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Stimulation of RNA Synthesis in L-929 Cells by Rabbit Tumor Necrosis Factor[1] (40449)

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Carswell *et al.* (1) 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. Corynebacteria and Zymosan could substitute for BCG in priming mice for release of TNF by endotoxin. TNF caused a hemorrhagic necrosis of various tumors in mice with no apparent effects on the host. Tumor cells in culture were also inhibited or killed by TNF whereas normal cells were not affected. Helson *et al.* (2) later reported that murine TNF had an inhibitory effect on human melanoma cells growing in culture showing that this substance was not species specific. This report concerns the effect of rabbit TNF on a transformed mouse cell (L-929) in culture and demonstrates a marked stimulation of RNA synthesis. RNA synthesis in normal mouse embryo cells was not affected by TNF treatment.

Materials and methods. Preparation of rabbit serum containing TNF. New Zealand white female rabbits (NZW) weighing from 2 to 3 kg were bled by cardiac puncture for a source of normal rabbit serum (NRS) for control experiments. Rabbit tumor necrosis factor (rabbit TNF) was then produced by the procedure described by Carswell *et al.* (1). Viable BCG (Tice strain, 3×10^8 viable organisms, or 7 mg killed *Corynebacterium parvum*, were injected into a marginal ear vein. The *C. parvum* was a generous gift from Dr. Whisnant, Burroughs Wellcome Laboratories, Triangle Park, NC. Fourteen days later, usually 100 μg of endotoxin from *Salmonella typhimurium*, virulent strain 7, was injected into the marginal ear vein. The endotoxin was a gift from Dr. Joseph Shands, University of Florida. The rabbits were exsanguinated by cardiac puncture 1½ hr later. Blood samples were also taken prior to injection of endotoxin and endotoxin was also given to rabbits without BCG or *C. parvum*. The bloods were allowed to clot and then centrifuged to remove the serum which was heated at 56° for 30 min and then stored in aliquots at −70°. To date, eight rabbits infected with BCG have been used as a source of rabbit TNF. Seven of them were challenged with 100 μg of endotoxin and the eighth with 150 μg. Only the rabbit receiving 150 μg of endotoxin died before the time of exsanguination although the other seven were in shock at that time. Sufficient blood was obtained from the heart of the dead animal for testing purposes; all eight rabbits demonstrated TNF activity in their sera. Rabbits receiving *C. parvum* and challenged with 100 μg endotoxin also produced TNF but of lower titer than BCG animals. Rabbits inoculated with endotoxin alone, or with BCG or *C. parvum* and *not* challenged with endotoxin, did not demonstrate any TNF activity in their sera. The experiments described in this study were performed with TNF obtained from rabbits inoculated with BCG and challenged 14 days later with 100 μg of endotoxin, exactly as produced by Carswell *et al.* (1).

Cells. L-929, a continuous transformed cell line which has lost contact inhibition of locomotion and anchorage dependency, was employed. They were grown in Eagle's minimal essential medium (MEM) supplemented with 10% bovine serum (BS) and 250 units of penicillin/ml and 125 μg of streptomycin/ml. The cells were passed weekly at 1:50 dilution. The mouse embryo fibroblasts were prepared by mincing near-term embryos, trypsinization (0.1% Difco trypsin with 0.04% ethylenediaminetetraacetic acid, sodium salt (EDTA)) for 60 min, followed by washing with Gey's balanced salt solution (BSS) and dispensing the cells in glass containers in fortified MEM with 10% fetal bovine serum (FBS) and an-

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tibiotics. Fortified MEM contained increased concentrations of essential amino acids, vitamins, and glutamine; non-essential amino acids and sodium pyruvate were also added (3).

Assay of TNF. TNF was assayed on L-929 cells essentially as described by Carswell *et al.* (1). Cells remaining on the glass or plastic following 48 hours exposure to TNF or NRS were trypsinized and counted with a Coulter counter, model ZBI. Viability of cells was determined by trypan blue exclusion using a light microscope.

(^3H) UDR incorporation. Ten μCi of [^{3}H] UDR (Amersham/Searle; 42 Ci/mmole) were added for 1 hr to cultures in 24 well Linbro TC plates (Linbro Scientific Co., Inc., Hamden, CO, model FB16-24TC). Medium was then aspirated, cells were washed once with Gey's BSS and then 3 times with cold 5% trichloroacetic acid (TCA). One ml of 5% TCA was then added to each well and the plates were heated for 60 min in a water bath at 80° to hydrolyze the RNA. Then 200 μl from each well was placed in Triton X-100 scintillation fluid and enumerated in a Beckman LS-230 liquid scintillation counter.

Results. Effect of various concentrations of TNF containing serum on the growth of L-929 cells: Assay of TNF. L-929 cells, 2.5×10^5 in 4 ml of growth medium, were plated in glass bottles with a surface area of 19 cm^2 and incubated at 37° for 6–8 hr to allow cell attachment. Then, one ml of various dilutions of rabbit serum containing TNF, or control rabbit serum, were each added to triplicate cultures. Following incubation for 48 hr, the monolayers were washed and the cells detached by trypsinization were enumerated with a Coulter counter, model ZBI. Viability was determined by trypan blue exclusion and indicated that greater than 95% of the attached cells were viable regardless of the concentration of TNF. However, cells in the supernatant were found to be greater than 95% non-viable. Figure 1 shows a dose response curve for one preparation of TNF. The 50% inhibitory dose (ID_{50}) was obtained at a serum dilution of 1:200; thus, this serum contained 200 ID_{50} units/ml. The same preparation of TNF was also employed on cultures of normal mouse embryo fibroblast cultures (Table I). No inhibition of growth of these normal cells was obtained which is a characteristic of TNF (1). Furthermore, only sera from animals receiving both the priming agent (BCG or *C. parvum*) and endotoxin showed inhibitory effects on L-929 cells. Similar dose response curves were obtained with other TNF preparations from rabbits primed with either BCG or *C. parvum.* The preparation used for most of the following experiments had a titer of 400 ID_{50} units/ml.

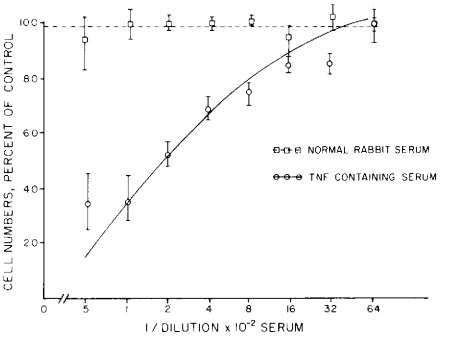

FIG. 1. The effect of various dilutions of TNF containing rabbit serum and normal rabbit serum on the growth of L-929 cells. Cultures were incubated for 48 hr, trypsinized, and cells enumerated in a Coulter counter. All cultures were done in triplicate; vertical bars represent ± SD.

TABLE I. THE EFFECT OF TNS AND NRS ON SECONDARY MOUSE EMBRYO CELLS.

Dilution of TNS or NRS	Number of cells (±SD) × 10^{-6} remaining at 48 hr following treatment[a]		
	TNS	NRS	(TNS/NRS) × 100
1:50	5.11 ± 1.1	4.41 ± 0.33	116 ± 15
1:100	4.85 ± 0.24	3.79 ± 0.75	128 ± 20
1:200	4.45 ± 0.56	4.66 ± 0.25	95.5 ± 6

[a] Secondary mouse embryo fibroblasts (3.4×10^5) were plated in plastic tissue culture flasks with fortified MEM and 10% FBS and 30 m*M* Hepes buffer. Triplicate cultures were used for each dilution of TNS and NRS. The cells were incubated for 48 hr at 37° and then counted using the Coulter counter. In the absence of rabbit serum, the cell counts were $4.77 \pm 0.54 \times 10^6$.

Growth curve of L-929 cells in the presence of TNF. Replicate cultures of 2.5×10^5 L-929 cells were plated in the presence of a 1:200 dilution of rabbit serum containing TNF (2 units). Control cultures received a 1:200 dilution of normal rabbit serum. At various periods of time, thereafter, replicate cultures were washed, trypsinized, and the cells counted with a Coulter counter. The results,

Fig. 2, indicated that this dilution of TNF seemed to prevent growth of the cells. However, after 24 hr in culture the number of viable cells in TNF treated cultures slowly declined. This is consistent with the observation of Carswell *et al.* (1) that the killing effects of TNF were delayed for 16 hr. Moreover, dead cell debris in the supernatant fluid from treated cultures increased with time indicating that some cells may have divided and subsequently died giving an illusion of a cytostatic effect. Attempts were made to enumerate these dead cells but this proved impossible since the cells were in various stages of disintegration (i.e., whole cells, nuclei and cell fragments were seen).

The effect of TNF on RNA synthesis in L-929 cells and normal mouse embryo fibroblasts. Since TNF treatment prevented L-929 cells from growing, and killed a portion of them depending upon the dose employed, we studied the time course of RNA synthesis in treated and control cultures. Triplicate cultures of 5×10^4 L-929 cells were seeded in 24 well Linbro plates and allowed to attach for 6 hr. Cultures then received a 1:200 dilution (2 units) of TNF containing serum or normal rabbit serum. At various times thereafter, cells were labeled with [^{3}H]-uridine for 1 hr. The cells were then washed once with cold BSS and three times with cold 5% TCA and RNA was hydrolyzed with 5% TCA by heating the trays for 1 hr at 80°. Aliquots of the hydrolysate were then counted. Another set of triplicate cultures was employed for cell number and viability determinations. Figure 3A shows a dramatic six-fold increase in [^{3}H]uridine incorporation into RNA between 24 and 30 hr after treatment. The increase in RNA synthesis was apparent almost from the time of addition of TNF. Since TNF has a killing effect on these cells, the dashed line in Fig. 3 represents a stimulation index corrected for cell numbers. When normal mouse embryo cells were treated in an identical way (Fig. 3B) there was no alteration in RNA synthesis. Figure 3C demonstrates the effect of TNF on RNA synthesis in a TNF resistant line of L-929 cells. These resistant cells represent the progeny of a few surviving cells which grew in the presence of 2 units of TNF. The stimulation index of this cell population varied between 0.9 and 2.3

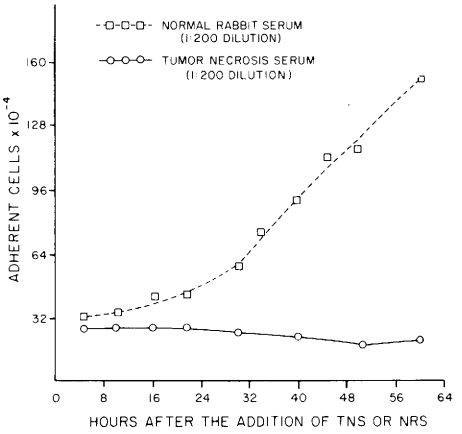

FIG. 2. The effect of TNF containing rabbit serum on L-929 cell growth. Adherent cells were counted following trypsinization at the indicated times. All cultures were done in triplicate.

over a 50-hr period. This is significantly lower than the sensitive L-929 cells and corrected values (for cell numbers) showed very little killing due to TNF.

The effect of actinomycin D on TNF-treated L-929 cells. In order to determine if the increase in RNA synthesis was related to the killing effect of TNF, we inhibited RNA synthesis with actinomycin D. L cells, 5×10^4, in 24 well Linbro plates in the presence of a 1:200 dilution of TNF or NRS were treated 12 hr later with 1 μg/ml of actinomycin D. This dose of actinomycin was shown to inhibit 95% of RNA synthesis within 1 hr. Twelve hours later (24 hr after addition of TNF) and near the peak of RNA synthesis, cell numbers and viability determinations were made as shown in Table II. Cells treated with TNF alone, actinomycin D alone, or actinomycin and NRS showed little inhibition of growth at this time. However, cultures treated with both TNF and actinomycin D were completely killed. Thus, the inhibition of RNA synthesis by actinomycin D seemed to enhance the killing effect of TNF. Similar findings were obtained with cycloheximide, an inhibitor of protein synthesis (data not shown).

Discussion. Endotoxin induced tumor necrosis factor (TNF) shows discriminating toxicity for some transformed cells (1, 2, 4). For example, Old (4) reported that 21 human tumor cell lines were screened for their sen-

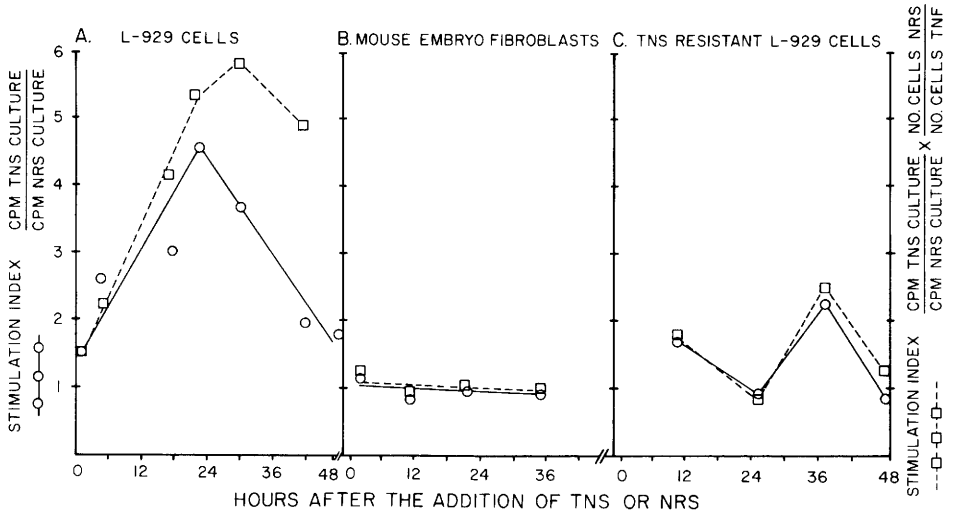

FIG. 3. The effect of serum containing tumor necrosis factor on [^{3}H]-uridine incorporation in: A. L-929 cells, B. Mouse embryo fibroblasts, and, C. TNS resistant L-929 cells. Solid lines (O——O) indicate the increase in RNA synthesis of TNF stimulated cultures as compared to control cultures with normal rabbit serum. Broken lines (□-----□) show the corrected values when the stimulation in RNA synthesis is corrected for the number of cells remaining on the plastic at the indicated times. Superimposition of the two lines indicate that TNF had no effect on cell numbers in the case of mouse embryo fibroblasts and TNF resistant L-929 cells.

TABLE II. THE EFFECT OF ACTINOMYCIN D TREATMENT ON TNF TREATED L-929 CELLS.

Treatment	Concentration	Cell counts ($\times 10^{-4}$) ±SD	Percent inhibition of cell growth[b]
TNF	2 units	8.95 ± 0.60	17.1
NRS	—[a]	10.08 ± 0.16	0
Actinomycin D	1 μg/ml	7.68 ± 0.77	28.8
Actinomycin D + TNF	1 μg/ml 2 units	0	100
Actinomycin D + NRS	1 μg/ml —[a]	6.69 ± 1.04	38.1

[a] The normal rabbit serum was used at the same dilution as the TNF containing rabbit serum.

[b] The inhibition of cell growth was measured with a hemacytometer chamber using trypan blue exclusion to determine cell viability. The cell counts were made 24 hr after the addition of actinomycin D. The dose of actinomycin D employed, 1 μg/ml, shuts off 95% of RNA synthesis within one hr.

sitivity to TNF. TNF was cytostatic for 10, cytotoxic for 1 and had no effect on 10 others with their assay system. He also stated that 6 cell lines derived from normal tissue were not affected by TNF thereby confirming and extending the observation that it has no effect on normal cells. The reasons for the variable effects of TNF on tumor cells is unknown but may emphasize that cancer is most likely a disparate group of diseases. TNF apparently recognizes some common feature on some transformed cells, but not all, and this feature is missing from normal cells.

We observed both a cytostatic and cytolytic effect of rabbit TNF on L-929 cells. In the presence of TNF the L-929 cells did not increase in numbers but the cells remaining on the glass seemed to be viable since they excluded trypan blue; these observations indicated cytostasis. Cell debris did appear later in the supernatant and accumulated with time suggesting that cell division and cell death were both occuring. The appearance of cell debris and the eventual decrease in cell numbers indicated that cytolysis also resulted and that the cytolytic event did not occur simultaneously in cells but progressed slowly with time. Furthermore, mitotic figures were seen in stained preparations. Time lapse cinema-

tography also showed cells dividing in the presence of TNF, but after dividing, one or both of the daughter cells became highly granular, rounded up and detached from the glass. These observations are consistent with the results shown in Fig. 2 which demonstrates a fairly flat or slightly declining line for TNF treated cells. Preliminary studies have also shown that DNA synthesis, as measured by incorporation of [^{3}H]thymidine, was similar in control and TNF treated cultures for the first 30 hr and then slowly began to decrease thereafter. It is probable that our population showed heterogeneity relative to its sensitivity to TNF; the observation that we could readily isolate TNF resistant cells from these cultures gives evidence to this possibility.

The reason for the increase in RNA synthesis is not clear at present. The slowly progressing events following TNF treatment and the acceleration of death by inhibitors of macromolecular synthesis can be interpreted as if the cell is actively resisting destruction. It is possible that increased synthesis of RNA is a compensatory mechanism on the part of the cell to repair or replace some essential component that is blocked or destroyed by TNF. It is also possible that the large increase in [^{3}H]-uridine incorporation into TCA precipitable counts may be due to a change in the uridine pool. A decrease in endogenous UTP production would yield a pool with a higher specific activity due to [^{3}H]-uridine conversion to [^{3}H]UTP and therefore more labelled UTP would be available to the cell. This could also reflect an actual change in pool size due to an increase uptake of [^{3}H]-uridine and conversion to [^{3}H]UTP.

The slowly progressing cytostatic and cytolytic events of TNF on target cells is similar to that described for lymphotoxin, a cytotoxic protein (lymphokine) which seems to be elaborated by T lymphocytes *in vitro* following mitogenic stimulation (5, 6). Lymphotoxin also stimulates RNA synthesis in target cells and the cytolytic events are accelerated by treatment with actinomycin D (6, 7). These authors also suggest that the increase in RNA synthesis is compensatory for cellular damage. Other similarities between TNF and lymphotoxin exists: for example both lack species specificity. However, a major difference exists in that lymphotoxin is not discriminatory for transformed cells but is lytic for both normal and neoplastic cells (8). The discriminatory cytostatic and cytolytic effects of TNF for some tumor cells makes studies of this natural substance of considerable importance, both for the exploitation of its potential in therapy of neoplastic diseases and for our understanding of the nature of oncogenically transformed cells.

Summary. BCG or *Corynebacterium parvum* primed rabbits injected with endotoxin contained a serum substance called tumor necrosis factor (TNF) that killed transformed mouse L-929 cells but not secondary normal mouse embryo fibroblasts. L-929 cells treated with TNF showed a six-fold stimulation of RNA synthesis that reached a maximum at approximately the same time as they started to die. Non-sensitive normal mouse embryo fibroblasts as well as TNF resistant L-929 cells did not show this large stimulation of RNA synthesis. When actinomycin D was employed to inhibit RNA synthesis, in the presence of TNF, a synergistic effect on killing of the transformed cells occurred.

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