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Rabbit Tumor Necrosis Factor: Mechanism of Action

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Rabbit tumor necrosis factor (TNF) was examined for effects on normal and transformed cells in culture. Several assays for killing of L-929 cell targets were developed, and their sensitivities were compared. Normal cells were not killed by TNF, and the discrimination between normal and transformed cells was shown not to be due to a cell cycle-dependent mechanism. TNF killing of L-929 cells was delayed for 10 to 12 h and thereafter showed concentration and time-dependent increases in cytolysis. Actinomycin D or cycloheximide treatment of L-929 cells resulted in an enhancement of the rate of cell killing as well as a shortening of the preceding lag period. TNF killing of L-929 cells was temperature dependent; cells were considerably more resistant to lysis at 25°C and showed enhanced killing at 39°C as compared to 37°C controls. The slope of the dose curve showed less than single-hit kinetics. A model for cell killing whose general features incorporate both the specificity and catalytic properties of an enzymatic reaction is proposed for TNF action.

Carswell et al. (2) have described a tumor necrosis factor (TNF) in the serum of endotoxin-treated mice, rats, and rabbits which had been previously infected with *Mycobacterium bovis* strain BCG. TNF caused a hemorrhagic necrosis of various tumors in mice with no apparent effects on the host (2). TNF also showed cytostatic and cytocidal activity against several transformed cell lines in culture, but normal mouse embryo fibroblasts were not similarly affected (2, 10, 16, 17, 19, 20b). Six additional cell cultures derived from normal human tissues were also reported to be unaffected by TNF (18), although details were not provided. For this report we tested primary rabbit kidney cells, hamster and chicken embryo fibroblasts, and a human diploid fibroblast line (Flow 7000) and found them to be insensitive to the in vitro killing effects of TNF. No normal cells have yet been described which are sensitive to TNF. TNF apparently acts in response to a common feature of some transformed cells which is missing in normal cells. In this report we examine the kinetics of cytolysis by TNF and describe some additional aspects of its action as an attempt to define the nature of the apparent discriminatory cytotoxicity.

MATERIALS AND METHODS

Preparation of TNF-containing serum. Rabbit TNF was produced by the procedure of Carswell et al. (2). Viable *M. bovis* BCG (Tice strain, 3×10^8 organisms) was injected into the marginal ear vein of New Zealand white female rabbits. Fourteen days later, 100 μg of endotoxin from *Salmonella typhimurium*, virulent strain 7, was injected into the ear vein. Rabbits were exsanguinated 1.5 h later, the blood was allowed to clot, and the serum was collected as the source of crude TNF. TNF prepared in this manner was capable of causing hemorrhagic necrosis of Meth A tumors in BALB/c mice.

Cells. L-929 is a transformed cell line originally derived from the C3H strain of mouse. B16 C3(melanoma from C57BL/6 mouse), HeLa (derived from a human cervical carcinoma), AV3 (human amnion), and Flow 7000 (diploid human embryonic foreskin) fibroblasts (purchased from Flow Laboratories, McLean, Va.) were also employed. These cells were grown in minimal essential medium with 10% bovine serum and passaged weekly in glass bottles. Primary cell cultures from mouse, chicken, and hamster embryos and rabbit kidneys were prepared by mincing near-term embryos or mature kidneys and trypsinizing (0.1%) (ISI Biologicals, Cary, Ill.), with 0.004% ethylenediaminetetraacetic acid (Fisher Scientific Co., Fairlawn, N.J.) in phosphate-balanced saline, for 60 min. Cells were collected by centrifugation, and trypsin was removed by washing with Gey's balanced salt solution. Cells were then suspended in fortified minimal essential medium (6) with 10% fetal bovine serum and dispensed in glass bottles.

Assays for TNF. TNF was assayed by several methods which are similar to those developed to study the actions of other cytotoxins, most notably lymphotoxin (11, 13, 21). In the morphological microassay, cultures of cells were established in 96-well flat-bottomed trays (3040; Falcon Plastics, Oxnard, Calif.) at 50,000 cells per well and incubated in a humidified atmosphere at 37°C with 5% CO_2, unless noted otherwise. Cells were added in a volume of 0.1 ml to dilutions of TNF or control rabbit serum in medium in a volume of 0.1 ml. Forty-eight hours after addition of TNF, medium was decanted, residual cells were stained with crystal violet and morphologically intact cells in representative microscopic fields were counted. TNF killing was also determined by establishing cells

in 25-cm^2 flasks for 28 h followed by the addition of TNF or control serum and incubation for 48 h. Cell killing was expressed as a survival ratio, S/S_0, where S_0 = number of cells in control culture and S = number of cells in experimental cultures. A photometric method of measurement was later adopted because of its rapidity and accuracy. This procedure has been described in detail elsewhere (20a, 20b). Briefly, the method employs a photocell to measure light intensity through the stained wells of the microtiter trays as a quantification of monolayer destruction. In a modification of this basic assay, cells were preestablished in 0.1 ml of medium overnight. TNF dilutions and actinomycin D (Sigma Chemical Co., St. Louis, Mo.), at 1 μg/ml final concentration, were added to a final volume of 0.2 ml. Residual cells were stained 18 h later.

For the cytotoxic release assay, L-929 cells were grown for 3 days in the presence of 1.0 μCi of [^{3}H]-thymidine per ml (6 Ci/mmol; Schwarz/Mann, Orangeburg, N.Y.) or 1.0 μCi of ^{32}P per ml (carrier free, New England Nuclear Corp., Boston, Mass.). Cells were washed three times with warm minimal essential medium before replating in 25-cm^2 flasks. Cytolysis was measured at various times by sampling portions of centrifuged supernatant fluids for scintillation counting. The specific lysis was computed by the formula: Specific lysis = (experimental − spontaneous release)/(100% release − spontaneous release). The 100% releasable counts per minute were determined by incubating target cells in 0.5% sodium dodecyl sulfate for 30 min. Spontaneous release was 8 to 14% of maximal release. The means and standard deviations of triplicate samples were determined. Matthews and Watkins (17) have used a similar procedure using ^{51}Cr release.

In the "end-label" method, cells were established in microtiter trays as for the morphological assay. At 44 h after TNF addition the cultures were pulsed with 0.5 μCi of [^{3}H]thymidine per ml (56 Ci/mmol; Schwarz/Mann) for 4 h. Cells were collected onto glass fiber filter strips with an automated sample harvester, and radioactivity was counted. The means and standard deviations of triplicate samples were determined.

RESULTS

Assays for TNF. We measured the killing effects of TNF on L-929 cells by three methods which generated sigmoidal dose-response curves. Two assays, which measured TNF activity by cell recovery (Fig. 1A) or by ability to incorporate radioactive isotope (Fig. 1B), showed similar sensitivities with a 50% survival dose at a TNF dilution of approximately 1:1,600. These methods measured two aspects of TNF action, cell destruction and growth inhibition.

A cytotoxic release assay (Fig. 1C) was somewhat more sensitive than these first techniques; the 50% survival dose occurred at a 1:6,400 dilution of TNF serum. This method did not suffer from the failure to distinguish between cytostasis and cytolysis inherent in the first two assays since the total amount of label incorporated by cells remained constant throughout the incubation period. Cell killing was measured independently of a background of cell proliferation since, within limits, the percentage of cell killing was independent of cell numbers (Table 1).

While studying another area of TNF action on cells, we noted that cells treated simultaneously with TNF and actinomycin D showed considerably enhanced cell killing effects, in terms of both onset of killing and dose of TNF (19). These effects were exploited in a modification of the morphological micromethod by including actinomycin D in culture with TNF. Due to the growth inhibition by actinomycin D, the cells were rendered nondividing, thereby providing an assay which measured only the cytolytic effects of TNF and which was comparatively rapid and very sensitive. A comparison of cell killing in the presence of TNF with or without actinomycin D is included in Fig. 1D. The dose curve showed the characteristic sigmoidal shape generated by the other methods. The 50% survival dose occurred at a TNF dilution of 1:10^6, which was almost 10^3 times more sensitive than the standard 48-h assay, although this assay was read in 18 h. The amount of actinomycin D we employed (1 μg/ml) inhibited ribonucleic acid synthesis by greater than 95%. Cycloheximide could be used at a concentration of 5 μg/ml, an amount which inhibited protein synthesis by greater than 90%, with similar acceleration of killing kinetics (data not shown).

Kinetics of TNF-induced cell killing. Figure 2 shows growth curves for L-929 cells cultured in the presence of various doses of TNF serum or normal rabbit serum as a control. L-929 cells were seeded in 5-ml aliquots into 25-cm^2 flasks at 5 × 10^4 cells per ml. Twenty-four hours later, at zero time, TNF was added, and cell numbers were determined by microscopic counting at the indicated times. TNF serum showed a concentration and time dependence of killing and growth inhibition.

Figure 2 defines another characteristic of TNF killing: a lag period of approximately 10 h preceded cell killing. Decreasing amounts of TNF serum extended the lag period, but concentrations of TNF serum as high as 1:50 did not shorten it (data not shown). The kinetics of TNF cell killing in the presence of actinomycin D was accelerated, and a lag period of approximately 5 h occurred in which no cells died (see Fig. 5).

Sensitivity to TNF of other cell types. Several cell lines were screened in an attempt to examine more closely the apparent selectivity of TNF action. Figure 3 shows the dose-response curves for some of these cells. We found that, as had been initially reported (2), TNF was not

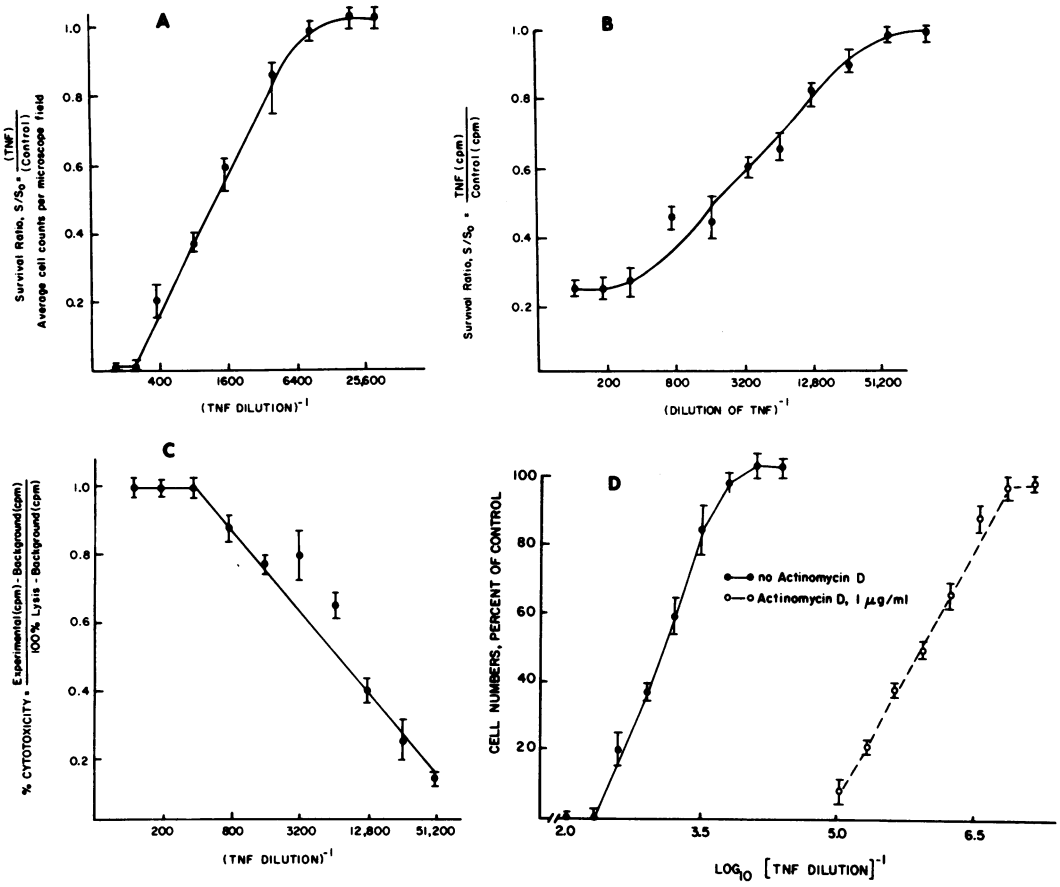

FIG. 1. *Dose-response curves for TNF killing of L-929 cells by three assay methods. Killing is expressed as a survival ratio, S/S_0, for TNF-treated cultures. Methods: (A) micromorphological; (B) end-label pulse with [^{3}H]thymidine; (C) cytotoxic release from isotopically prelabeled cells (^{32}P); (D) micromorphological, with and without actinomycin D at 1 µg/ml.*

TABLE 1. *Effect of plating density on TNF killing of normal and transformed cells*[a]

Cell type	Ratio of [^{3}H]thymidine in TNF/control cultures at plating density (cells per culture):		
	50×10^3	25×10^3	12×10^3
Mouse embryo fibroblasts	1.17	1.33	1.82
L-929	0.21	0.20	0.21

[a] Numbers represent the ratio of [^{3}H]thymidine incorporation in TNF/control cultures at the end of 48 h. Cultures were treated with TNF serum at a dilution of 1:200 at the indicated plating densities.

toxic to normal mouse embryo cells. We extended these observations to include primary cells from rabbit kidney, hamster and chicken embryo fibroblasts, and a human diploid fibroblast line, Flow 7000. Erythrocytes from human, mouse, and chicken were also insensitive to the cytolytic effects of TNF (data not shown).

Two transformed human cell lines, HeLa and AV3, showed similar sensitivity to rabbit TNF action, although these cells were less sensitive than mouse L-929. Interestingly, normal cells (Fig. 3 and Table 1) showed an apparent stimulation of growth at the higher concentrations of TNF. We assumed the effect was the result of growth-promoting factors in the crude serum, unrelated to TNF.

Effect of seeding densities. The specificity of TNF action could be due to the fact that normal cells, by virtue of their density-dependent growth properties, might be refractory to TNF action because they were relatively quiescent as a result of the high initial seeding densities employed. We therefore seeded cells at concentrations so that multiple rounds of division would be required to reach confluency. These results are shown in Table 1. TNF in-

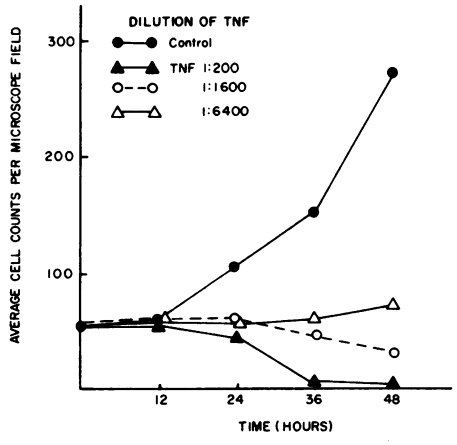

FIG. 2. *Growth curve for L-929 cells in the presence of various doses of TNF or normal rabbit serum. Cells were seeded in 5-ml aliquots in 25-cm² flasks at 5 × 10⁴ cells per ml. Twenty-four hours later, TNF was added.*

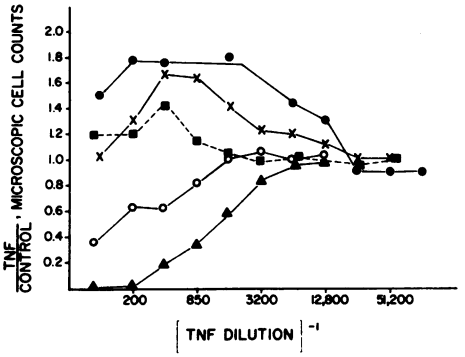

FIG. 3. *TNF sensitivities of several cell types, normal and transformed. Cell numbers were determined by cell counting by the micromorphological method.* (●) *Mouse embryo fibroblasts;* (×) *human diploid fibroblasts;* (○) *HeLa cells;* (▲), *L-929 cells;* (■) *B16C3 cells.*

hibited L-929 cells by 80% at all seeding densities employed. Mouse embryo fibroblasts, on the other hand, were resistant to TNF whether they went through two (12.5×10^3 cells per culture), one (25×10^3 cells per culture), or less than one (50×10^3 cells per culture) division. The stimulatory effect of TNF serum on normal cells was more pronounced on metabolically active, dividing cultures (e.g., mouse embryo fibroblasts at 12.5×10^3 cells per well). Since cells were seeded in the presence of TNF, this experiment indicated that TNF serum did not detectably reduce the plating efficiency of the normal cells. We have verified that L-929 cells were not inhibited in their plating efficiency 3 h after seeding in the presence of TNF.

Effect of serum concentration. Since cell growth and division were not discriminating factors in the action of TNF, we considered the effect of cell growth rate on sensitivity to TNF. L-929 cells were seeded in microtiter plates at an initial density of 12.5×10^3 cells per well in various concentrations of serum. The results (Fig. 4) showed that cells grown in low serum, 2.5%, were more resistant to TNF than cells grown in higher serum. This effect could be overcome by adding increasing amounts of TNF.

Temperature dependence of TNF action. The kinetics of TNF killing at various temperatures in the presence of actinomycin D are shown in Fig. 5. Despite their greatly enhanced susceptibility to TNF when cultured in the pres-

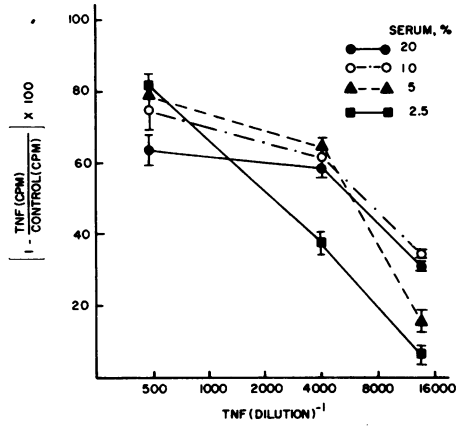

FIG. 4. *Serum dependence on TNF killing, measured by cytotoxic release on L-929 cells. Vertical bars indicate ± standard deviation.*

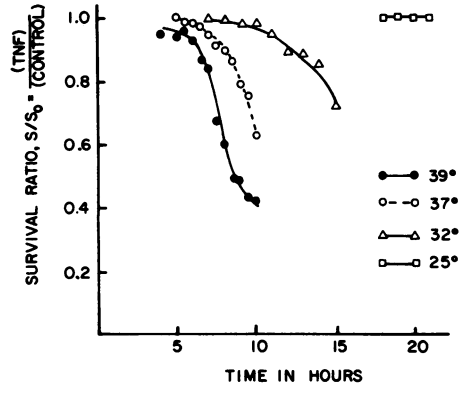

FIG. 5. *Temperature dependence of TNF killing in the presence of actinomycin D. Cultures of L-929 cells were prepared as described for the microphological methods. TNF serum at a dilution of 1:100 and actinomycin D at 1 μg/ml were added, and plates were incubated at various temperatures. At indicated times, plates were stained and cells were counted photometrically. Photometric measurement determined the extent of cell killing by the increase in light transmission through the stained wells of the microtiter plates.*

ence of actinomycin D, L-929 cells were refractory to the lytic effects of TNF at 25°C. As the temperature was increased, the onset of cell killing was accelerated as well as the rate at which the cells died. Cells cultured at 39°C showed substantially more lethality than those at 37°C at the end of 10 h. The inhibition of TNF killing at 25°C or lower was also observed for cells treated in the absence of actinomycin D (17; data not shown).

DISCUSSION

The slowly progressing cytostatic and cytocidal effects of TNF are similar to those described for lymphotoxin, a cytotoxic protein which seems to be elaborated by T lymphocytes in vitro after mitogenic stimulation (14, 20). A major difference, however, is that lymphotoxin is not discriminatory for transformed cells but is lytic for both normal and neoplastic cells (9, 21). Lymphotoxin also lyses erythrocytes (23), but we found no lysis of these cells by TNF. Some similarities exist between lymphotoxin and TNF action. Both lack species specificity, and both result in a paradoxical increase in RNA synthesis in treated cells (14, 19, 20), observations which suggested an active cellular repair process in these cells. Enhancement of TNF cytotoxicity by inhibitors of transcription or translation would indicate that a cell's ability to respond to TNF damage by the proposed repair mechanism may also be an important factor in its apparent sensitivity.

A soluble supernatant fraction from endotoxin-activated peritoneal macrophages, which killed malignant but not normal cells, has been described (5) and shown to have arginase activity (4). These results were reminiscent of earlier work describing an antilymphoma agent present in the serum of normal guinea pigs, later shown to be an asparaginase (1). Such a mode of action for TNF, i.e., nutritional depletion, would not be consistent with the kinetics just described for TNF action, nor would such a mechanism explain the acceleration of TNF kinetics by actinomycin D or cycloheximide. Furthermore, media which had been dialyzed against TNF serum for 4 days did not show any L-929 inhibitory activity, nor was there a decrease in 18 amino acids when assayed directly. Supplementation of media with additional vitamins and amino acids did not inhibit TNF action (20b). We therefore feel that TNF must interact directly with the sensitive cell.

Transformed cells differ from normal cells in several respects, one of the most pronounced being the loss of density-dependent growth control. We questioned whether this phenotypic difference might serve as the basis for the selectivity of TNF action. Normal cells plated in the presence of TNF serum and allowed to go through multiple rounds of division were still resistant to the cytostatic and lytic effects of TNF (Table 1). Transformed cells can be made to slow down their movement through the cell cycle by low serum or low temperature. Under these conditions, L-929 cells showed decreased sensitivity to TNF (Fig. 4 and 5). We feel, however, that a cell cycle-dependent mechanism for TNF action is unlikely since cell killing proceeded at an accelerated rate in the absence of major anabolic activity when treated with the inhibitors actinomycin D or cycloheximide (Fig. 1D). Mitomycin C-blocked cells have also been reported to remain susceptible to TNF killing (17), further strengthening this argument. Moreover, the inhibitor data suggest that TNF exerts its effects by actively degrading or inactivating an essential cell component, perhaps structural. The cycloheximide sensitivity would suggest a protein target.

An explanation for the serum dependency of TNF action is suggested by work done by Cikes and Klein (3), who have described the reexpression of surface antigens after refeeding of stationary-phase YAC lymphoma cells. Serum-starved cells may simply have diminished numbers of TNF binding sites. As was suggested for lymphotoxin action (15), a serum component might also be directly required for TNF activity.

Since the loss of TNF activity on cells at lower temperatures is apparently not due to the effects of temperature on cell growth, we suggest that other temperature-dependent cellular activities may be required for expression of TNF toxicity. Relevant metabolic processes could include, among others, uptake or processing of TNF. The lag period which precedes the onset of cell death supports the contention that there is an intervening event(s) between the proposed binding of TNF to the cell and cell disruption. Of course, the temperature dependence of cytolysis may be the result of a temperature effect on TNF itself.

The slope of the dose curves can be expressed as the fractional change in cell killing compared to the log increase in dose, measured over the linear portion of the curve. Theoretical considerations (7, 8) derived from the Poisson distribution show that under these conditions a slope of 0.705 (fraction of cells dying over a one-decade change in TNF concentration) would be expected for single-hit kinetics, i.e., one molecule of TNF may kill one cell. Since TNF dose-response curves consistently have slopes much less than 0.705 (in the range of 0.35 to 0.55), one interpretation is that one molecule of TNF can kill more than one cell. One possibility is that TNF itself may have enzymatic activity or that

an additional effector may be involved at some stage of cytolysis. This would help explain the temperature effects on TNF activity. We propose a model for cell killing whose general features incorporate both the specificity and the catalytic properties of an enzymatic reaction for TNF cytolysis.

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