

Inhibition of Tumor Necrosis Factor Production by Lymphocytes from Anti-TNF Antibody-Treated, Cardiac-Allografted Rats

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Presented at the Annual Meeting of the Association for Academic Surgery, Hershey, Pennsylvania, November 10-13, 1993

Tumor necrosis factor- α (TNF) is a multifunctional cytokine involved in the immunopathologic consequences of allograft rejection. We have previously demonstrated that anti-TNF antibody treatment prolongs cardiac allograft survival in rats. To elucidate the mechanism of anti-TNF antibody in modulating the immune response, we investigated TNF production by spleen and lymph node cells from anti-TNF antibody-treated Lewis rats who received MHC-mismatched Brown Norway rat cardiac allografts. In 10 untreated rats, cardiac allografts were rejected at 6.8 ± 0.6 days after transplantation (mean \pm SD). Anti-TNF antibody treatment enhanced graft survival to 12.7 ± 1.4 days ($P < 0.001$ vs controls). In other anti-TNF antibody-treated recipients spleen and lymph node cells were isolated on Day 5 after transplant. TNF production was measured and showed significantly less TNF than those from untreated (no anti-TNF antibody), transplanted recipient rats (28.7 u/ 10^6 spleen cells vs 76.4 u/ 10^6 spleen cells at 2 hr and 4.6 u/ 10^6 lymph node cells vs 9.2 u/ 10^6 lymph node cells at 24 hr). Furthermore, following lipopolysaccharide stimulation, spleen cells from anti-TNF-treated rats again produced significantly less TNF than those from untreated transplanted rats (68.9 u/ 10^6 cells vs 189.4 u/ 10^6 cells at 2 hr). Finally, with allogeneic cell stimulation, anti-TNF treated rats again produced significantly less TNF than untreated transplanted rats (spleen cells, 2.2 u/ 10^6 cells vs 40.4 u/ 10^6 cells at 24 hr; lymph node cells, 1.2 u/ 10^6 cells vs 22.2 u/ 10^6 cells at 72 hr). These findings suggest that anti-TNF antibody treatment may not only neutralize TNF activity, but also suppress TNF production itself, providing a new insight into the regulation of TNF by anti-TNF antibody. © 1994 Academic Press, Inc.

INTRODUCTION

Tumor necrosis factor- α (TNF) is produced primarily by activated monocytes and macrophages [1], but can

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also be produced by a broad range of cells including human T and B lymphocytes, [2, 3] NK cells [4], and mast cells [5]. TNF has been shown to mediate many host responses in bacterial infection, ischemia-reperfusion, and delayed-type sensitivity reactions [6]. TNF is a mediator of fatal bacteremic shock and antibody therapy against TNF has been shown to attenuate the life-threatening effects of endotoxin in baboons [7] and mice [8].

TNF is increasingly being recognized as an important cytokine involved in the development of allograft rejection. TNF is released following transplantation and increased circulating TNF levels have been reported during episodes of renal and liver allograft rejection in patients [9, 10]. TNF exerts multiple stimulatory effects on T-cells by binding to a specific TNF receptor [11], increasing the expression of HLA antigens and high-affinity IL-2 receptors [12]. In addition, TNF can act as a costimulator of IL-2-dependent T-cell proliferation and can enhance antigen-induced T4 helper T-cell proliferation [13].

Previous studies from this laboratory demonstrated that anti-TNF antibody therapy prolonged cardiac allograft survival in rats [14] and that the combination of anti-TNF antibody and subtherapeutic cyclosporine further improved allograft survival [15]. These findings indicate that anti-TNF antibody facilitates the prolongation of allograft acceptance and led us to study its mechanism of action in delaying allograft rejection in a rat model. In this present study, we investigated the regulation of TNF production by spleen cells and lymph nodes cells in anti-TNF-treated cardiac allografted rats.

MATERIALS AND METHODS

Animals. Inbred male Lewis (LEW) and Brown Norway (BN) rats weighting 200 to 300 g were obtained from Harlan Sprague-Dawley Inc. (Walkersville, MD).

Culture medium and reagents. RPMI 1640 medium with 2 mM L-glutamine (GIBCO) was supplemented with 10% heat-inactivated fetal calf serum, 0.1 mM non-essential amino acids, 10 mM Hepes buffer, 100 μ /ml penicillin, and 100 μ g/ml streptomycin. Lipopolysaccharide (LPS) from *Escherichia coli* serotype 0111:B4 (SIGMA) was diluted in RPMI medium.

Treatment protocol. A model of cervical heterotopic cardiac transplantation using LEW as the recipient and BN as the donor, developed in our laboratory, was used in this study [16]. Rats were randomly assigned to treatment groups including: (1) allograft implantation plus a single dose (1 ml) of anti-TNF by intraperitoneal injection on the day of transplantation ($n = 10$) (the preparation and specificity of this antiserum has been previously described [17]); (2) allograft implantation only, without anti-TNF treatment ($n = 8$); and (3) normal LEW rats without transplantation or anti-TNF treatment as controls ($n = 6$).

Mixed lymphocyte culture. Spleen cells and lymph node cells were isolated on Day 5 after transplantation. The effect of anti-TNF antibody treatment on the primary immune response was examined in one-way mixed lymphocyte culture. Splenic or lymph node cells from allograft-transplanted LEW rats with or without anti-TNF treatment were the responders. Irradiated spleen or lymph node cells from BN rats were the stimulators. Cells were processed through a stainless steel mesh and suspended in RPMI 1640 medium. Erythrocyte free suspension was obtained following brief treatment with water and then isotonicity recovered by equal volume of $2\times$ PBS. Responding cells (3×10^5) mixed with irradiated allogeneic stimulator cells (3×10^5) or reagents were plated in quadruplicate in 96-well flat-bottomed microtiter plates in complete medium and incubated at 37°C in a 5% CO_2 atmosphere. For the TNF production studies, the supernatants were collected at designed time points and stored at -20°C .

TNF assay. The amount of TNF in one-way mixed lymphocyte culture conditional medium was determined by using the WEHI-164 clone 13. An equal volume (100 μl) of conditional medium in serial twofold dilutions or TNF standard (Genzyme) dilutions was added in 96-well microtiter plates. WEHI-164 cells were seeded at a density of 5×10^4 cells/well in a 100- μl volume in RPMI 1640 containing 1% FCS and incubated at 37°C for 20 hr in a humidified CO_2 incubator. MTT tetrazolium was added, the cells were further incubated for 4 hr at 37°C , and then isopropanol with 0.04 N HCl was added. After dissolving the dark blue forazan crystals, the plates were read on a BIO-Kinetics reader using a test wavelength of 550 nm. Data were expressed as u/ml of TNF calculated by Macintosh Elisa Analysis DELTA SOFT.

Data analysis. Student's t test analysis or analysis of variance was used, where appropriate, to compare differences between experimental groups. Differences were considered to be statistically significant at a confidence limit of 95% ($P < 0.05$). Animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Pub. No. 80-23, revised 1978).

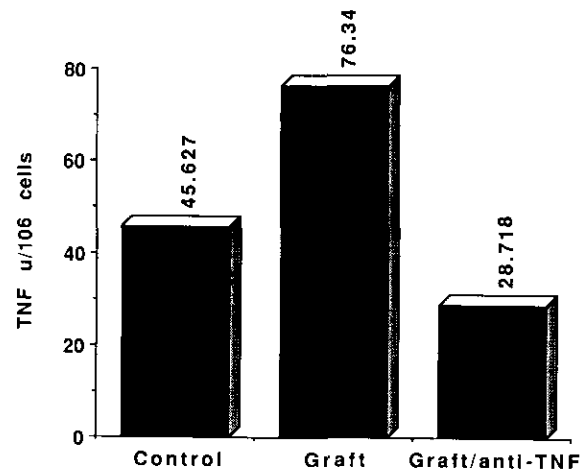


FIG. 1. TNF production *in vitro* by spleen cells from heart-transplanted rats with or without anti-TNF treatment *in vivo*. Control was from normal untreated LEW rat without graft. Supernatants were collected after spleen cells were incubated for 2 hr without treatment. TNF is expressed in u/10⁶ cells. Similar results were obtained in another experiment.

RESULTS

Allograft survival. Anti-TNF antibody treatment resulted in significantly extended periods of graft survival in Lewis rats who received MHC-mismatched Brown Norway rat cardiac allografts. In 10 untreated rats, cardiac allografts were rejected at 6.8 ± 0.6 days after transplantation (mean \pm SD). Anti-TNF antibody treatment (1 mg ip) administered to 8 LEW recipients on the day of transplantation had BN cardiac allograft survival enhanced to 12.7 ± 1.4 days ($P < 0.001$ vs controls).

Inhibition of TNF production without treatment in vitro. The effect of anti-TNF antibody treatment *in vivo* on lymphocytes was assessed by TNF production obtained in spleen and lymph node cells from anti-TNF antibody-treated and untreated LEW rats with cardiac allografts. Anti-TNF antibody treatment was administered only on the day of transplantation and the cells were isolated on Day 5 after transplantation and washed two times before culturing. There was no added anti-TNF antibody in the culture medium. As shown in Fig. 1, spleen cells from anti-TNF antibody-treated LEW rat with allograft produced less TNF than that from untreated rat (28.7 u/10⁶ cells vs 76.4 u/10⁶ cells in 2 hr). TNF production by lymph node cells from anti-TNF antibody-treated LEW rat with allografts produced less TNF than that from untreated rats (4.6 u/10⁶ cells vs 9.2 u/10⁶ cells in 24 hr) (Fig. 2).

Production of TNF with LPS stimulation. Isolated spleen and lymph node cells were exposed to LPS at a concentration of 10 or 100 ng/ml and TNF secretion in the supernatants was examined. Results showed that spleen cells from anti-TNF antibody-treated rat with cardiac allografts produced much less TNF than those

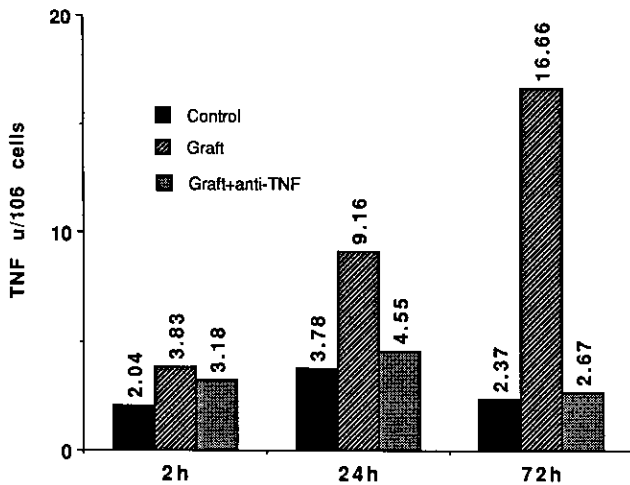


FIG. 2. Production of TNF *in vitro* by lymph node cells from heart-transplanted rats with or without anti-TNF treatment *in vivo*. Supernatants were collected at designed time points after cells were incubated without treatment *in vitro*. TNF is expressed in $u/10^6$ cells.

from untreated rats at 2 hr of incubation ($68.9 u/10^6$ cells vs $189.4 u/10^6$ cells with 10 ng/ml LPS; $557.5 u/10^6$ cells vs $760.5 u/10^6$ cells with 100 ng/ml LPS) (Fig. 3). TNF production in lymph node cells from anti-TNF antibody-treated rats with cardiac allografts, after LPS stimulation, was lower than that from untreated rats at 72 hr of incubation ($3.2 u/10^6$ cells vs $7.9 u/10^6$ cells with 10 ng/ml LPS; $8.8 u/10^6$ cells vs $26.5 u/10^6$ cells with 100 ng/ml LPS) (Fig. 4).

TNF production in response to allogeneic stimulation. In order to evaluate the effect of anti-TNF antibody treatment on lymphocyte response to allogeneic antigen,

mixed lymphocyte cultures were performed. Irradiated BN rat spleen or lymph node cells were used as stimulators and spleen or lymph node cells from anti-TNF antibody-treated LEW rats with cardiac allografts were responders. With allogeneic stimulation, cells from anti-TNF antibody-treated transplanted LEW rats produced less TNF than those from untreated rats (spleen cells, $2.2 u/10^6$ cells vs $40.4 u/10^6$ cells in 24 hr; lymph node cells, $1.2 u/10^6$ cells vs $22.2 u/10^6$ cell in 72 hr; full results in Tables 1 and 2).

DISCUSSION

Previous data from our laboratory showed that anti-TNF antibody treatment can prolong cardiac allograft survival in LEW rat recipients with BN rat heart grafts [18]. Our further study demonstrated that anti-TNF antibody combined with a subtherapeutic dose of CsA also significantly prolonged allograft survival [19]. To investigate the mechanism of this beneficial effect of anti-TNF antibody treatment in allograft rejection, we conducted studies *in vitro* to examine if anti-TNF antibody suppressed lymphocyte proliferation in one-way MLR using LEW lymph node cells as responder and irradiated BN lymph node cells as stimulators. While Shalaby reported that addition of rabbit anti-rHuTNF- α antibody alone significantly abolished the proliferative activity in the MLR in humans [20], we found that the addition of anti-TNF antibody to primary rat MLR cultures failed to suppress lymphocyte proliferation in our rat MLR system (data not shown). Therefore, in this study, we investigated TNF production and regulation by spleen cells and lymph nodes cells from anti-TNF-treated car-

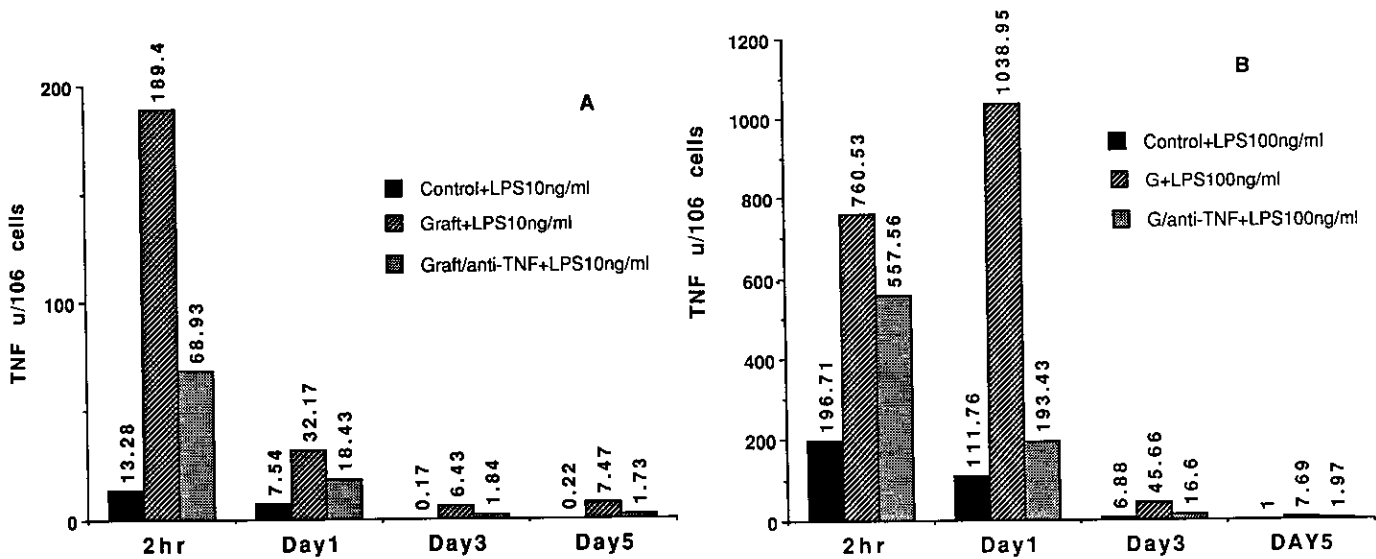


FIG. 3. Significant differences in kinetics of TNF production with LPS stimulation by splenocytes from BN heart-transplanted LEW rats with or without anti-TNF treatment *in vivo*. Supernatants were collected at designed time points after spleen cells were incubated with 10 ng/ml LPS (A) and 100 ng/ml LPS (B). TNF is expressed in $u/10^6$ cells.

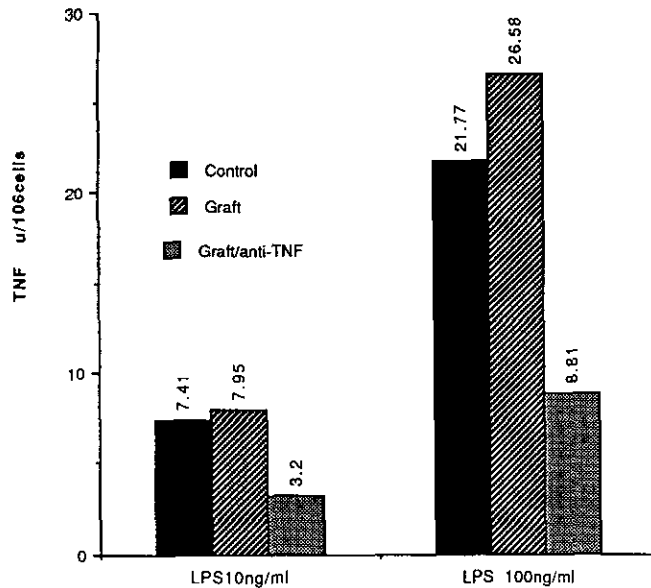


FIG. 4. Significant differences in LPS induction of TNF production *in vitro* by lymph node cells from BN heart-transplanted LEW rats with or without anti-TNF treatment *in vivo*. Supernatants were collected after cells were incubated with 10 or 100 ng/ml LPS for 72 hr. TNF is expressed in u/10⁶ cells.

diac-allografted rats. The results clearly indicate that TNF production *in vitro* is remarkably inhibited after anti-TNF antibody treatment *in vivo*, in response to both LPS and antigen stimulation.

The role of TNF as an immunoregulatory molecule has been the subject of several recent investigations. Anti-TNF antibody has been shown to neutralize TNF cytotoxic activity in mouse and human cells. Grau *et al.* demonstrated that a single injection of anti-TNF- α prevented local macrophage accumulation in brain and lymphoid tissues and fully protected infected mice from cere-

TABLE 1

Inhibition of TNF Production by Splenocytes from Anti-TNF-Treated Rat with Allograft

Group	TNF (u/10 ⁶ cells)			
	2 hr	Day 1	Day 3	Day 5
Control + BNx	1.64	1.95	0.59	0.03
Graft + BNx	3.39	40.37	23.72	1.25
Anti-TNF/Graft + BNx	2.78	2.22	0.62	0.06

Note. Significant differences in TNF production *in vitro* by spleen cells from allografted LEW rats stimulated with irradiated BN cells (BNx) with or without anti-TNF treatment *in vivo*. Controls were from normal untreated LEW rat without a BN allograft. Supernatants were collected after spleen cells were incubated for 2 hr without treatment.

TABLE 2

Inhibition of TNF Production by Lymph Node Cells from Anti-TNF-Treated Rat with Allograft

Group	TNF (u/10 ⁶ cells)			
	2 hr	Day 1	Day 3	Day 5
Control + BNx	0.046	0.335	1.507	0.617
Graft + BNx	0.064	1.122	22.187	14.72
Anti-TNF/Graft + BNx	0.094	0.405	1.157	0.697

Note. Significant differences in TNF production *in vitro* by lymph node cells from allografted LEW rats stimulated with irradiated BN cells (BNx) with or without anti-TNF treatment *in vivo*. Controls were from normal untreated LEW rat without a BN allograft. Supernatants were collected after cells were incubated at designed time points without treatment *in vitro*.

bral malaria [21]. Imagawa *et al.* reported that anti-TNF antibody can suppress circulating levels of TNF and that TNF levels rise once immunotherapy is terminated [22] and it has been reported that antibody against murine TNF- α prevents the cutaneous and intestinal lesions of acute phase, graft vs host disease and reduced overall GVH mortality [23].

In our experiment, spleen cells and lymph node cells from cardiac allografted rats stimulated by allogeneic antigen *in vivo* produced more TNF than those from normal, untransplanted (unstimulated) rats. However, after anti-TNF antibody treatment *in vivo*, spleen cells and lymph node cells from cardiac-allografted rats demonstrated much less TNF production than untreated rats. This effect of anti-TNF antibody was upon TNF production and not cell proliferation. However, there are differences between our *in vivo* study and others. There was no added anti-TNF antibody in our culture medium, whereas the suppression of cell proliferation with anti-TNF antibody treatment noted in prior studies [20] may be due to the anti-TNF used in the MLR, as that was strictly an *in vitro* study. Furthermore, there may be differences between rat and human MLR systems.

In conclusion, these results imply that despite prior stimulation, anti-TNF antibody pretreatment acts to suppress TNF production and that neutralization of TNF and suppression of proliferation are not the only mechanisms of action of anti-TNF antibody. Anti-TNF antibody treatment suppressed lymphocytic TNF production in response to endotoxin and allogeneic challenge. These findings give a new insight into the regulation of TNF production by anti-TNF antibody. While further studies are required to delineate anti-TNF effect on lymphocyte subpopulations and cytokine production related to allograft presentation, these data suggest an important mechanism by which anti-TNF antibody may be immunoregulatory.

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