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Enhancement of Cell-Mediated Cytotoxicity by Recombinant Tumor Necrosis Factor (TNFα) (42812)

YOSHINORI SHIMAMOTO,[1] SATHIT PICHYANGKUL, AND AMANULLAH KHAN

Department of Immunotherapy, The Cancer Center at Wadley Institutes of Molecular Medicine, Dallas, Texas 75235

Abstract. The effects of recombinant tumor necrosis factor (rTNFα) on the immune responses were investigated. A single iv injection of rTNFα (6×10^3U) caused regression of sarcoma-180 transplanted into BALB/c nu/+ mice, but failed to regress this tumor in nu/nu mice. A higher dose of rTNFα (2×10^4U) was necessary to induce antitumor effect in nu/nu mice. A host-related factor seemed to be involved in mediating tumor regression. Therefore, the effects of rTNFα on various T-dependent immune responses, including delayed footpad reaction (DFR), cell mediated cytolysis (CMC), and plaque-forming cells (PFC) were examined in BALB/c mice, immunized ip with chicken erythrocytes (CRBC). A single injection of rTNFα, at the time of the antigen administration, induced the augmentation of CMC to CRBC in a dose-dependent manner. DFR and PFC were not affected in optimal immunization procedures. The TNFα injection, at or after the time of antigen administration, was more effective in inducing augmentation of CMC. The increase in CMC by TNFα was mediated by nonadherent, Thy 1.2, Lyt 2.2 positive cells and neutralization of TNFα by the anti-TNFα monoclonal antibody abolished the effect on CMC. These results indicated that the human recombinant TNFα induced changes in the T-cell-mediated responses.

Tumor necrosis factor (TNFα) is an active antitumor substance. It is released into the serum of Bacillus Calmette–Guérin (BCG)-primed mice, following challenge with endotoxin (1). In addition to its direct cytotoxicity, TNFα also possesses other activities including inhibition of lipoprotein lipase, stimulation of collagenase and prostaglandin E_2, stimulation of bone resorption and inhibition of bone formation, and activation of polymorphonuclear cells (2–5). Recently, immunomodulatory properties of TNFα have received much attention (6–8). There are, however, little data available regarding the *in vivo* effects of TNFα on the immune responses. The antitumor effect of TNFα was stronger in BALB/c nu/+ mice suggesting that the host immune factors may contribute to the antitumor effect (9). We report the effects of recombinant human TNFα (rTNFα) on T-cell-dependent responses *in vivo*.

[1] Fellow in Department of Immunotherapy at Wadley Institutes. Present address: Saga Medical School, Department of Internal Medicine, Nabeshima, Saga 840-01, Japan.

Materials and Methods. *rTNFα preparation.* Recombinant human TNFα was produced in *Escherichia coli* by recombinant technology (10). It was purified by CM-Sephadex, Blue agarose column chromatography and high-performance liquid chromatography (HPLC) on TSK-DEAE-5PW column. The purified rTNFα was found to be over 95% pure, as determined by SDS–polyacrylamide gel electrophoresis and gel filtration on HPLC. The cytotoxic activity of TNFα was measured on L-929, using a semiautomated computer-assisted microassay (11). The unit of rTNFα activity was defined as the reciprocal of the highest dilution that caused 50% cytotoxicity as compared to the control (11). The purified rTNFα had specific activity of 1×10^7 U/mg. The amount of endotoxin in the recombinant preparation used was found to be 0.25 ng/mg of protein by limulus amoebocyte lysate (LAL) test (Whittaker, MA, Bioproduct, Walkersville, MD).

Animals. Eight-week-old athymic homozygous (nu/nu) and heterozygous (nu/+) mice of BALB/c background and BALB/c mice were purchased from Harlan Sprague–

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Dawley (Indianapolis, IN). There were six to eight mice in each group.

Tumors. Sarcoma-180, an allotransplantable murine tumor, was maintained in RPMI 1640 medium, supplemented with 10% fetal calf serum (FCS), 50 μg/ml gentamycin (Gibco, Grand Island, NY), 1 m*M* L-glutamine, and 1 m*M* sodium pyruvate (Whittaker, MA, Bioproducts, MD). The tumor cells (1×10^7) were injected sc into BALB/c nu/nu or nu/+ mice. Seven days after tumor inoculation, rTNFα was given as a single iv injection. Tumor size was expressed as the product of the longest diameter and shortest diameter.

Antigen and immunization. Chicken red blood cells (CRBC), in Alsever's solution, were purchased from Hazleton (Denver, PA), washed three times with phosphate-buffered saline (PBS), and suspended at the desired concentration in PBS. BALB/c mice were immunized ip with 10^8 CRBC and rTNFα was given as a single iv injection on the same day, unless otherwise indicated.

Assay for delayed footpad reaction. Delayed footpad reaction (DFR) was elicited by injecting 10^8 CRBC in 50 μl of 0.9% NaCl solution into the left hind footpad, 5 days after immunization, unless otherwise indicated. The right hind footpad was injected with 50 μl of 0.9% NaCl solution as a control. Swelling of the footpads was measured with a dial thickness gauge 24 hr later. The DFR was expressed as the difference in the thickness between the left and right hind footpads (12–14).

Assay for cell-mediated cytolysis. CRBC were labeled with ^{51}Cr as described by Perlmann and Perlmann (15) and used as target cells. Spleens were obtained 5 days after immunization, teased, and pressed gently between two glass slides in RPMI 1640 medium. Cell suspensions were passed through a layer of gauze to remove large fragments. To assess the cytotoxic activity, 2×10^7 spleen cells were mixed with 1×10^6 ^{51}Cr-labeled CRBC in 2 ml of RPMI 1640 supplemented with 10% FCS in roller tubes (Falcon 2027), as described previously (12, 14, 16). The amount of ^{51}Cr released in the supernatant was determined by a well-type gamma counter after a 6-hr incubation at 37°C in a humidified atmosphere with 5% CO_2. Cytotoxic activity of effector cells was expressed as percentage cytotoxicity in the following manner:

$$\text{Percentage Cytotoxicity} = \frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Maximum release} - \text{spontaneous release}} \times 100.$$

Maximum release represents the amount of ^{51}Cr released from target cells alone, into distilled water. Spontaneous release represents

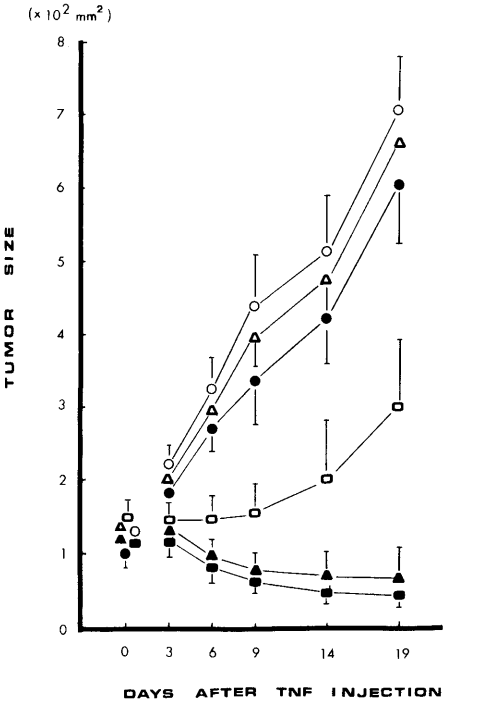

FIG. 1. Effect of TNFα on sarcoma-180 transplanted into BALB/c nu/nu or nu/+ mice. Sarcoma-180 (1×10^7 cells) were inoculated sc 7 days prior to the administration of rTNFα. rTNFα (6×10^3 U/mouse; ▲, nu/+; △, nu/nu or 2.4×10^4 U/mouse: ■, nu/+; □, nu/nu) was given as a single iv injection on Day 0. Control mice received saline (●, nu/+; ○, nu/nu). The results represent the mean value of six to eight animals and standard error. The tumor size of rTNFα-treated nu/+ mice differs significantly after Day 6 from nu/+ controls ($P < 0.001$). The tumor size of rTNFα-treated (24,000 U/mouse) nu/nu mice differs significantly after Day 6 from rTNFα-treated (6000 U/mouse) and control nu/nu mice ($P < 0.02$).

the amount of ^{51}Cr released in the absence of effector cells.

Assay for antibody plaque-forming cells. Antibody plaque-forming spleen cells were counted 5 days after immunization by the hemolytic plaque technique of Cunningham and Szenberg (17).

Preparation of nonadherent cells. Spleen cells were suspended in RPMI 1640 (5×10^6/ml) and incubated for 60 min at 37°C in petri dishes (Falcon 1029). After incubation, free cells were harvested. The process was repeated one more time. The nonadherent cells, thus collected, were used as effector cells in the cytotoxicity assay.

Treatment with anti-Thy 1.2, Lyt 2.2 antibody and complement. The anti-Thy 1.2 or anti-Lyt 2.2 monoclonal antibody was purchased from New England Nuclear (Boston, MA) or Cedar Lane (Ontario, Canada). Spleen cells were suspended at 2×10^7/ml in anti-Thy 1.2 antibody at a dilution of 1:100 or in anti-Lyt 2.2 antibody at a dilution of 1:20. The suspension was incubated for 60 min at room temperature, washed once, resuspended in 1:10 dilution of rabbit complement (Pel-Freez, Ar) and incubated for 60 min at 37°C. The suspension was then washed twice with RPMI 1640 medium and used as effector cells. These treatments caused 95% cytotoxicity to thymocytes and 30% with anti-Thy 1.2 or 10% with anti-Lyt 2.2 antibody to the spleen cells obtained from normal BALB/c mice, as measured by the trypan blue dye exclusion method.

Monoclonal antibody against rTNFα. Monoclonal antibody against rTNFα was produced by fusing NS-1 cells with the splenocytes of BALB/c mice immunized with recombinant TNFα. As a result, six monoclonal antibodies were produced (18). One of these antibodies, AT-1, had neutralizing activity against rTNFα and was used in this study.

Statistical analysis. The statistical significance of the data was determined by Student's *t* test.

Results. *Effect of rTNFα on sarcoma-180.* In BALB/c nu/+ mice, both doses of TNF (2.4×10^4 and 6×10^3 U/mouse) caused regression of sarcoma-180 (Fig. 1). However, in nu/nu mice, the low dose of rTNFα (6×10^3 U/mouse) showed no effect, but the high dose of rTNFα caused inhibition of tumor growth. This suggested that the T-cell-mediated immune responses may be implicated in the antitumor effect of rTNFα.

Effect of rTNFα on various immune responses. Various immune responses were measured in BALB/c mice following treatment with rTNFα on the day of immunization. DFR, CMC, and PFC to CRBC were assessed 5 days after immunization and are shown in Fig. 2. DFR and PFC were not affected by the rTNFα in this immunization procedure. On the other hand, CMC was

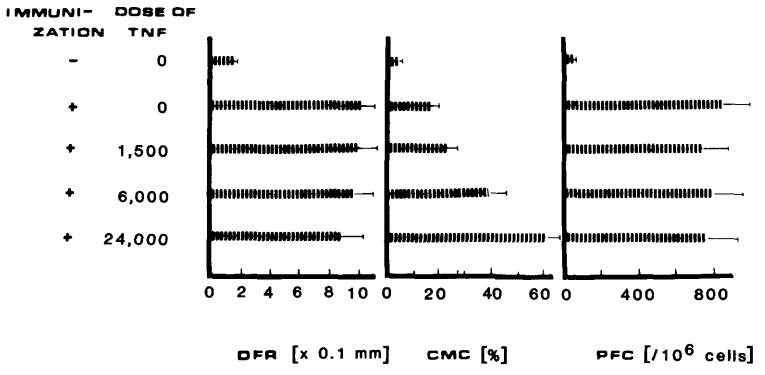

FIG. 2. Effect of rTNFα on various immunologic reactions. The BALB/c mice were immunized ip with 10^6 CRBC and injected iv with various doses of rTNFα on the same day. DFR, CMC, and PFC were assessed 5 days after immunization. The results represent the mean value of six to eight animals and standard error. CMC in rTNFα-treated mice (6000 and 24,000 U/mouse) was significantly increased, compared to the immunized controls ($P < 0.02$).

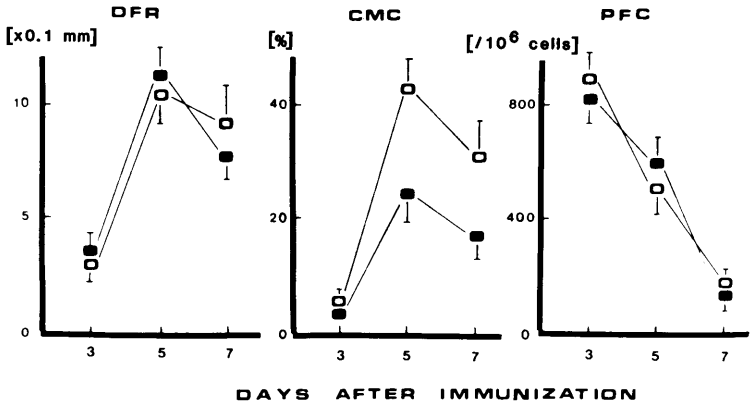

FIG. 3. Time course of rTNFα effect on the immunologic response. The mice were immunized ip with 10^8 CRBC and injected iv with 6000 U/mouse of rTNFα (□) on the same day. Control mice received saline (■). DFR, CMC, and PFC were assessed on Days 3, 5, and 7. The results represent the mean value of six to eight animals and standard error. CMC in rTNFα-treated mice was significantly increased on Days 5 ($P < 0.01$) and 7 ($P < 0.02$) compared to the immunized controls.

augmented with rTNFα. A dose of 2.4×10^4 U/mouse of rTNFα induced three times higher CMC than that of normal immunized controls.

In order to examine the effect of rTNFα over a broader period of time, immune responses were assessed 3, 5, and 7 days after immunization and rTNFα treatment. The increase in CMC occurred on Days 5 and 7 with maximum increase on Day 5 (Fig. 3).

The timing of administration of rTNFα. The effect of timing of administration of rTNFα was investigated in relation to the day of immunization. The mice were immunized with CRBC on Day 0. A dose of 6 $\times 10^3$ U/mouse of rTNFα was administered on Days −4, −1, 0, +1, and +4 in relation to immunization. The immune reactions were assessed 5 days after immunization (Table I). DFR and PFC were not affected by the timing of administration of rTNFα. The administration of rTNFα on or after the day of immunization induced augmentation of CMC, but not on the days before immunization. Thus, the administration of rTNFα at or after the time of immunization enhanced CMC.

Role of nonadherent and Thy 1.2, Lyt 2.2 positive cells. The augmentation of CMC by rTNFα was found to be caused by the popu-

TABLE I. TIMING OF ADMINISTRATION OF rTNFα[a]

Immunization	Days of treatment	Dose of TNF (U/mouse)	DFR (×0.1 mm)	CMC (%)	PFC (10^6 cells)
−	—	0	0.2 ± 0.1	2.0 ± 0.1	5 ± 2
+	—	0	11.2 ± 0.9	20.1 ± 1.8	597 ± 51
+	−4	6000	11.0 ± 1.2	23.9 ± 2.7	476 ± 40
+	−1	6000	12.3 ± 0.6	23.2 ± 3.7	525 ± 28
+	0	6000	9.8 ± 0.8	37.4 ± 4.5[b]	557 ± 54
+	+1	6000	10.7 ± 1.5	34.7 ± 3.8[c]	546 ± 41
+	+4	6000	8.7 ± 0.7	32.5 ± 3.1[d]	587 ± 61

[a] The mice were immunized ip with 10^8 CRBC on Day 0. rTNFα (6000 U/mouse) was administered on Day −4 or −1 before immunization, on Day 0 of immunization, or on Day +4 or +1 after immunization. DFR, CMC, and PFC were assessed 5 days after immunization. The results represent the mean value of six to eight animals and standard error.

[b] $P < 0.001$, compared to the immunized controls.

[c] $P < 0.01$, compared to the immunized controls.

[d] $P < 0.01$, compared to the immunized controls.

TABLE II. DEPENDENCE OF CMC AUGMENTATION ON THE NONADHERENT AND Thy 1.2, Lyt 2.2 POSITIVE CELLS[a]

Immunization	TNF treatment	Treatment of spleen cells	CMC (%)
−	−	None	1.8 ± 0.3
+	−	None	17.0 ± 2.4
+	+	None	36.3 ± 3.5[c]
+	−	Nonadherent	20.9 ± 1.4
+	+	Nonadherent	41.8 ± 3.7[d]
+	−	Anti-Thy 1.2 + C[b]	7.1 ± 1.2[e]
+	+	Anti-Thy 1.2 + C	10.2 ± 1.3[f]
+	−	Anti-Lyt 2.2 + C	8.2 ± 1.7[e]
+	+	Anti-Lyt 2.2 + C	10.5 ± 2.5[f]

[a] The mice were immunized with 10^8 CRBC and injected iv with rTNFα (6000 U/mouse). CMC was assessed 5 days after immunization.
[b] Complement.
[c] $P < 0.005$, compared to the immunized controls.
[d] $P < 0.001$, compared to the immunized nonadherent controls without treatment of TNF.
[e] $P < 0.005$, compared to the immunized controls.
[f] $P < 0.01$, compared to the immunized TNF-treated mice.

lation of spleen cells which did not adhere to the plastic dishes. This effect was diminished by treatment with anti-Thy 1.2 or anti-Lyt 2.2 antibody and complement (Table II).

Effect of anti-rTNFα antibody on the augmentation of CMC by rTNFα. A neutralizing anti-rTNFα monoclonal antibody (18) was used to establish that the augmentation of CMC was caused by rTNFα. Recombinant tumor necrosis factor alpha, given with control mouse ascites fluid, increased CMC, while rTNFα treated with monoclonal antibody failed to augment CMC. The monoclonal antibody alone did not affect CMC (Table III).

Discussion. The antitumor activity of native TNFα has been described by many investigators in murine and human tumor cells (1, 9, 19, 20). It was found to lack species specificity (9, 21, 22). The recombinant human TNFα also showed the antitumor effect of murine and human tumors, both *in vitro* and *in vivo* (23–26). Human recombinant TNFα caused regression of murine sarcoma-180 transplanted into BALB/c nu/+ mice (Fig. 1). In BALB/c nu/nu mice, however, a higher dose of rTNFα was necessary that in nu/+ mice, to obtain the antitumor effect. Similarly, Haranaka and his coworkers (9) had reported a difference in the curative effects of murine TNFα on the Meth A sarcoma in BALB/c nu/+ mice and nu/nu mice. The effect was stronger in BALB/c nu/+ mice than in nu/nu mice after a single iv injection of murine native TNFα. This finding was consistent with our results using recombinant human TNFα (Fig. 1). Therefore, it appeared that rTNFα either induced or required a T-cell-dependent host factor for its effect. To further elucidate this relationship, the effect of rTNFα on the host immune responses was investigated.

In the present study, CRBC were used for immunization since this antigen could induce DFR, CMC, and PFC simultaneously. These immune reactions were reported to be

TABLE III. EFFECT OF ANTI-TNF ANTIBODY ON THE ENHANCEMENT OF CMC BY rTNFα[a]

Immunization	Treatment	CMC
−	—	1.0 ± 0.1
+	—	19.9 ± 1.7
+	Ab	18.9 ± 1.5
+	TNF	45.7 ± 3.8[b]
+	TNF + Ab	23.4 ± 2.7[c]

[a] rTNFα (6×10^4 μg/ml) was incubated at 37°C for 1 hr with the ascitic fluid (×100) containing monoclonal antibody against rTNFα, or the control ascitic fluid, and then at 4°C for 30 min. This treatment neutralized 99% of rTNFα activity. The mice immunized with 10^8 CRBC were injected iv with rTNFα (6000 U/mouse), antibody (Ab), or rTNFα + Ab on the day of immunization. CMC was assessed 5 days after immunization.
[b] $P < 0.001$, compared to the immunized control.
[c] $P < 0.005$, compared to the immunized TNF-treated mice.

T-cell-dependent or controlled by different T-cell populations (12, 14, 16). Recombinant TNFα was shown to augment the CMC to CRBC but had no effect on DFR and PFC in optimal immunization procedures. The increased CMC, induced by rTNFα, was mediated by nonadherent Thy 1.2, Lyt 2.2 positive cells (Table II).

Endotoxin (lipopolysaccharide) has been shown to modulate host immune response, including DTH, PFC, and CMC (27–30). To ensure that the effect of TNFα was not due to endotoxin contamination in rTNFα preparation, the anti-TNFα monoclonal antibody was used to neutralize rTNFα activity. It was found that neutralization of rTNFα with the monoclonal antibody abolished the augmentation of CMC (Table III), suggesting that rTNFα is the molecule responsible for CMC augmentation. Although endotoxin contamination in TNF preparation was small, there is another possibility that the augmentation of CMC might be due to the release of endogenous TNFα by endotoxin as well as the previous studies which showed the modulation of host immune responses. TNFα is known to be one of the endogenous mediators of endotoxin (31). However, it is unlikely that endogenous TNF production plays a role in CMC development, because anti-TNFα monoclonal antibody failed to abolish CMC generation to CRBC (Table III).

Recently, it was reported that TNFα increased the expression of HLA-DR antigens and IL-2 receptors on activated T cells (6). As a result, TNFα-treated T cells show an enhanced proliferation response to IL-2 (6). Ranges *et al.* (8) reported that TNFα augmented cytotoxic T-lymphocyte responses *in vitro*. This fact agreed with our studies *in vivo*. The role of TNFα in immunoregulation is important in understanding the mechanisms of action of TNFα.

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