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Immunology

An endotoxin-induced serum factor that causes necrosis of tumors

(activated macrophage)

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ABSTRACT **In studying "hemorrhagic necrosis" of tumors produced by endotoxin, it was found that the serum of bacillus Calmette–Guérin (BCG)-infected mice treated with endotoxin contains a substance (tumor necrosis factor; TNF) which mimics the tumor necrotic action of endotoxin itself. TNF-positive serum is as effective as endotoxin itself in causing necrosis of the sarcoma Meth A and other transplanted tumors. A variety of tests indicate that TNF is not residual endotoxin, but a factor released from host cells, probably macrophages, by endotoxin. Corynebacteria and Zymosan, which like BCG induce hyperplasia of the reticulo-endothelial system, can substitute for BCG in priming mice for release of TNF by endotoxin. TNF is toxic *in vitro* for two neoplastic cell lines; it is not toxic for mouse embryo cultures. We propose that TNF mediates endotoxin-induced tumor necrosis, and that it may be responsible for the suppression of transformed cells by activated macrophages.**

One of the best-known enigmas of cancer biology is the "hemorrhagic necrosis" of tumors induced by endotoxin (1–5). We report here that endotoxin acts indirectly by causing the host to release a substance, which we name tumor necrosis factor (TNF), that is selectively toxic for malignant cells.

MATERIALS

BCG (bacillus Calmette–Guérin, Tice strain) was obtained from the Institute for Tuberculosis Research (University of Illinois Medical Center, Chicago, Ill.); *Corynebacterium granulosum* (Réticulostimuline, no. 90808) from the Pasteur Institute (Paris, France); *Corynebacterium parvum* from the Burroughs Wellcome Laboratories (Triangle Park, N.C.); Zymosan (from *Saccharomyces cervisiae*) from Nutritional Biochemical Corporation; endotoxin (lipopolysaccharide W from *Escherichia coli*) from Difco (Detroit, Mich.); BCG old tuberculin (O.T.) from Jensen-Salisbury Laboratories (Kansas City, Mo.); mixed bacterial vaccine (MBV = heat-killed *Streptococcus pyogenes* and *Serratia marcescens*) from Bayer Co. (Wuppertal, Germany); *Brucella abortus* vaccine from the U.S. Department of Agriculture (Ames, Iowa); and poly(I)·poly(C) from P-L Biochemicals (Milwaukee, Wisc.).

RESULTS

Assay for tumor necrosis factor (TNF) in serum

The criterion adopted as a standard for assaying TNF in the sera of mice subjected to various treatments (see below) was visual observation of necrosis in a subcutaneous transplant of BALB/c sarcoma Meth A. Fig. 1 illustrates the grades of response (− to +++) elicited in individual (BALB/c × C57BL/6)F_1 mice by administration of serum containing TNF. In the maximum (+++) response, the major part of the tumor mass is destroyed, leaving only a peripheral rim of apparently viable tumor tissue. In about 25% of mice treated with 0.5 ml of TNF-positive serum, the tumor regresses. Regression is not seen in control untreated mice under these conditions. Mice receiving TNF-positive serum show no marked signs of toxicity.

Necessity for treatment with both BCG and endotoxin for the production of TNF in the serum

In the studies summarized in Table 1, TNF was demonstrable in the serum of BCG-infected mice given endotoxin, but not in the serum of mice given either BCG alone or endotoxin only.

Conditions for optimal production of TNF

Dose of BCG. An inoculum of 2×10^7 viable organisms was chosen because we found that this gives maximal reticulo-endothelial system (RES) stimulation and sensitization to endotoxin lethality (6).

Interval Between BCG Infection and Administration of Endotoxin. We chose an interval of 14–21 days because this is the time of maximal RES stimulation, as judged by enlargement of the spleen and liver and by phagocytic indices.

Table 1. Necrosis of sarcoma Meth A* produced *in vivo* by serum from BCG-infected CD-1 Swiss mice treated with endotoxin

Serum† from mice treated with:		TNF assay: Necrotic response‡			
		+++	++	+	−
BCG§	Endotoxin¶	Number of mice			
−	−				9
+	−			2	7
−	+				9
+	+	171	109		

* 7-day subcutaneous transplants of BALB/c sarcoma Meth A in (BALB/c × C57BL/6)F_1 mice; initial inoculum 2×10^5 cells; approximate diameter of tumor mass at time of assay, 7–8 mm.
† Pooled sera from female CD-1 Swiss donors; 0.5 ml iv per tumor-bearing recipient.
‡ For scoring of the necrotic response, see Fig. 1.
§ Viable organisms (2×10^7) iv per mouse 14 days before exsanguination.
¶ Twenty-five micrograms iv per mouse 2 hr before exsanguination.

Abbreviations: TNF, tumor necrosis factor; BCG, bacillus Calmette–Guérin; iv, intravenous; RES, reticulo-endothelial system; MEF, mouse embryo fibroblasts.

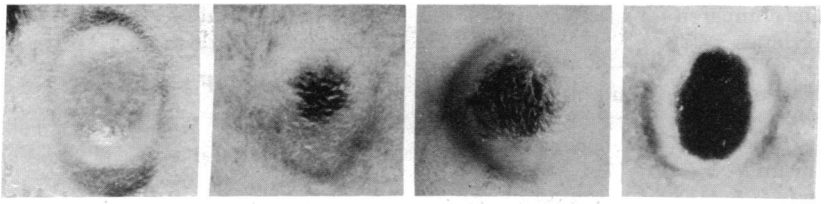

FIG. 1. Grading of the necrotic responses of BALB/c sarcoma Meth A (7-day subcutaneous transplants) 24 hr after administration of 0.5 ml of TNF-positive serum intravenously (iv). Histology shows corresponding devastation of tumor cells; hemorrhage is only a minor feature at this stage.

Dose of Endotoxin. In the range of 0.25–25 μg per mouse, the highest dose was most consistently effective in eliciting TNF in BCG-infected mice.

Time of Serum Collection. Intervals of 30 min to 4 hr after endotoxin (in BCG-infected mice) were investigated. The optimal time for collecting serum was found to be 2 hr. Although at this time the mice were acutely shocked, their blood volume was still sufficient for a good yield, whereas later they were moribund with circulatory collapse. (Clotting of blood from donors yielding TNF is minimal or absent; in fact poor clotting is a good indication that a particular serum will show TNF activity. We refer to the supernate obtained after centrifugation of pooled blood as "serum," although its composition may be closer to plasma.)

Conditions for optimal assay of TNF

(*a*) The BALB/c ascites sarcoma Meth A (inoculated subcutaneously in counted numbers) was chosen for the standard TNF assay because we knew it to be susceptible to "hemorrhagic necrosis" produced by endotoxin, and because we are familar with its highly consistent growth characteristics. Although the assay can be conducted equally effectively with Meth A in syngeneic BALB/c hosts, the (BALB/c × C57BL/6)F_1 hybrid was preferred because these mice were available in greater numbers. (*b*) As in the case of endotoxin itself, TNF produced its most consistent effect on well-established (7-day) transplants, less effect on 6-day transplants and virtually none on 5-day transplants. (*c*) In the standard assay, the necrotic response generally corresponds with the volume of TNF-positive serum administered in the range 0.1 ml (usually negative)–0.5 ml (+++ reaction). (*d*) We found 24 hr to be the optimal time to score TNF reactions. Reactions are already evident after 3–4 hr.

Other priming and eliciting agents

As Table 2 shows, *C. granulosum*, *C. parvum*, and Zymosan are as effective as BCG as priming agents for TNF release by endotoxin. These agents have in common their capacity to produce marked RES hyperplasia. Two agents in addition to endotoxin, mixed bacterial vaccine and poly(I)·poly(C), elicited TNF in BCG-primed mice, whereas old tuberculin and *B. abortus* did not.

Estimation of residual endotoxin in TNF-positive sera

Two assays were used to detect residual endotoxin in TNF-positive sera. In the standard rabbit pyrogenicity assay (7), the two separate pools of TNF-positive sera tested showed endotoxin levels of ≦0.01 μg/ml and 0.022 μg/ml. Sera from normal mice, endotoxin-treated mice, or BCG-treated mice (Table 1) were non-pyrogenic. In the *Limulus* assay (8), endotoxin levels estimated at 1 μg/ml were found in the serum of endotoxin-treated mice, whether pretreated with BCG or not. These amounts of residual endotoxin in TNF-positive sera are less than 0.1–1% of the amount necessary to produce comparable hemorrhagic necrosis in Meth A.

Effect of TNF on other tumors

A high degree of sensitivity to TNF, comparable to that of Meth A, was observed with the following transplanted tumors, growing subcutaneously in the indicated mouse strains: sarcomas S-180 (CD-1 Swiss) and BP8 (C3H); leuke-

Table 2. Assays for TNF in the serum of mice treated with various priming and eliciting agents

Treatment of serum donors*		TNF assay: Necrotic response†			
		+++	++	+	−
Priming agent	Eliciting agent‡	Number of mice			
BCG (2×10^7 viable organisms)	Endotoxin (25 μg)	171	109		
	Mixed bacterial vaccine (5 μl)	10	6	3	
	poly(I)·poly(C) (200 μg)		2		9
	Old tuberculin (50 μg)				9
	B. abortus (1×10^8 killed organisms)				11
	BCG (1×10^8 viable organisms)			2	9
C. granulosum (700 μg)		8			
C. parvum (1000 μg)	Endotoxin (25 μg)	24	7		
Zymosan (2000 μg)		4			

* CD-1 Swiss mice received the eliciting agent 14 days after the priming agent (both iv) and were exsanguinated 2 hr later.
† For scoring of the necrotic response, see Fig. 1 and Table 1.
‡ Doses are given in parentheses.

Table 3. Mouse strains compared for their capacity to produce TNF

Strain of serum donors	Treatment of serum donors*: BCG (2×10^7 viable organisms) + endotoxin (25 μg) — TNF assay: Necrotic response† +++	++	+	—	Treatment of serum donors*: *C. granulosum* (700 μg) + endotoxin (25 μg) — TNF assay: Necrotic response† +++	++	+	—
	Number of mice							
CD-1 Swiss	171	109			8			
C57BL/6	4				4			
SJL/J	3			1	4			
AKR/J	4				3	1		
A/J				4		3		1

* Donor mice received endotoxin 14 days after BCG or *C. granulosum* (both iv) and were exsanguinated 2 hr later.
† For scoring of the necrotic response, see Fig. 1 and Table 1.

mias EL4 (C57BL/6), ASL1 (A strain), RADA1 (A strain), RLδ1 (BALB/c), and EARAD1 (C57BL/6 × A)F_1; and mastocytoma P815 (DBA/2). The reticulum-cell sarcoma RCS5 (SJL), which disseminates widely, was resistant. Among primary spontaneous neoplasms, AKR leukemias show intermediate sensitivity (indicated by reduction in the size of spleen and lymph nodes), and mammary tumors of (C3H/An × I)F_1 origin were only slightly responsive. Meth A growing in the ascites form was highly sensitive to TNF given intraperitoneally, even in recipients with advanced disease.

Production of TNF by different mouse strains (Table 3)

Strains C57BL/6, SJL/J, and AKR/J produced TNF roughly as well as Swiss mice. A/J mice failed to produce TNF when primed with BCG but did so to some extent when primed with *C. granulosum*.

TNF production in other mammals (Table 4)

Both rats and rabbits produced TNF; as in the mouse, both BCG and endotoxin were required to induce appreciable amounts of TNF. Rabbits are particularly sensitive to endotoxin, so the dose was adjusted accordingly. The serum of BCG-infected rabbits that died less than an hour after endotoxin showed little or no TNF activity.

Activity of TNF against cells in culture (Fig. 2)

This was tested with Meth A cells, L cells (NCTC Clone 929), and mouse embryo fibroblasts (MEF). The L cells proved most sensitive, Meth A sarcoma cells somewhat less so, and MEF virtually insensitive. The criterion employed was the count of viable cells after 48-hr exposure. Judging by proportional viability counts (trypan blue exclusion or phase microscopy), the effect of TNF on Meth A appears primarily cytostatic, whereas L cells die within the 48-hr test period. The toxicity is delayed; no effect of TNF is demonstrable in the first 16 hr of exposure. Measurable toxicity for L cells was demonstrable with dilutions of TNF-positive serum as high as $1:10^4$. Toxicity was not abolished by heating the TNF serum to 56° for 30 min. Sera from normal mice, or mice treated with either BCG or endotoxin alone, tested under the same conditions as TNF-active sera, showed no toxicity. Endotoxin itself, in concentrations as high as 500 μg/ml, was not toxic for L cells. Rabbit and rat TNF sera had the same pattern of toxicity as mouse TNF, being highly toxic for L cells but not for MEF.

Correlation of toxic effects of TNF-positive sera *in vitro* and *in vivo*

In a broad range of tests, there has been no discrepancy between the TNF activity of sera against Meth A *in vivo* and their toxicity for L cells *in vitro*.

DISCUSSION

The inhibitory effect of bacterial products on human cancer has long been known (9). The counterpart in laboratory animals has been regarded as the "hemorrhagic necrosis" of transplanted tumors caused by material from Gram-negative bacteria. Much early work culminated in Shear's isolation of a tumor-necrosing "polysaccharide" (10), now recognized as endotoxin. The fact that endotoxin does not kill tumor cells in culture indicated that its action must be indirect, and lent credence to Algire's conclusion (11) that hemorrhagic necrosis might be secondary to endotoxin-induced hypotension leading to circulatory stasis and ischemia in the tumor. The discovery of TNF provides a more obvious rationale for the indirect action of endotoxin, namely, that endo-

Table 4. TNF release in rats and rabbits

Treatment of serum donors*: BCG	Endotoxin	Serum donors: CD rat — TNF assay: Necrotic response† +++	++	+	—	NZW rabbit — TNF assay: Necrotic response† +++	++	+	—
		Number of mice							
—	—				8			2	6
+	—				8			1	7
—	+			1	7			1	7
+	+	5	6	1	2	11‡	23‡	10‡	7‡

* BCG (7×10^7 viable organisms iv per rat or 3×10^8 iv per rabbit) was given 14 days before endotoxin. Endotoxin (250 μg iv per rat or 100 μg iv per rabbit) was given 2 hr before exsanguination.
† For scoring of Meth A necrotic response, see Fig. 1 and Table 1.
‡ Heat-inactivated sera from 17 different rabbits assayed in three mice each, making a total of 51 assay mice. Each mouse was injected with 0.5 ml of rat serum or 1 ml of rabbit serum.

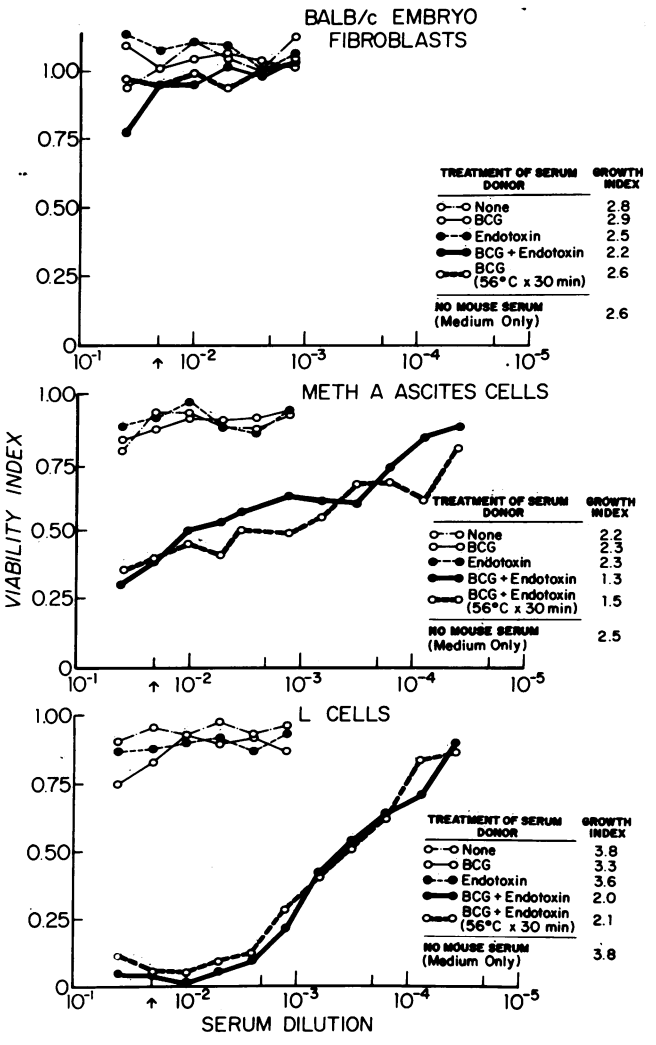

FIG. 2. Inhibition of growth of cultured cell lines by TNF-positive serum. L cells (NCTC Clone 929) and BALB/c embryo fibroblasts (MEF) were grown as monolayers, and Meth A cells in suspension. Culture medium: Eagle's minimum essential medium plus nonessential amino acids, 10% heat-inactivated fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). One milliliter of cell suspension (2×10^5 MEF or L cells per ml; 2.5×10^4 Meth A cells per ml) was plated with 1 ml of the mouse serum to be tested, in serial dilution (abscissa), or 1 ml of culture medium (standard). Incubation: 48 hr in 5% CO_2 in air at 37°. Viability index = number of viable cells present in culture with test serum, divided by number in culture with medium alone. Insets: growth index = total number of cells (viable and dead) after 48 hr in culture in 1/50 mouse serum (↑) divided by number of cells plated. (Replicate plates; duplicate readings.)

toxin causes the host to release a factor which is toxic for the tumor. The conclusion that TNF found in the serum mediates tumor necrosis produced by endotoxin is further supported by the fact that both agents, TNF and endotoxin, act against a similar spectrum of tumors and at a similar phase of their growth.

Partial characterization of TNF indicates a glycoprotein with a molecular weight of about 150,000 which migrates with α-globulins (12). This glycoprotein has the characteristic properties of TNF-positive serum, i.e., necrosis of Meth A *in vivo* and toxicity for L cells *in vitro*. It does not contain the sugar 2-keto-3-deoxyoctonate (KDO) or the 3-D-myristomyristic fatty acid characteristic of the Lipid A moiety of endotoxin from *E. coli* (13). Can TNF be identified with any of the factors already known to be elicited by endotoxin? So far we can exclude interferon, which, although abundant in TNF sera, is absent from partially purified TNF. C-reactive protein has been ruled out, and the stability of TNF at 56° excludes any heat-labile factor.

The cellular origin of TNF is uncertain, but the fact that macrophage-inciting agents are necessary for its demonstration in serum points to this cell as the source. This interpretation is strongly supported by recent observations we have made on TNF-donor mice (S. Sternberg, unpublished data). In the greatly enlarged spleens of BCG-infected mice, microscopy shows massive hyperplasia of macrophages; two hours after administration of endotoxin, at the time when TNF is abundant in the blood, there is pyknosis and disruption of this cell population.

One of the most provocative findings about macrophages in recent years is that when nonspecifically "activated" by agents such as BCG, endotoxin, and certain protozoa, they acquire selective toxicity for malignant cells (14–17). The fact that TNF *in vitro* showed discriminatory toxicity for transformed cells in our present study might suggest that TNF mediates the selective cytotoxicity of activated macrophages.

To explore the full potential of TNF, a more plentiful source will be required. Extraction from liver and spleen of mice with RES hyperplasia produced by BCG or *C. parvum* is one possibility; but the fact that the rat and rabbit produce TNF suggests the use of larger animals for preparing TNF in quantity.

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