/ml) followed previously described procedure (28, 29) and isotype-identical nonreactive mAbs were used as negative controls. Stained cells were analyzed on a dual-beam cytofluorograph (System 50H; Ortho Diagnostics Systems, Westwood, MA).

Cytogenetic Analysis. Z-6, Z-43, and Z-55 cells were fed with fresh RPMI-1640 medium supplemented with 20% FCS 24 h before harvesting. Cells were then spun down and exposed to hypotonic solution (0.06 M, KCl) for 10 min at room temperature. After centrifugation at 1,700 rpm for 5 min, the supernatant was discarded, the cell pellet was fixed in a mixture of methanol and acetic acid (3:1 by volume), and washed three times with a fixative. Fixed cells were dropped on glass slides, and air-dried chromosome preparations were made. 5–6-d-old slides were G-banded following the routinely used technique (30). 50 G-banded metaphase spreads from each cell line were evaluated under the microscope and 7–10 karyotypes were prepared using the automatic karyotyping system (Genetiscan; PSI, Houston, TX).

DNA Analysis for Immunoglobulin Heavy Chain Joining Region (J_H) Rearrangement and for the Presence of EBV. DNA was prepared by proteinase K (Bethesda Research Laboratories, Bethesda, MD) digestion in SSCE buffer (0.15 mol/liter sodium chloride, 0.015 mol/liter sodium citrate, 0.01 mol/liter EDTA). 15 μg of DNA from each cell line were digested with restriction endonucleases under conditions recommended by the supplier (International Biotechnologies, Inc., New Haven, CT), electrophoresed on 0.8% agarose gel, blotted, and hybridized as described (31). The probes were labeled by oligoprimer extension to a sp act of 1–3 × 10^9 cpm/μg of DNA (32). After hybridization, the filters were washed at 60°C for 60 min with a solution of 0.1 × SSC (1× SSC =

[1] *Abbreviations used in this paper:* BM, bone marrow; J_H, immunoglobulin heavy chain joining region; LT, lymphotoxin; RCV, relative cell viability; rh, recombinant human.

0.15 M NaCl + 0.015 M NaCitrate) and 0.1% SDS, dried, and autoradiographed. The probes used were the following: H-a 2.9 kb Hind III/EcoRI insert (kindly provided by Dr. A. Deisseroth, M. D. Anderson Cancer Center) for the J_H rearrangement, and EBV 5.0-kb segment containing the coding region for the nuclear antigen-1 (generously provided by Dr. J. Hearing, State University of New York, Stony Brook, NY) (33).

LT and TNF Bioassays. Mouse connective tissue cell line L-929 (CCL 1) was obtained from American Type Culture Collection, (Rockville, MD).

TNF secretion by B lymphoblastoid cells was determined in culture supernatants, using bioassay as previously described (34). Briefly, 0.2 × 10^5 cells in 0.1 ml of the MEM medium containing 10% FCS, glutamine (2 mM), penicillin (500 U/ml), and gentamycin (5 μg/ml) were plated in 96-well plates (Falcon Labware, Oxnard, CA). After overnight incubation, media was removed and a serial dilution of the test sample along with actinomycin D (1 μg/ml) was layered in a total final volume of 0.1 ml, and then the incubation at 37°C was continued for the next 18 h. Thereafter, the media was removed and viable cells were monitored by crystal violet staining according to the procedure as described (22). The plates were read for optical density by an autoplate reader. Percent relative cell viability (RCV) was calculated as optical density in the presence of LT/TNF as test sample divided by optical density in the absence of test sample (media) multiplied by 100. 1 U of TNF/LT was the amount needed for 50% RCV. For neutralization of biological activity, test sample was incubated with the TNF or LT-specific antibodies (35) for 30 min at 37°C, and then assayed for remaining activity by the bioassay.

Radioreceptor Assay. Receptor binding assays were carried out essentially as previously described (27, 36). TNF was labeled with $Na^{125}I$ using the Iodogen method as described previously (36). Briefly, 10 μg of TNF in a 20 μl volume was placed onto a film of 50 μg of Iodogen and incubated for 10 min at 4°C in the presence of 1 mCi of carrier-free $Na^{125}I$. Free iodine was removed by gel filtration on a PD-10 (Sephadex G-25; Pharmacia LKB Biotechnology AB, Uppsala, Sweden) column equilibrated with PBS containing 0.1% gelatin. More than 96% of the iodine in the final product was incorporated into the protein as determined by TCA precipitation. The specific activity of the labeled TNF ranged from 20 to 30 mCi/mg. Standard binding assays were performed in flexible 96-well plates precoated with 0.2 ml of FCS for 24 h at 4°C. The binding medium (RPMI-1640) contained 10% FCS. Cells (10^6/0.1 ml) were incubated with increasing amounts of ^{125}I-TNF in the absence (total binding) or presence of 1,000 nM unlabeled ligand (nonspecific binding) for 90 min at 4°C. The cells were washed three times with ice-cold medium (PBS containing 0.1% BSA) at 4°C, and the cell-bound radioactivity was determined in a gamma-counter (Cobra-Auto-Gamma, Packard Instrument Co., Meriden, CT). Each determination was performed in triplicate. Specific binding of the ^{125}I-labeled TNF was calculated by subtraction of nonspecific binding from the total binding. The dissociation constant (K_d) and the number of receptors were obtained by Scatchard analysis (37).

Clonogenic Assay. The clonogenic assay was performed as previously described (38). B lymphoblastoid cells were cultured in 0.8% methylcellulose (Fluka Chemical Corp., Ronkonkoma, NY), 10% FCS (Flow Laboratories) and alpha medium (Gibco Laboratories, Inc.) at 2–4 × 10^4 cells/ml. The culture mixture was placed in 35-mm petri dishes (Nunc Inc., Naperville, IL) in duplicate and maintained at 37°C with 5% CO_2 in air in a humidified atmosphere. Colonies were counted after 7 d using an inverted microscope. A colony was defined as a cluster of more than 40 cells.

Thymidine Incorporation Assay. This assay was carried out essentially according to a previously described procedure (39). To determine the effect of human LT, cells (10 × 10^3/well) were plated in 0.2 ml of the medium (RPMI-1640 plus 10% FCS) in 96-well plates (Falcon Labware) along with variable concentrations of LT. After 5 d of incubation, [^{3}H]TdR incorporation was examined. During the last 6 h, [^{3}H]TdR (6.7 Ci/nmol; New England Nuclear, Boston, MA) was added to each well (0.5 μCi/well). Thereafter, cells were harvested with the aid of a cell harvester (PHD; Cambridge Technology, Inc., Watertown, MA) and lysed by washing with distilled water. Radioactivity bound to the filter was measured in a liquid scintillation counter (Model 1600 TR; Packard Instrument Co.). Each determination was made in five replicates.

Results

Cell Characterization Studies. The three lines were characterized by morphological, immunophenotypical, cytogenetic, and receptor binding studies. All cells from our three cell lines have lymphoblast morphology. Their doubling time in liquid culture is ∼24 (Z-6), 16 (Z-43), and 12 h (Z-55). The cells form clusters that can be easily broken by repeated pipetting and if left in culture, clusters would be formed again. All cell lines were mycoplasma-free, as tested by a rapid mycoplasma detection system (Gen-Probe, San Diego, CA).

Immunophenotype studies revealed that the three cell lines expressed B cell (CD9, CD10, CD19, CD20, CD21, CD22) but not T cell (CD2, CD3, CD7) or myeloid (CD13, CD14, CD33) surface antigens (Table 1), indicating that Z-6, Z-43, and Z-55 are B cells. It is interesting that CD21, which is expressed in the three cell lines, is a receptor for the gp350/220 envelope glycoprotein of EBV (40, 41).

Cytogenetic analysis showed that the lymphoblastoid cell line Z-6 was exclusively tetraploid, and not a single metaphase cell showed near-diploid or diploid chromosome constitution. Giemsa-banding pattern revealed a male XY chromosome constitution with only three copies of chromosome 6 and a translocation between the 1q and 15q. This only submetacentric marker chromosome was of the morphology of a chromosome No. 2. A typical G-banded karyotype showing this marker chromosome from three additional cells is shown in Fig. 1 *a*. In cell line Z-43, the stem line chromosome number varies from 46 to 48. The Giemsa-banding pattern revealed three cell populations with respect to both structural and numerical chromosome abnormalities. A derivative chromosome No. 2 was present in every metaphase spread with the XX sex chromosome constitution. The distal end of the q arm of this chromosome 2 was replaced by an unidentified chromosome segment. Other cell populations have either trisomy of chromosomes 12, 15, or both. A typical G-banded karyotype of this cell line is shown in Fig. 1 *b*. All of these abnormalities, individually or in combination, are clonal. Lymphoblastoid cell line Z-55 showed 47 chromosomes in all metaphase spreads. Giemsa-band analysis revealed complete or partial trisomy of chromosome 3 and an XY chromosome constitution (Fig. 1 *c*). There were three types of metaphases with respect to chromosome 3 abnormalities. One third of the cells had complete trisomy of chromosome 3, and another one-third cell population had partial trisomy of chromosome 3,

Table 1. *Surface Marker Analysis of the B Lymphoblastoid Cell Lines*

	Percent positive cells											
Cell line	CD2	CD3	CD7	CD9	CD10	CD19	CD20	CD21	CD22	CD13	CD14	CD33
Z-6	0	8	0	53	5	94	94	87	15	0	0	0
Z-43	0.3	0	0	51	13	82	91	56	28	0	0	0.9
Z-55	0	5	4	48	9	81	89	60	38	0	0	2

Figure 1. (*a*) A G-banded karyotype from the cell line Z-6 showing tetraploid chromosome constitution. Only chromosome 6 is present in three copies. A translocation involving the long arm of chromosome 1 and the long arm of 15 is a characteristic feature of this cell line. (*Bottom*) This marker chromosome from three additional metaphase spreads is shown (*arrows*). (*b*) A G-banded karyotype of the cell line Z-43 showing a derivative chromosome No. 2 (*arrows*) and trisomies of chromosomes 12 and 15. All other chromosomes appear to have normal G-banding pattern and are present in disomic form. (*Bottom*) Derivative and a normal chromosome 2 and trisomies of 12 and 15 from another metaphase spread. (*c*) A G-banded karyotype of a cell from the cell line Z-55 showing partial trisomy of chromosome 3 and the XY chromosome constitution. All other autosomes are present in two copies each with a normal banding pattern. (*Bottom*) Two karyotypes showing complete trisomy of chromosome 3 and partial trisomy of No. 3 with a translocation in the short arm from two additional metaphase spreads.

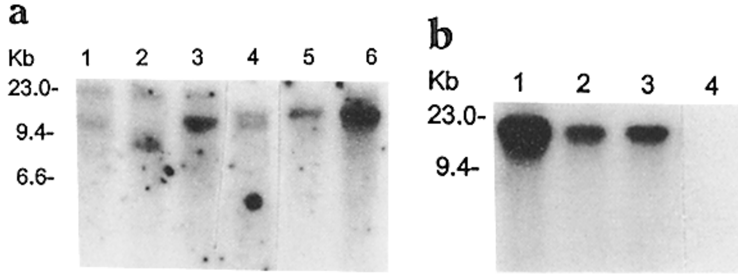

Figure 2. (*a*) Southern blot of DNA digested with EcoRI and hybridized with a J_H probe. (Lane *1*) Z-43 cells; (lane *2*) acute lymphoblastic leukemia patient; (lane *3*) Z-55 cells; (lane *4*) Z-6 cells; (lane *5*) HL-60 cells (acute promyelocytic leukemia cell line); (lane *6*) chronic myelogenous leukemia patient. The germline position for the J_H band is seen in lanes *5* and *6*. (*b*) Southern blot of DNA digested with EcoRI and hybridized with an EBV probe. (Lane *1*) Z-43 cells; (lane *2*) Z-6 cells; (lane *3*) Z-55 cells; (lane *4*) HL-60 cells (acute promyelocytic leukemia cell lines).

and a third chromosome 3 was deleted in the short arm. The short arm of the extra chromosome 3 was replaced by an unidentified segment with two distinct bands. All other chromosomes showed normal G-banding patterns. In conclusion, the lymphoblastoid cell lines (Z-6, Z-43, and Z-55) have their own chromosomal characteristics and can be easily distinguished from each other. Two of them are of male origin (Z-6 and Z-55) and the third of female origin (Z-43).

To establish that these are monoclonal B cell lines, we performed a DNA analysis of their J_H. In all three lines, rearranged J_H bands were found by Southern blot analysis of DNA digested with HindIII, EcoRI, and BamHI restriction enzymes. The rearranged J_H bands were of distinct size in each of the cell lines. Southern blot analysis of DNA digested with EcoRI is depicted in Fig. 2 *a*. DNA from a patient with acute lymphoblastic leukemia was used as a positive control (Fig. 2 *a*, lane *2*), and DNA from HL-60 cells and from a patient with chronic myelogenous leukemia were used as negative controls. Lanes *1* (Z-43), *2* (Z-55), and *4* (Z-6) show J_H rearrangement. These results prove that each line is a monoclonal B cell line.

The presence of EBV was demonstrated in these cell lines by Southern blotting as demonstrated in Fig. 2 *b*. The DNA of the cell lines was digested with EcoRI and hybridized with an EBV probe. Lanes *1* (Z-43), *2* (Z-6), and *3* (Z-55) show detection of EBV DNA. Lane *4* is a negative control of HL-60 cells.

All Three Cell Lines Produce LT and Express TNF/LT Receptors. The requirements that must be fulfilled for a given molecule to act as an autocrine growth factor include the capability of the cell to produce the cytokine, the presence of cytokine receptors on the surface of the cell, the ability of a cell to proliferate in response to the specific cytokine, and finally inhibition of cell growth by antibodies against that cytokine.

The supernatants from all three cell lines employed in these studies were examined for their ability to constitutively secrete LT and TNF. The results of these experiments are shown in Table 2. All the three cell lines were found to produce LT and not TNF. The levels of LT secreted by Z-6 cell line (192 U/ml) were significantly higher than those produced by Z-55 (78 U/ml) or Z-43 (48 U/ml). Besides producing

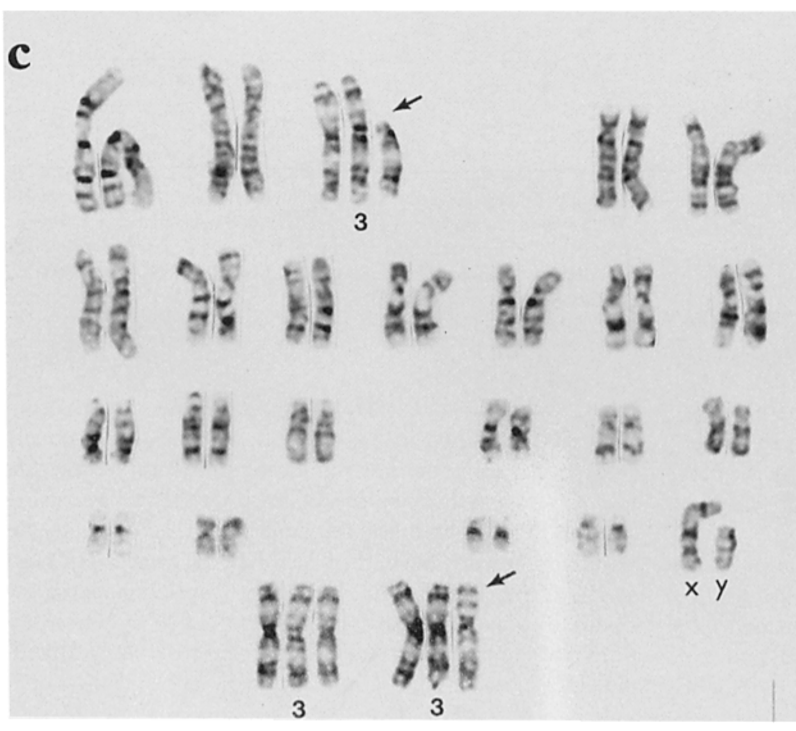

Table 2. *Constitutive Production of TNF and LT from B Lymphoblastoid Cell Lines*

Cell line	TNF/LT U/ml	TNF	LT
Z-6	192	–	+ +
Z-43	48	–	+ +
Z-55	78	–	+ +

Cells (10^6 cells/ml) were cultured for 72 h at 37°C and then the conditioned media was analyzed for the cytotoxic factor on actinomycin D treated L-929 cells according to the procedure as described (22). Anti-LT and anti-TNF antibodies were used to distinguish between TNF and LT. All determinations were made in duplicate.

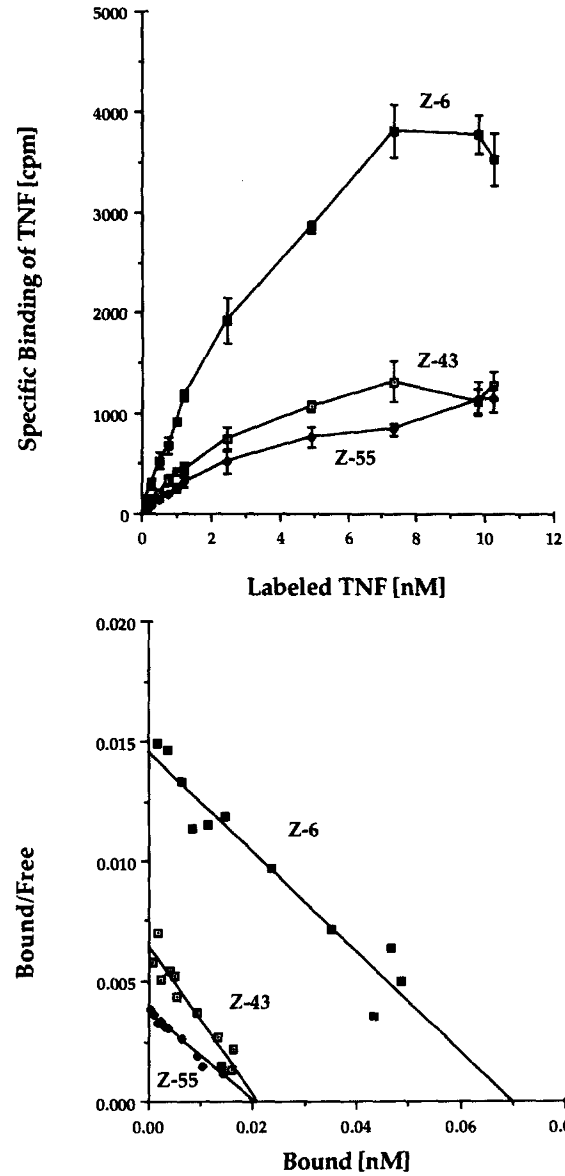

Figure 3. Saturation of specific binding of ^{125}I-TNF to our B lymphoblastoid cell lines. 10^6 cells were incubated with increasing amounts of ^{125}I-TNF-α in the presence or absence of 1,000 nM unlabeled ligand for 90 min at 4°C in a total final volume of 0.1 ml as described in Materials and Methods. (*Top*) Specific binding plotted as a function of concentration of the labeled ligand; (*bottom*) results of Scatchard analysis. Each point represents the mean of three determinations.

LT, we found that all three cell lines expressed the receptors for TNF/LT (Fig. 3). All cell lines bound labeled TNF, and a saturating binding was observed that could be reversed with unlabeled TNF (*top*). The highest amount of TNF binding occurred with Z-6 cell line, whereas binding with Z-43 and Z-55 was very similar. Scatchard analysis (*bottom*) revealed high-affinity binding with receptor density of 4197, 1258, and 1209 sites/cell on Z-6, Z-43, and Z-55 cell lines, respectively. It is interesting that a large number of binding sites per cell on Z-6 cell line corresponded with the higher amount of LT production.

Previously, our laboratory has shown that both TNF and LT share a common receptor on certain cell types (42). We, therefore, examined whether these cells express receptors that bind both LT and TNF. The results of these experiments are shown in Fig. 4. It is clear that labeled TNF binds to all three cell lines and that its binding can be displaced by both unlabeled TNF and LT, thus suggesting a common receptor for the two cytokines.

Recently, two different receptors for TNF/LT have been identified with a molecular mass of 60 and 80 kD (43–50). The 60-kD receptor, which is expressed primarily on epithelial cells, has been shown to be involved in the antiproliferative effects of TNF/LT, whereas the 80-kD form of the receptor, which is expressed mainly on myeloid cells, has been shown to transmit proliferative signals in certain cell types (51). In our studies, therefore, we also examined the type of receptor expressed by our lymphoid cell lines. The results of these experiments are depicted in Fig. 5. These results show that antibodies specific for the p60 form of the receptor are unable to block the binding of TNF, whereas the binding is completely blocked by antibodies to the p80 form of the TNF receptor, indicating that these cells express only the p80 form of the TNF receptor. No receptor blocking was observed with the preimmune serum.

Effect of LT on B Lymphoblastoid Cell Proliferation. Since we found that our cell lines produce LT and express LT receptors, we investigated the effect of exogenous and endogenously produced LT on these lymphoblastoid cell proliferations. Addition of exogenous LT stimulated Z-6, Z-43, and Z-55 cell multiplication in a dose-dependent fashion as assessed by a clonogenic assay at concentrations ranging from 25 to 500 U/ml, by up to 100, 150, and 75%, respectively (Fig. 6). A similar but less significant stimulatory effect was found with a well-established EBV B line that was used as control. LT stimulated Raji cell proliferation, however, in contrast to our recently established lines, Raji cell growth plateaued at 100 U/ml (Fig. 6). Similar results were obtained in the thymidine incorporation assay. Addition of LT at concentrations ranging from 25 to 1,000 U/ml increased cellular thymidine incorporation in Z-6 cells, in a dose-dependent manner by up to 65% (Fig. 7).

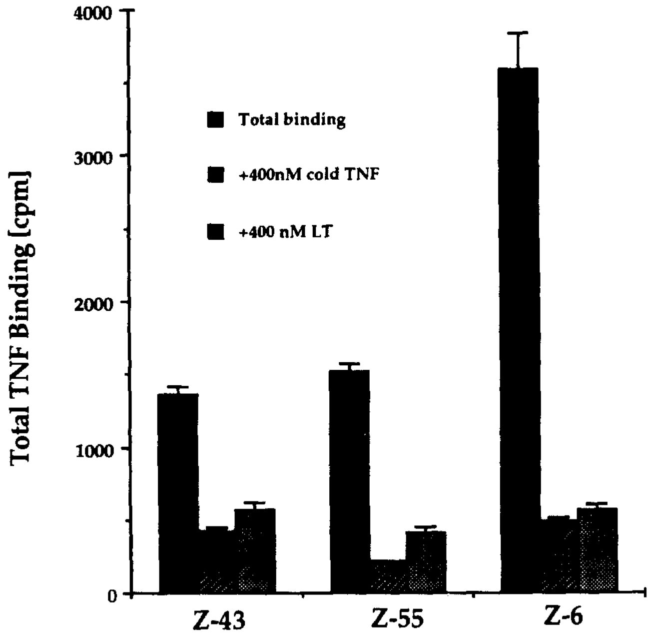

Figure 4. Displacement of TNF binding by LT on the three B lymphoblastoid cell lines. 10^6 cells were incubated with ^{125}I-TNF-α (0.5 × 10^6 cpm) in the presence or absence of 400 nM unlabeled either TNF or LT for 90 min at 4°C in a total final volume of 0.1 ml as described in Materials and Methods. Each point represents the mean of three determinations.

These results indicated that LT stimulates proliferation of the B cell lines. However, our data also indicate that these cells constitutively produce LT. We, therefore, examined the effect of antibodies against LT on these EBV B cell lines. Anti-

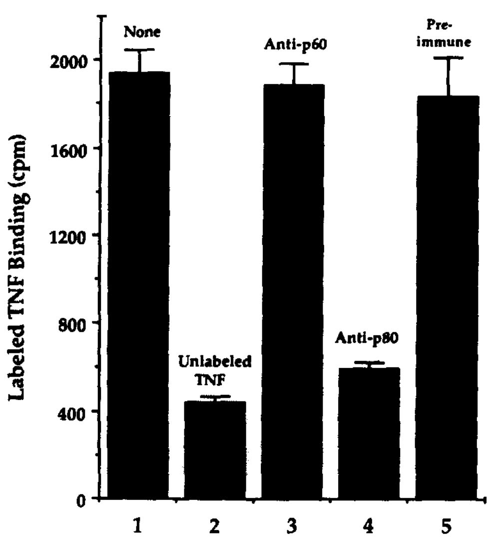

Figure 5. Inhibition of TNF binding to B lymphoblastoid cell line z-6 by antibodies specific for two different types of TNF receptors. 10^6 cells were incubated with ^{125}I-TNF-α (0.5 × 10^6 cpm) in the absence (total binding) or presence of either 400 nM unlabeled TNF (nonspecific binding) or 2.4 μg/ml anti-p60 (p60 receptor) or 2.4 μg/ml anti-80 (p80 receptor) antibodies for 90 min at 4°C in a total final volume of 0.1 ml as described in Materials and Methods. Each point represents the mean of three determinations.

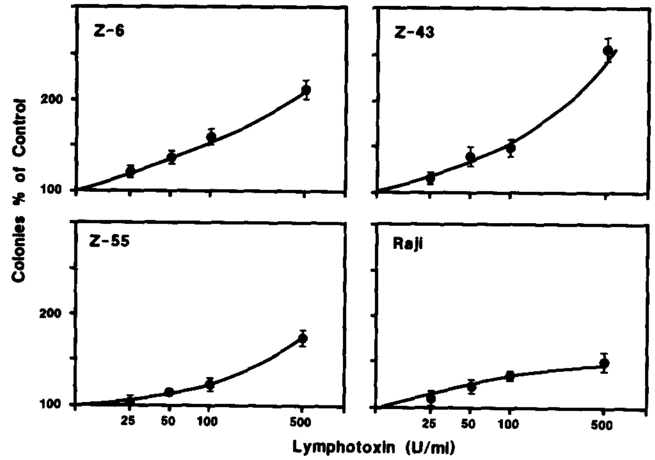

Figure 6. Effect of LT on Z-6, Z-43, Z-55, and Raji cell proliferation. Cells were plated in a clonogenic assay as described in Materials and Methods. Data are presented as means ± SD of percent control colony numbers grown in the absence of exogenous LT. Data of duplicate cultures from two separate experiments of each of the cell lines are presented. Means of colony numbers in control duplicate dishes of Z-6 were 186 and 223; of Z-43 were 359 and 436; of Z-55 were 546 and 659; and the means of colony numbers of Raji cells were 702 and 682. Z-6, Z-43, and Z-55 were plated at a density of 4 × 10^4 cells/ml and Raji cells at a density of 2 × 10^4 cells/ml. Raji cells—an EBV B lymphoblastoid cell line (2)—were obtained from the American Type Culture Collection (Rockville, MD) and used as control.

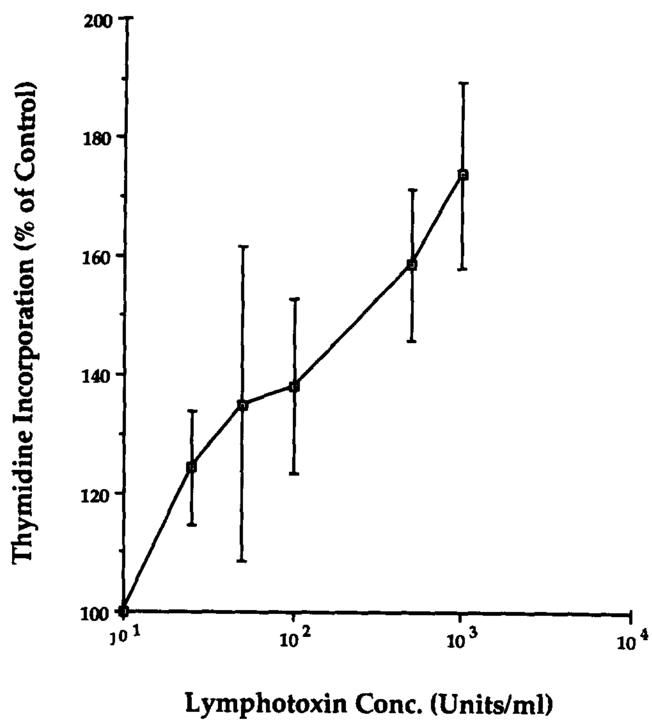

Figure 7. Effect of different concentrations of lymphotoxin on thymidine incorporation by human B lymphoblastoid cell line Z-6. Cells (10^4) were incubated with different combinations of human LT in a total final volume of 0.1 ml for 5 d at 37°C. During the last 6 h, cells were pulsed with thymidine (0.5 μCi) and incorporation determined as described in Materials and Methods. Each point represents the mean of five determinations.

LT neutralizing antibodies significantly inhibited B lymphoblastoid cell proliferation (Fig. 8). Increasing concentrations of anti-LT antibodies suppressed Z-43 and Z-55 colony growth in a dose-dependent manner. The inhibitory effect of the neutralizing antibodies was partially reversed by a low concentration of LT which did not affect cell growth (Fig. 9), suggesting that endogenously produced LT may stimulate B lymphoblastoid cell growth in an autocrine fashion.

Effect of TNF on Z-6, Z-43, and Z-55 Cell Proliferation. Because TNF and LT share the same receptors (42) even in our cell lines as indicated above, we therefore evaluated the effect of TNF on the proliferation of the B cell lines. TNF (1,000 U/ml) inhibited Z-6 and Z-43 cell proliferation by 32 and 37%, respectively (68 ± 8 and 63 ± 7% control), whereas it had no effect on Z-55 cell growth (99 ± 5% of mean colony numbers in control cultures). LT partially (Z-6) and completely (Z-43) reversed the suppressive effect of TNF. However, TNF partially suppressed the stimulatory effect of LT on Z-55 cells (Fig. 10).

Effect of Soluble TNF Receptors on Lymphoblastoid Cell Proliferation. TNF soluble receptors are important cytokines that may serve as naturally occurring inhibitors of both LT and TNF. We therefore investigated their effect on the three EBV B lines. Both p60 and p80 soluble TNF receptors inhibited the three cell lines' proliferation (Fig. 11) by 48 and 45% (2–6), 41 and 20% (2–43), and 30 and 54% (2–55), respectively, at a concentration of 0.1 mg/ml. LT (100 U/ml) partially reversed the suppressive effect, thus proving its specificity.

Discussion

TNF and LT (also termed TNF-β) are polypeptide hormones that share a 30% sequence homology and possess multiple biological activities, many of which overlap to various degrees (52–62). Unlike TNF, which is produced by different cells including macrophages (54, 62), lymphocytes, Kupffer's cells, smooth muscle cells, and other cells (62), the production of LT is restricted to lymphocytes or cells derived from lymphoid progenitors (62–65). Because human LT was first isolated from a lymphoblastoid cell line (22, 23), and since all lymphoblastoid B cells that we tested constitutively produce LT (Aggarwal, B. B., unpublished data), we investigated the role of LT in different EBV-infected lymphoblastoid B cell lines. These lines originated from BM low-density cells of leukemia patients who had an EBV infection before diagnosis of leukemia. The three cell lines had a karyotype that was different from the patient's leukemic cells and they expressed B cell, but not T cell or myeloid surface markers.

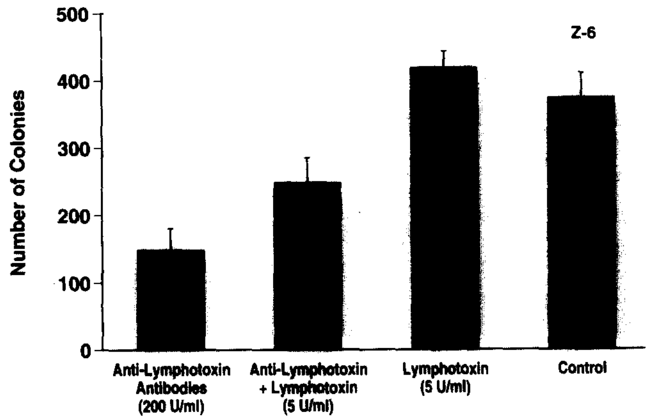

Figure 9. Effect of anti-LT neutralizing antibodies on Z-6 cell proliferation. Data are presented as means ± SD of total colony numbers from two identical experiments.

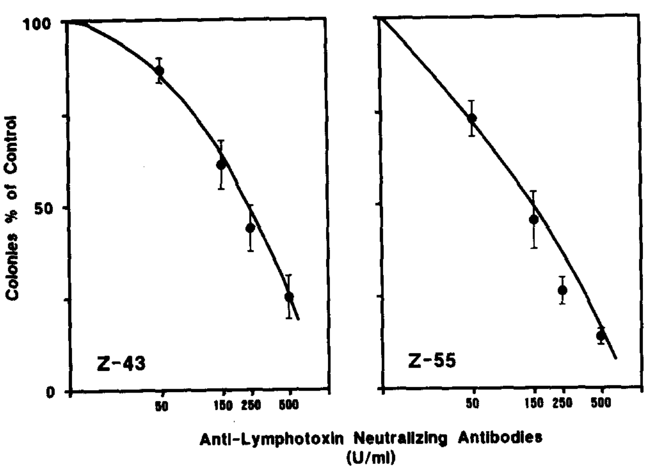

Figure 8. Effect of anti-LT neutralizing antibodies on Z-43 and Z-55 colony proliferation. Data from two separate experiments are presented as means ± SD of percent of control colony numbers. Means of colony numbers in control duplicate cultures of Z-43 were 225 and 341, and of Z-55 were 456.5 and 597.

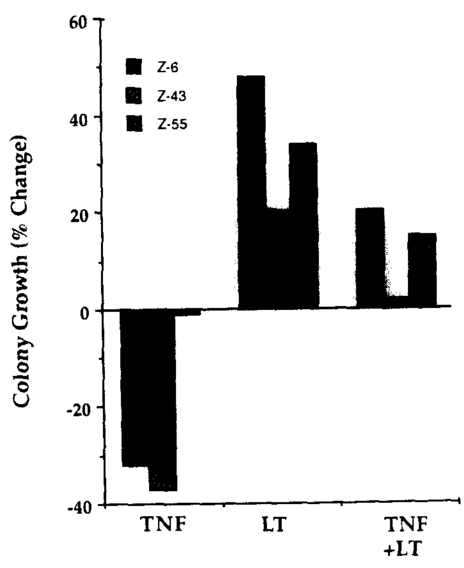

Figure 10. Effect of TNF on Z-6, Z-43, and Z-55 cell proliferation. Data from two separate experiments on each cell line are presented as percentage of change in colony growth. Mean numbers of colonies in control cultures of Z-6 were 303 and 287; of Z-43 were 1591 and 522; and of Z-55 were 568 and 1503. TNF was added to culture at a concentration of 1,000 U/ml, and LT was added at a concentration of 100 U/ml.

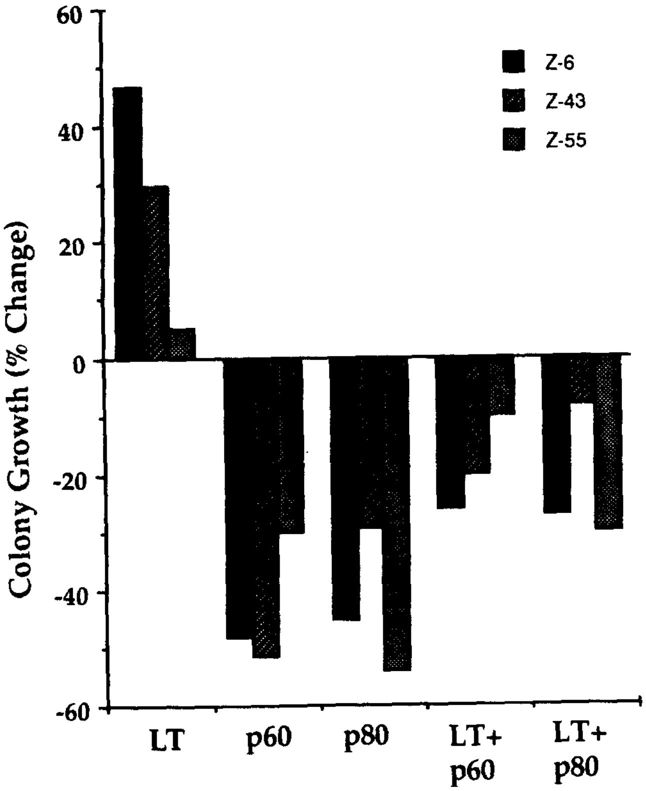

Figure 11. Effect of soluble TNF receptors (p60 and p80) on Z-6, Z-43, and Z-55 cell proliferation. Data from two separate experiments are presented as percentage of change in colony growth. Mean numbers of colonies in control cultures of Z-6 were 125 and 131; of Z-43 were 1218 and 1710, and of Z-55 were 1223 and 1268. LT was added to culture at a concentration of 100 U/ml, whereas p60 and p80 soluble TNF receptors were added at 0.1 mg/ml.

In all three lines we found rearranged J_H bands and EBV DNA. Supernatant obtained from all the lines contained high concentrations of LT, but not TNF when studied in our bioassay. The latter results agree with a previous report (25).

All three of our lymphoblastoid cell lines expressed high-affinity TNF/LT receptors. Labeled TNF binding could be reversed by both unlabeled TNF as well as LT, thus providing its specificity. Recently, two immunologically distinct TNF receptors with approximate molecular masses of 60 (p60) and 80 kD (p80) have been identified (43–49). Both receptors are expressed to varying degrees in different cell types, and both TNF and LT bind to these receptors (59, 60). We used anti-p60 and anti-p80 antibodies to determine the presence of these receptors in our lines, and found that the three cell lines express mainly the p80 type of TNF/LT receptor. We then tested the effect of LT on these B lymphoblastoid cells. Addition of exogenous LT exerted a stimulatory effect on the three lines in a dose-dependent manner. Anti-LT neutralizing antibodies significantly inhibited the growth of cells, and this suppressive effect was partially reversed by exogenous LT. These results suggest that the autonomously produced LT serves as a growth factor in these EBV-infected B cells and stimulates their growth in an autocrine fashion.

Since both TNF and LT share common receptors, and because TNF was reported to either inhibit (66) or stimulate B cell proliferation (67–69), we studied the effect of this cytokine in our cell lines. TNF exerted a mild inhibitory effect on Z-6 and Z-43 cells, and its suppressive effect was reversed by exogenous LT. However, TNF at a concentration of 1,000 U/ml had no effect on Z-55 colony proliferation, whereas exogenous TNF partially reversed the stimulatory effect of LT. These results reflect a possible competitive interaction between TNF and LT, whereas both bind to the same receptors (42).

We then studied the effect of soluble TNF receptors on our B lymphoblastoid cell lines. Truncated fragments of the extracellular domains of the TNF receptors initially designated TNF-binding proteins, have been found in human serum and urine (42–50, 70–73). These TNF-binding proteins bind both TNF and LT and inhibit the effects of TNF on target cells in culture (43, 72, 73). We found that both p60 and p80 soluble TNF receptors inhibited B lymphoblastoid cell proliferation and that LT reversed their inhibitory effect.

EBV infects more than 90% of the human population worldwide and persists for life in infected hosts (74). Upon primary infection, EBV may cause infectious mononucleosis, a self-limiting lymphoproliferative disorder (75–77). It is important that EBV also has been associated with certain malignant disorders such as undifferentiated nasopharyngeal carcinoma, Burkitt's lymphoma, and Hodgkin's disease (78–82). Highly lethal EBV-associated lymphoblastoid diseases have been described in severe congenital or acquired immunodeficiency (78, 83, 84), and in recipients of BM transplantation (78, 83, 85). Moreover, recent reports suggest that lymphomas complicating AIDS may be induced by EBV (84), and that EBV may be implicated in the pathogenesis of angioimmunoblastic lymphadenopathy and angioimmunoblastic lymphadenopathy-like lymphoma (86, 87). The mechanism underlying the aberrant lymphoproliferative stimulus induced by EBV is unclear.

Our investigations demonstrate that LT stimulates EBV-infected B cells in an autocrine fashion. Anti-LT neutralizing antibodies and soluble TNF receptors inhibit their proliferation by either binding and neutralizing LT, or by preventing the interaction between LT and its receptors. It is therefore possible that these molecules may have salutary effects in EBV-associated disorders, and their usefulness merits exploration in future therapeutic trials.

We thank Wanda Quezada for her secretarial assistance, and Mary Blake and Nicholas M. Brown for their technical assistance.

This study was supported in part by grants from the Leukemia Research Foundation, the John S. Dumm Research Foundation, the American Cancer Society (CH 531), and by the Clayton Foundation for Research.

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Received for publication 2 November 1992.

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