

NONHEMATOPOIETIC CELLS SELECTED FOR RESISTANCE
TO TUMOR NECROSIS FACTOR PRODUCE
TUMOR NECROSIS FACTOR

BY BERISH Y. RUBIN,* SYLVIA L. ANDERSON,* SUSAN A. SULLIVAN,*
BARBARA D. WILLIAMSON,† ELIZABETH A. CARSWELL,‡ AND
LLOYD J. OLD‡

*From the *Department of Lymphokine Biology, New York Blood Center, New York 10021; and
the †Laboratory of Experimental Cancer Therapy, Memorial Sloan-Kettering Cancer Center,
New York, 10021*

Tumor necrosis factor (TNF) was first identified during a study of the antitumor activity present in sera of mice infected with bacillus Calmette-Guerin and subsequently injected with endotoxin (1). TNF is a product of activated macrophages that has antitumor activity in vivo and in vitro (2). TNF has also been implicated in the mediation of other activities associated with endotoxin, e.g., shock, cachexia (3). Human and mouse TNFs have been cloned and show ~80% homology (2, 3). Human lymphoblastoid cell lines also produce molecules [lymphotoxin and TNF(LuKII)] with the full range of TNF activities (4, 5). Lymphotoxin has been cloned and shows ~30% homology with TNF (6).

The standard in vitro assay for TNF involves mouse L cells, a transformed fibroblast cell line (1). L cells can be selected for their resistance to TNF by repeated exposure to TNF, and these resistant cells have been useful in distinguishing TNFs from other cytotoxic molecules (5). TNF-sensitive L cells have receptors for TNF (7-10), and competition analysis studies show that mouse and human TNF and TNF(LuKII) bind to the same receptor (7). In contrast, TNF-resistant L cells (L_r) lack demonstrable cell surface receptors for TNF (7).

In the present study, we report that TNF-resistant L cells produce a factor with the properties of mouse TNF.

Materials and Methods

TNFs and Antibodies to TNFs. The production and purification of mouse TNF and TNF(LuKII) were as described (11, 12). The serum of a rabbit immunized with pure TNF(LuKII) served as a source of polyclonal antibody to TNF(LuKII). Human rTNF and rabbit polyclonal antibody to pure human rTNF were kindly provided by Dr. Michael Palladino, Genentech Inc. (South San Francisco, CA).

Production of L_r Cytotoxic Factor. L_r cells seeded at a density of 5×10^5 cells in 150-cm² flasks were grown for 3 d in Eagle's MEM (EMEM) containing 10% FBS. The medium was then removed and replaced with EMEM containing no FBS. These cells were allowed to incubate for 4 d at 37°C at which time the medium was harvested. This medium served as a source of the L_r cytotoxic factor. When necessary, the medium was concentrated using an Amicon cell (Amicon Corp., Lexington, MA) containing a YM-10 membrane.

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SDS-PAGE. SDS-PAGE was carried out in 18-cm slab gels according to published methods (13).

In Vitro Cytotoxicity/TNF Assay. L cell cytotoxicity assays were performed as described (12). Samples diluted twofold in 96-well microtiter plates were sterilized by ultraviolet radiation and TNF-sensitive L cells were added to each well at a density of 2×10^4 cells/well in 100 μ l. After incubation for 2 d at 37°C, the plates were examined under a microscope and the percentage of dead cells was determined. The number of units in a sample was calculated as the reciprocal of the highest dilution that killed 50% of the cells. All assays were run in parallel with a TNF laboratory standard, and all titers are expressed in laboratory units.

Radioiodination of TNF(LuKII). TNF(LuKII) was labelled with 125 I using 1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycoluril (Iodogen; Pierce Chemical Co., Rockford, IL) as described (7).

Assay for Binding of 125 I-labelled TNF(LuKII) to Cells. TNF-sensitive L cells (L_s) seeded in 25-cm² flasks at a density of 2×10^6 cells/flask were allowed to adhere to the flasks for 24 h. The cells were then incubated at 4°C for 4 h with either 75 U/ml of radiolabelled TNF(LuKII) alone (control) or with 75 U/ml of radiolabelled TNF(LuKII) and increasing amounts of the L_r cytotoxic factor. After 4 h, the cells were washed three times with cold PBS, scraped from the flask in PBS, and centrifuged at 15,000 g for 5 min over a cushion of PBS containing 5% sucrose. The sucrose cushion was then removed and the cell pellets were counted in a gamma counter.

Results

TNF-resistant L cell lines were derived from the TNF-sensitive L(M) cloned line by passage in partially purified TNF from mouse serum (11) or purified TNF(LuKII) from the LuKII lymphoblastoid cell line (12). During initial passage, >99% of the cells are killed by TNF, but after three to six additional passages in TNF-containing medium, resistant L cells begin to predominate. During early phases of TNF-resistance, cells revert to TNF-sensitivity if passaged in the absence of TNF. However, after prolonged culture in TNF, the TNF-resistant phenotype is stable and does not require continued exposure to TNF. L cells made resistant to mouse TNF show crossresistance to TNF(LuKII) (and vice versa) and to human rTNF. TNF-sensitive and TNF-resistant L cells have the same fibroblast morphology by light and electron microscopy. Because identical results were obtained with L cells made resistant to mouse TNF or TNF(LuKII), we refer to the separately derived stable TNF-resistant L cell line as L_r . The L_r cells used in these studies have been passaged for one year in TNF-free media. L_s refers to TNF-sensitive L cells.

To determine whether the absence of demonstrable TNF receptors on L_r cells is due to their production of a factor that blocks TNF receptors, we compared conditioned media from L_r and L_s cultures for their ability to compete with radiolabelled TNF in TNF-binding assays. Supernatants from L_r cultures but not L_s cultures showed strong TNF receptor-blocking activity. To determine whether the TNF-blocking factor in L_r supernatants has cytotoxic activity, we tested L_r culture supernatants for the presence of a factor toxic to L_s cells. Fig. 1 shows that L_r cells produce a factor that is released into the culture media and kills L_s cells. L_r cells are not sensitive to L_r media, and L_s cells do not produce cytotoxic factors for L_s or L_r cells. The L_r cytotoxic factor was size-fractionated on a G-100 Sephadex column, and was observed to have a molecular mass of 43 kD under nondenaturing conditions (Fig. 2). SDS-PAGE of this factor reveals recovery of activity at a molecular mass of 16 kD under denaturing conditions (Fig. 3). These characteristics of the L_r factor closely resemble mouse TNF and

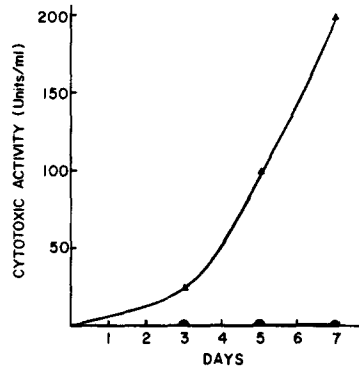


FIGURE 1. Accumulation of a cytotoxic factor in media conditioned by L_R cells. Cells seeded at a density of 2.5×10^5 in 75-cm^2 flasks were allowed to grow for 7 d. On days 3, 5, and 7, a sample of media was removed from cultures of L_R (\blacktriangle) and L_S cells (\bullet), and the cytotoxic activity present in the media was measured in an in vitro cytotoxicity/TNF assay.

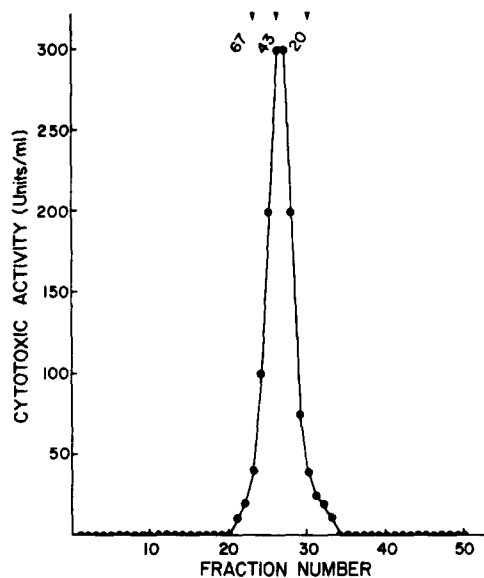


FIGURE 2. Molecular mass determination of the cytotoxic activity present in L_R -conditioned medium. Medium conditioned by L_R cells for 4 d was concentrated 10-fold using an Amicon cell containing a YM-10 membrane, and was then applied to a $1 \times 50\text{-cm}$ column bed of G-100 superfine Sephadex (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated with PBS (20 mM sodium phosphate, pH 7.0, 0.15 M NaCl). 1-ml fractions were collected and assayed for the presence of the cytotoxic factor. The following proteins provided molecular mass markers: BSA (67 kD), ovalbumin (43 kD), and soybean trypsin inhibitor (20 kD).

human TNF (2, 3). Neutralization tests with rabbit antisera to human rTNF (which has neutralizing activity for mouse TNF) or to TNF(LuKII) are summarized in Table I. The cytotoxic activity of the L_R factor is neutralized by antibody against rTNF but not by antibody against TNF(LuKII). Competitive assays with the L_R factor indicate that it quantitatively competes for the same receptor as TNF(LuKII) (Fig. 4), human rTNF, and non recombinant mouse TNF. In the standard in vivo TNF assay, the L_R factor induces necrosis of Meth A sarcoma (Table II). The L_R factor also shows the characteristic TNF pattern of reactivity

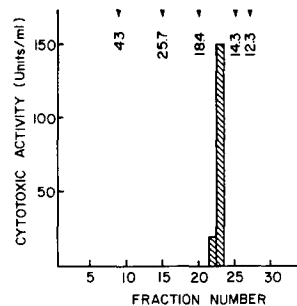


FIGURE 3. Recovery of cytotoxic activity from L_R -conditioned medium after SDS-PAGE. A 50- μ l sample of concentrated L_R -conditioned medium containing 700 cytotoxic units, adjusted to contain 0.1% SDS and 0.1 M 2-ME was incubated for 10 min at room temperature, and then applied to a 12% SDS-polyacrylamide gel. After electrophoresis, the gel was sliced and the activity was eluted and assayed. The following proteins provided molecular mass markers: ovalbumin (43 kD), α -chymotrypsinogen (25.7 kD), β -lactoglobulin (18.4 kD), lysozyme (14.3 kD), and cytochrome c (12.3 kD).

TABLE I
Neutralization Assays of the L_R Cytotoxic Factor with Antisera to TNF or TNF (LuKII)

Factor added	Titer of TNF or L_R cytotoxic factor in presence of:		
	No antiserum	Antiserum to TNF(LuKII)	Antiserum to rTNF
	<i>U/ml</i>		
TNF(LuKII)	16,000	<6	16,000
rTNF	20,000	20,000	<6
Mouse serum TNF	20,000	20,000	<6
L_R cytotoxic factor	3,200	3,200	<6

Samples of TNF(LuKII), human rTNF, and mouse serum TNF were mixed with either 20,000 neutralizing units of rabbit polyclonal antibody directed against pure TNF(LuKII), 20,000 neutralizing units of rabbit polyclonal antibody directed against human rTNF, or no antibody. The L_R cytotoxic factor was mixed with either 5,000 neutralizing units of antibody directed against TNF(LuKII), 5,000 neutralizing units of antibody directed against rTNF, or no antibody. These samples were allowed to incubate for 1 h at 37°C and then assayed for the presence of either TNF or the L_R cytotoxic factor.

on a panel of TNF-sensitive and TNF-resistant human cell lines (5, 14), and has synergistic cytotoxic activity with interferon (5).

Discussion

Mouse L cells selected for resistance to TNF were found to produce a cytotoxic factor. This factor has biochemical and immunological characteristics of monocyte-derived TNF, is capable of competing with TNF for receptor binding, and causes the necrosis of the Meth A sarcoma in the *in vivo* TNF assay.

Several cell types of hematopoietic origin, including macrophages, lymphocytes, and lymphoblastoid cells, produce factors with TNF activity (2). Our finding that nonhematopoietic cells of fibroblastic origin produce a molecule with TNF activity indicates that TNF and TNF-related molecules may have a role as mediators in cells outside the immune system. In this respect, the TNF family of molecules resembles the interferon family, in that many cell types have the capacity to produce interferons. IL-1, another mediator of macrophage origin, is also known to be produced by nonimmunological cells. It will be

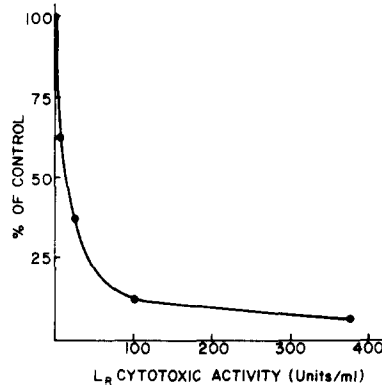


FIGURE 4. Competitive binding of ^{125}I -labelled TNF(LuKII) by the L_R cytotoxic factor. Receptor binding studies were performed as described in Materials and Methods. All values presented are expressed as a percentage of the radiolabelled TNF(LuKII) bound in the absence of the L_R factor.

TABLE II
Induction of Hemorrhagic Necrosis of BALB/c Sarcoma Meth A by the L_R cytotoxic factor

Source of Material	Number of mice	Units injected	Necrotic reaction
L_R cells	3	2,500	++, ++, ++
L_S cells	3	—	—, —, —
rTNF	3	1,000	+++, +++, +++)
Mouse TNF	5	4,800	+++, +++, +++, ++, ++
TNF(LuKII)	2	5,000	+++, ++

(BALB/c \times C57BL/6) F_1 female mice were injected intradermally with 5×10^5 Meth A BALB/c sarcoma cells. After 7 d, mice received a single intratumoral injection of concentrated media conditioned by L_R or L_S cells, human rTNF, mouse serum TNF, or TNF(LuKII). After 24 h, tumor hemorrhagic necrosis was scored according to Carswell et al. (1). All materials injected contained <45 ng of endotoxin as measured in Limulus amoebocyte lysate assays (Associates of Cape Cod, Inc., Woods Hole, MA).

important to determine the full range of cell types capable of TNF production, either constitutively, after induction with chemical agents, or after viral or bacterial infection.

Although we have repeatedly isolated TNF-resistant L cells from TNF-sensitive populations of L cells, we do not know whether the resistant variants preexist and are selected by TNF, or whether TNF resistance can be induced in sensitive cells. Attempts to isolate uniformly resistant cells from other TNF-sensitive cell types, e.g., BT-20 and HeLa, have not been successful. With the finding that TNF-resistant L cells produce TNF, the lack of demonstrable TNF-binding sites on these cells (7) could have several explanations, including saturation of TNF receptors, downregulation of TNF receptors, or selection of receptor-negative cells. Whatever the reason, the production of TNF by cells selected for resistance to TNF represents a novel mechanism of cellular resistance.

Summary

TNF-resistant lines of L cells can be derived from TNF-sensitive populations by repeated exposure to TNF, and these resistant L cells, in contrast to sensitive L cells and other types of cells, lack demonstrable cell surface receptors for TNF.

We have now found that TNF-resistant L cells produce a factor that is cytotoxic for L cells and has the following distinguishing characteristics of mouse TNF: it is a protein of 43 kD, composed of 16 kD subunits, that competes with TNF for receptor binding, induces hemorrhagic necrosis of the TNF-sensitive mouse sarcoma Meth A, has synergistic cytotoxic action with interferon, and its activity is neutralized by antibody to TNF. The two conclusions of this study are that cells selected for TNF resistance spontaneously produce a molecule resembling macrophage TNF, and that cells of nonhematopoietic origin are capable of producing TNF.

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