In Vivo Administration of Tumor Necrosis Factor-α is Associated with Antiviral Activity in Human Peripheral Mononuclear Cells (43236)

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Abstract. **Tumor necrosis factor-α (TNF-α) has a spectrum of biologic effects and has been shown to exert antiviral effects in fibroblasts *in vitro*. The *in vivo* administration of TNF-α (40–160 μg/m^2 intravenously over 2 hr) and its effects on vesicular stomatitis virus (VSV) replication in peripheral blood mononuclear cells (PBMC) from patients with malignancy was investigated. Blood was obtained before, during, and after infusion. The PBMC were separated and infected with VSV at a multiplicity of infection of 0.005 plaque-forming units/cell and virus yields were determined 72 h later. The TNF-α inhibited VSV yields by as much as 99% in a dose-dependent manner with the inhibition initially observed during the first hour of infusion. Despite a rapid reduction in TNF-α serum levels, the higher doses still produced antiviral effects 4 hr after the infusion. Sera obtained at identical times had no interferon activity. Human γ-interferon (25 μg/ml) added *in vitro* augmented the TNF-α-induced inhibitory activity in both magnitude and duration. Percentages of lymphocytes and monocytes in peripheral blood were reduced at 4 hr after TNF-α administration and the monocyte to lymphocyte ratio was diminished and temporally coincided with the loss of TNF-induced antiviral state. These data suggest that the *in vivo* administration of TNF has a direct inhibitory activity on VSV replication in human peripheral blood mononuclear cells that was enhanceable by γ-interferon and possibly monocyte mediated.** [P.S.E.B.M. 1991, Vol 197]

Tumor necrosis factor (TNF) is a cytokine that is primarily produced by cells of the monocyte/macrophage lineage (1, 2). It was originally recognized for its cytotoxic and antitumor properties (3, 4), but has been shown to have a spectrum of biologic effects. In addition to its ability to kill tumor cells, TNF has been shown to regulate macrophage-mediated cytotoxic effects (5), cell proliferation (6, 7), induction of interleukin 1 (8), and induction of granulocyte/monocyte colony-stimulating factor (9) and has also been reported to have some inhibitory effects on parasites (10, 11) and viruses (12–14).

TNF can be induced *in vitro* in response to lipopolysaccharide, mitogens including phytohemagglutinin, bacteria, phorbol esters, calcium ionophores, and viruses. In vivo induction can also be elicited by bacterial endotoxin, bacille Calmette-Guerin, and in response to infections with certain microorganisms, including viruses and protozoa (3, 10, 15, 16).

Recently, Kohase *et al.* (13) and Mestan *et al.* (12) showed that human recombinant TNF-α had an *in vitro* antiviral activity against encephalomyocarditis virus and herpex simplex virus in cultures of human fibroblasts. Moreover, Wong and Goeddel (14) demonstrated that TNF has an antihuman immunodeficiency virus activity *in vitro*, in HUT 78 cells, that was enhanceable by γ-interferon (17). Although initially it was thought that the TNF-induced antiviral effects were mediated by B cell-stimulating factor (B cell-stimulating factor 2)/β_2-interferon (13), additional evidence suggests that these effects are mediated via β-interferon (18).

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In light of the reported antiviral effects for TNF *in vitro*, in this study the antiviral effects of the *in vivo* administration of human TNF on vesicular stomatitis virus (VSV) replication in peripheral blood mononuclear cells (PBMC) from eight cancer patients were examined.

Materials and Methods

Patient Population. Patients included in this study were part of a clinical phase I study of TNF-α. They were all patients with solid tumors that had not responded to other regimens. The patients received only supportive medication in the 3 weeks before the initiation of therapy. Eight patients participated in the study, five males and three females; their mean age was 57 years and their mean weight was 72 kg. Recombinant TNF-α, provided by Knoll Pharmaceuticals, Whippany, NJ, was administered intravenously in a 2-hr infusion, in doses of 40, 80, or 160 $\mu g/m^2$.

Virus, Cells, and Virus Yield Assays. Heparinized blood was drawn before, during, and at selected times after TNF administration. The PBMC were then separated by the standard Ficoll-Hypaque technique. The PBMC (1×10^6, in duplicate) were infected with VSV at a multiplicity of infection of 0.005 plaque-forming units/cell in 1 ml of RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and L-glutamine (2 m*M*). Infected cells were incubated in 24-well plates in the presence or absence of 25 units of human recombinant γ-interferon (Genzyme, Boston MA), and recombinant α_2-interferon (Hoffman La-Roche, Nutley NJ), or recombinant β_{ser} interferon (Triton Biosciences, Almeda CA) and were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. VSV was harvested at 72-hr postinfection by two rounds of freezing and thawing at −70°C. VSV yields were determined by a virus yield plaque assay using WISH cells. Briefly, 50 μl of serial 10-fold dilutions were used to infect WISH cells, in quadruplicate, in 96-well tissue culture microtiter plates. After a 1-hr adsorption, the virus was aspirated and the plates were washed twice with medium and then monolayers were overlayed with Eagle's minimal essential medium with 2% carboxymethocellulose, 2% fetal calf serum, and 0.225% sodium bicarbonate. Plates were incubated in a CO_2 incubator described as above for 24 hr, the overlay was decanted, and cells were stained with 1% aqueous crystal violet for 30 min. Plates were then washed under running water and air dried. Plaques were scored using an inverted microscope.

TNF-α Enzyme Immunoassay. A specific sandwich enzyme-linked immunoabsorbent assay was used to monitor serum levels of biologically active TNF-α in patients. For that, a 96-well microtiter plate (Nunc) was coated overnight with 0.1 ml/well of anti-TNF-α mAb 199 (0.005 mg/ml) in phosphate-buffered saline (PBS). Postcoating was done with 1% bovine serum albumin in PBS for 0.5 hr at 37°C. The plate was washed five times with PBS containing 0.1% Tween 20 (PBS-T). For a standard curve, TNF-α was diluted in 20 m*M* Tris-HCl, 150 m*M* NaCl, and 1% bovine serum albumin (pH 7.4). TNF-α containing medium or serum (0.1 ml/well) was reacted for 3 hr at room temperature or overnight at 4°C. After washing, 0.1 ml of biotinylated anti-TNF mAb 195 (0.001 mg/ml)/well was added and incubated for 2 hr at room temperature. After three washes with PBS-T, 0.1 ml of 10,000-fold diluted streptavidin-peroxidase complex (Boehringer Mannheim)/well was added. After incubation for 0.5 hr at room temperature and washing with PBS-T, 0.1 ml of 0.42 m*M* 3,3′5,5′-tetramethylbenzidine (Boehringer Mannheim) in 0.1 *M* acetate-citrate buffer (pH 4.9) containing 0.004% H_2O_2 was added. The reaction was stopped after 10 min by the addition of 2.0 *N* sulfuric acid and the absorbance at 450 nm of each well was determined using an SLT Easy Reader.

The enzyme-linked immunoabsorbent assay has a detection limit of 5–10 pg/ml (two times the standard deviation) and a linear range up to 300 pg/ml. The specificity was demonstrated by the lack of any signal generated by 10 μg/ml TNF-β, γ-interferon, and interleukin 2.

Results

Pharmacokinetics of Intravenously Administered TNF. Pharmacokinetics of TNF were measured during intravenous infusions at three different dosages, 40, 80, and 160 $\mu g/m^2$. Serum samples at 1 hr into the infusion, at the end of infusion, and at selected times thereafter were tested for TNF by enzyme immunoassay. Data for six patients are presented in Figure 1. The peak of serum TNF was observed either at 1 or 2 hr during the infusion, then decreased gradually after the

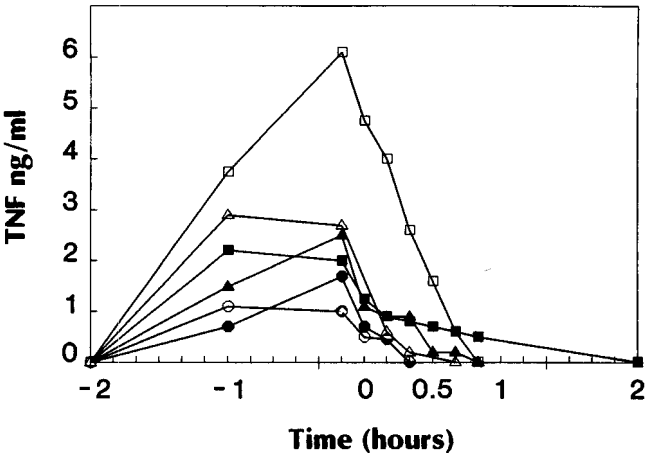

Figure 1. TNF serum levels following a single dose of 40 $\mu g/m^2$ (■, ▲, ○, ●), 80 $\mu g/m^2$ (△), and 160 $\mu g/m^2$ (□); levels were determined by enzyme-linked immunoabsorbent assay. TNF was administered in a 2-hr infusion, 0 hr marks the end of the infusion.

end of the infusion. With the exception of one patient by 1 hr after the end of infusion, TNF was no longer detected, regardless of the dose. The serum levels of TNF appeared to be dose dependent with the patient who received a dose of 160 $\mu g/m^2$ having had the highest blood level (6 ng/ml) followed by the 80 $\mu g/m^2$ and finally the 40 $\mu g/m^2$. The data from Figure 1 reflect the rapid elimination of TNF and/or increased tissue distribution.

Effect of TNF on VSV Replication in PBMC. The PBMC obtained from patients at selected time intervals before, during, and after TNF infusion were infected with VSV at a multiplicity of infection of 0.005/cell, and virus yields were determined 72 hr later. Virus yields were inhibited as early as 1 hr into the infusion when compared with VSV yields from PBMC obtained from the same patients before the administration of TNF (Table I). The percentage of inhibition of VSV yield at TNF doses of 40, 80, and 160 $\mu g/m^2$ for 1 hr during the infusion was 94, 96, and 98% respectively. Although the 40 $\mu g/m^2$ dose of TNF did not inhibit virus replication by 30 min after the end of infusion, the antiviral effects produced by the 80- and 160-$\mu g/m^2$ doses extended through 4 hr after the end of infusion. These data suggest that the *in vivo* administration of TNF confers antiviral protection to PBMC against *in vitro* viral infection.

Since interferon (IFN) had been shown previously to enhance the TNF-induced antiviral activity, various IFN preparations were added to the *in vitro* cultures. Because of the limited number of PBMC obtainable from single patients, different interferons were tested on PBMC from different subjects. Figure 2 illustrates that 25 units of IFN-γ significantly enhanced the TNF-induced antiviral activity in PBMC from patients infused with 80 and 160 $\mu g/m^2$. The combined antiviral effect was more than that induced by either TNF or IFN-γ alone. Recombinant IFN-α and β_{ser} had similar effects, also potentiating the TNF-induced antiviral effects (Fig. 3).

Table I. Effect of TNF-α on Vesicular Stomatitis Virus Replication in PBMC[a]

Sample	Dose ($\mu g/m^2$)	% Inhibition of virus yield[b]	Fold inhibition[a]
1 hr into infusion	40	93.68	11.4
	80	95.81	46.7
	160	97.89	54.7
½ hr postinfusion	40	39.47	6
	80	96.99	34
	160	98.96	122
4-hr postinfusion	40	4, −1180, −335	0.3
	80	55.75	1.8
	160	−8, 78.6, 97.1	5.6

[a] TNF was administered in a single 2-hr infusion at doses of 40, 80, or 160 $\mu g/m^2$. At selected times PBMC from patients were infected with VSV and VSV yield was determined 72 hr later.
[b] Geometric mean.

The antiviral effects induced by TNF lasted for approximately 4 hr after the infusion. Incubating the cells at 37°C for 24 hr before infecting them with VSV did not affect the pattern or magnitude of the TNF-induced effects (Fig. 2D). If the TNF-induced effects were IFN-mediated then a prolonged antiviral state would be expected in PBMC that were infected 24 hr later (19). Moreover, no interferon activity was detected in sera from those patients through 24 hr following TNF administration as determined by radioimmunoassay (using IFN-γ radioimmunoassay kits from Centocor Inc., Malvern, PA, and IFN-α radioimmunoassay kits from Novo Biolabs, Danbury, CT). Incubating PBMC with 10 units of IFN-β/ml for 2 hr, then removing it, and further incubating the cells for another 24 hr before challenging them with VSV reduced the viral yield by 1.5 log (data not shown). This argues against the fact that the TNF-induced antiviral effects in PBMC was IFN-β mediated. If the TNF antiviral effects had been mediated by IFN-β, then the antiviral state should have extended to 24 hr after exposure to TNF. These data indicate that the TNF-induced antiviral activity appeared not to be mediated by IFN.

Lymphocyte/Monocyte Involvement in TNF-Induced Effects. The profiles of the white blood cells counts of the patients under study are shown in Table II. TNF did not significantly change the total cell counts in peripheral white blood cells when measured at 4 hr after the end of the TNF infusion. However, in five of eight patients the percentage of lymphocytes and monocytes was drastically reduced and was compensated for by an increase in the percentage of neutrophils.

Since the percentages of lymphocytes and monocytes were reduced and both cells are represented in the PBMC fraction of the patients' blood, it is thus possible that a relative change in the ratio of both cells to each other during the course of TNF treatment might influence the TNF-induced effects. Table III shows the ratio of lymphocytes to monocytes before TNF administration and 4 hr after the end of the infusion. TNF increased the lymphocyte to monocyte ratio. The increase appeared to be dose dependent, as it was more pronounced with the 160-$\mu g/m^2$ TNF dose. The relative increase in the lymphocyte to monocyte ratio was less prominent at the 80 $\mu g/m^2$ of TNF, and there was no significant change at the 40-$\mu g/m^2$ dose. The relative change in cell ratio temporally coincided with the loss of the antiviral effect induced by TNF, suggesting a possible relationship between these two phenomena.

Discussion

TNF-α, a lymphokine produced primarily by cells from the monocyte/macrophage lineage, has been shown to mediate a number of biologic activities (20).

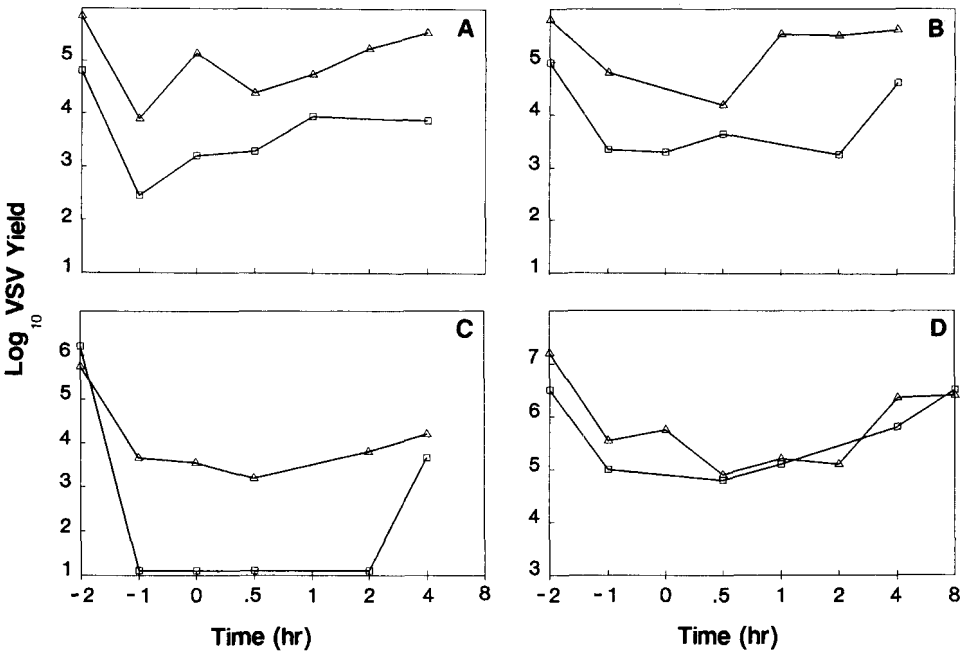

Figure 2. Effect of *in vivo* administration of TNF on VSV replication in PBMC from four different patients. TNF, 80 $\mu g/m^2$ (A and B) or 160 $\mu g/m^2$ (C and D), was administered during a 2-hr infusion. PBMC obtained before, during, and after TNF administration were infected with VSV in the absence (△) or presence (□) or 25 units of human IFN-γ; VSV yields were determined 72 hr later. Cells (D) were either infected directly with VSV (△) or incubated first for 24 hr before they were infected (□).

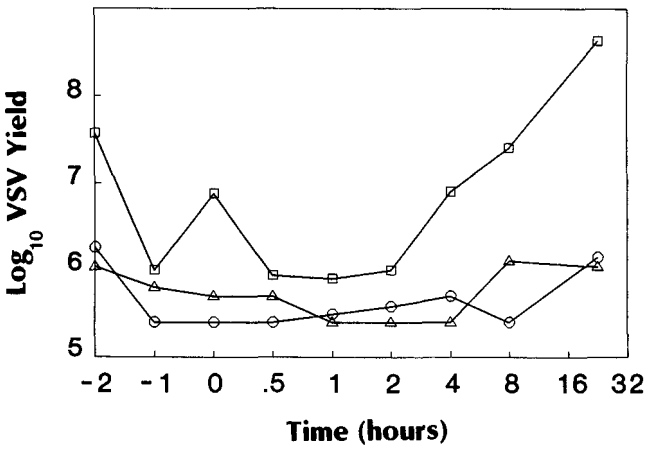

Figure 3. Effect of IFN-α and IFN-β on the TNF-induced antiviral effect. Experimental procedure is the same as that described for Figure 2. IFN were added *in vitro* following VSV infection; TNF alone (□), TNF with IFN-α (○), and TNF with IFN-β (△).

Table II. White Cell Count Profiles for Patients on TNF

Patient	Dosage ($\mu g/m^2$)	Total white count[a] (×1000)		Neutrophil[b] (%)		Lymphocytes[b] (%)		Monocytes[b] (%)	
		Pre-	4 hr	Pre-	4 hr	Pre-	4 hr	Pre-	4 hr
1	160	4.3	4.3	68	89	16	9	10	1
2	160	8.0	10.6	77	98	19	2	3	0.1
3	160	3.3	.3	45	89	19	9	27	1
4	80	13.3	12.3	83	69	6	18	5	6
5	80	5.8	7.3	80	96	7	2	11	2
6	40	5.9	6	81	81	13	13	4	4
7	40	4.4	8.4	60	95	33	2	8	1
8	40	7.8	11.2	69	69	25	25	4	4

[a] Normal range of total white cell count ranges from 2.5×10^3 to 10.5×10^3.

[b] White cell count profiles from normal individuals for neutrophils are 37–76%, for lymphocytes 25–55%, and for monocytes 0–10%. Note there was a pronounced decrease in lymphocyte and monocyte percentages in five of eight patients after TNF administration. This was compensated for by an increase in the percentage of neutrophils.

Among those is the recently described antiviral activity. TNF-induced antiviral activity was demonstrated *in vitro* in fibroblasts, against encephalomyocarditis and herpes simplex virus (12, 13), in a human leukemic cell line HUT 78 against human immunodeficiency virus (17), and in a renal carcinoma cell line against VSV, encephalomyocarditis, and herpes simplex virus (14). The data presented in this study demonstrate that the *in vivo* administration of recombinant TNF-α was associated with the production of an antiviral activity in human PBMC, and that the antiviral state extended beyond the time frame in which TNF was detected in patients' sera.

In human fibroblasts *in vitro*, it appears that TNF exerts its antiviral activity through induction of β-interferon (18). Reis *et al.* (18) showed that the antiviral activity induced by TNF in human fibroblasts was neutralized by monoclonal antibodies specific to IFN-β but not by rabbit antibodies against recombinant B

Table III. Relative Changes in Lymphocyte to Monocyte Ratios after TNF infusion[a]

Patient	Dose ($\mu g/m^2$)	Lymphocyte/monocyte	
		Pre-	4 hr
1	160	1.6	9
2	160	6.3	20
3	160	0.7	9
4	80	1.2	3
5	80	0.6	1
6	40	3.3	3.3
7	40	4.1	2
8	40	6.1	6.1

[a] TNF increased the lymphocyte to monocyte ratios in a dose-dependent manner.

cell-stimulating factor 2. However, others have shown that the TNF-induced antiviral activities were not exclusively mediated by IFN-β (12). Using anti-IFN-α or anti-IFN-γ, Mestan *et al.* (12) did not affect the TNF-mediated antiviral activities of TNF, whereas anti-IFN-β partially reversed the TNF effects. Complete blocking of the TNF effects was not possible even with high doses of anti-IFN-β, suggesting a direct role of TNF itself in the induction of the antiviral state. The latter study, however, was conducted in a human laryngeal carcinoma cell line. The same investigators and others have demonstrated that there is a selective cytolytic effect of TNF on virus-infected cells (12, 21) that was accelerated by γ-interferon (17, 22). In the present study, the antiviral activity induced by TNF appears not to be mediated by IFN. Since no interferon activity was detected in the patients' sera, the antiviral state was short-lived and the antiviral state was not affected by prior incubation of the cells at 37°C for 24 hr, allowing an optimal IFN-induced antiviral state to develop before infecting the cells with VSV. Moreover, IFN-β is not known to be produced by peripheral blood mononuclear cells. Thus, in this system the TNF-induced antiviral state would appear to be due to TNF alone and not due to the combined effect of TNF and TNF-induced IFN-β as observed in other systems. This would explain the short-lived antiviral state observed in PBMC in contrast to that reported in fibroblast and renal and laryngeal carcinoma cell lines *in vitro*.

The short-lived antiviral state had a temporal correlation with the decreased percentages of the lymphocytes and monocytes in peripheral blood. However, the ratio of monocytes to lymphocytes at 4 hr after the end of the TNF infusion was decreased in a dose-dependent manner, suggesting a possible role for monocytes in the TNF-induced antiviral effect. It is possible that the *in vivo* administration of TNF exerts its antiviral function by first activating the monocytes, and the latter selectively kill infected cells, or by possibly inducing the release of other monokines that would in turn activate cytotoxic T cell activities against virus-infected cells. Alternatively a synergistic action between TNF and IFN-γ, produced constitutively from lymphocytes at low concentrations beyond the sensitivity of the assays used for IFN detection, might explain the antiviral state induced by TNF. Despite the disappearance of the antiviral state induced by TNF at 4 hr, it is possible that the antiviral activity still existed in the subjects due to possible margination of the monocytes to the tissues from the peripheral blood.

The enhancing effects of IFN-γ on the *in vivo* TNF-induced antiviral activity is not surprising since similar interaction between both lymphokines in other *in vitro* systems has been reported (23). In addition, IFN-γ enhances synergistically the antiproliferative activity induced by TNF (24) and induces tumoricidal activity of resident peritoneal macrophages *in vitro* (5). The combination of both lymphokines might thus prove useful in combination therapy for malignant neoplasia not responding to conventional methods of chemotherapy or theoretically against virus infections. This combination could possibly be limited however by *in vivo* toxic reactions.

The assay system described in this report might be useful in the evaluation of *in vivo* induction of an antiviral state that might be induced by certain lymphokines, immunomodulators, and antiviral agents in humans.

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