Journal of Cellular Biochemistry 36:353–367 (1988)
Growth Regulation of Cancer 121–135

Human Cytokines, Tumor Necrosis Factor, and Interferons: Gene Cloning, Animal Studies, and Clinical Trials

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Presented is a comprehensive program designed to isolate human cytokine genes and investigate their relative induction, and to analyze cytokine activities in cell culture, animal tumor models, and human clinical trials. Human cytokine cDNAs have been isolated from a cDNA library made from normal human peripheral blood leukocytes (PBLs) treated with Sendai virus and the relative induction of tumor necrosis factor (TNF), alpha and gamma interferons (IFN-α, IFN-γ), and interleukin-1 beta IL-1β) genes has been analyzed. In the Sendai virus-induced PBL system, IL-1β mRNA was shown to be approximately twofold higher than TNF or IFN-α mRNA whereas IFN-γ mRNA was 50–100-fold lower than TNF or IFN-α mRNA. The cytotoxic activity of TNF was analyzed on several cell lines and IFN-α and IFN-γ were shown to potentiate TNF cytotoxicity about 2–200-fold depending on cell lines. The LD_{50} for recombinant TNF in BALB/c mice was determined to be 6×10^7 U/kg and the therapeutic dose of recombinant TNF in sarcoma 180 bearing BALB/c mice was 3×10^5 U/kg, indicating a wide therapetic index. Phase I clinical trials of recombinant TNF given I.V. indicated a tolerated dose of 150,000 U/kg with biphasic half-life (T-1/2) of 2 and 31 min following TNF injection. Phase II trials of TNF and trials of TNF combined with IFN-α are in progress. These studies indicate that cytokines such as TNF and IFN-α are subject to similar induction systems, potentiate each other's activities, and can be tolerated at specific doses for potential therapeutic use.

Key words: TNF, IFN, genes, clinical use

Cytokines are proteins involved in the modulation of the immune system [1] and are the targets of intense investigation due to their potential use for increasing our understanding of the immune system and for help in the development of new

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Received April 12, 1987; revised and accepted October 7, 1987.

drugs. Some cytokines permit the activation of key immune cells such as T-lymphocyte activation by interleukin-1 (IL-1) and B-lymphocyte activation by B-cell differentiation factor (BCDF) [1]. Other cytokines function as growth factors, such as interleukine-2 (IL-2), which is the growth factor for T-lymphocyte helper and killer cells, or B-cell growth factor (BCGF), which is involved in the proliferation of B-lymphocytes [1]. Several cytokines have cytotoxic activities, such as tumor necrosis factor (TNF), which is produced by activated macrophages and has been shown to be cytotoxic for various tumor cell lines [2]. Cytokines such as alpha interferon (IFN-α) are produced by peripheral blood leukocytes (PBLs) and induced by Sendai virus, as well as by a large array of other inducers [3]. IFN-αs have potent antiviral activity and antiproliferative activities against a variety of tumor cell lines [3] and have recently been licensed as the treatment of choice for hairy cell leukemia.

Due to the occurrence of low levels of natural cytokines either circulating in patients with various diseases or in human cell culture systems, alternative methods have been required to produce cytokines cost-effectively. Recombinant DNA technology [4] has permitted the isolation of human cytokine genes and large-scale production of the respective proteins. Human IFN-α genes have been isolated by a number of investigators from cDNA libraries [5,6] and human genomic libraries [7,8]. We have isolated a novel human IFN-α gene, designated IFN-αWA, from a human genomic library by using 17-base oligonucleotide probes [8–10]. IFN-αWA contains differences at five amino acid positions which are conserved for all other IFN-α genes. The IFN-αWA gene appears to be a natural hybrid between IFN-αF at the 5′-end and IFN-αH at the 3′-end [10]. Human TNF cDNA was isolated from tumor cell lines HL-60 [11,12] and U-937 [13], and the human TNF gene was initially isolated from a human genome library by using rabbit TNF cDNA as a probe [14]. We have isolated TNF cDNA from normal human peripheral blood leukocytes (PBLs) induced with Sendai virus [15–17].

Since the immune system involves a network of different cytokines, we have been interested in the availability of all the known cytokines as well as new ones. Such availability of various cytokines is critical for our studies since we believe that although some cytokines may have therapeutic use when delivered alone, many cytokines will function best in combination. Our approach has been to establish human cDNA libraries which are screened for known cytokines and stored on filters for future screening. We have created a cDNA library from human PBLs induced with Sendai virus. From this cDNA library we have isolated several cytokine cDNAs such as TNF [15–17], IFN-α, IFN-β, and IL-1β cDNAs. In addition, we are screening the cytokine-enriched cDNA libraries with cytokine cDNA probes as well as oligonucleotide probes by using various hybridization strategies so as to isolate related but new cytokines.

To facilitate cytokine cDNA isolation and analysis of cytokine activities, we have had the advantage of a comprehensive cancer research center which includes several critical capabilities. The blood bank at Wadley has been a source of PBLs which have been resource material for the molecular genetics department, which has isolated cytokine cDNAs. The departments of molecular genetics and immunology have collaborated to study cytokine expression and activities in tumor cell cultures and animals. The fermentation facility has permitted large-scale production of cytokines from genetically engineered bacteria and yeast. Wadley's Granville C. Morton Cancer and Research Hospital has permitted the clinical trials of the respective cytokines.

This paper describes the isolation of the cytokine cDNAs, the analysis of their induction by Sendai virus, and the testing of the TNF on cells, animals, and human clinical trials, alone and in combination with IFN-α. Hence, these studies relate to the analysis of the cytokine network and their potential therapeutic use.

METHODS AND RESULTS

Human Peripheral Blood Leukocyte cDNA Library

We have established a human peripheral blood leukocyte (PBL) cDNA library which has been utilized for the isolation of various human cytokine cDNAs. The human PBLs were cultured according to the method of Cantell et al. [18] and induced with Sendai virus. As indicated in Figure 1, PBL cultures containing approximately 10^7 cells/ml were primed with 100 U/ml of IFN-α at −2 hr. Sendai virus was added at 0 hr to a concentration of 200 hemagglutination U/ml. The PBL cultures were incubated at 37°C. Either cells were centrifuged at specified times and processed for RNA isolation, or supernatants were assayed for cytokine activities. Poly(A)$^+$ RNA was isolated from PBL cultures induced for 4 hr and cDNA was synthesized. In addition, poly(A)$^+$ RNA was stored at −70°C. The cDNA was transformed into *E. coli* and approximately 30,000 colonies were generated on filters [17].

Transformed colonies were screened on filters for IFN-α genes by using the IFN-αWA gene [8] as a probe. At least 15 colonies hybridized with the IFN-α probe and more than half of these clones appeared to contain IFN-αD based on hybridization and restriction analysis. One clone which was fully characterized was shown to contain IFN-αD by DNA sequence analysis and was used in experiments shown in

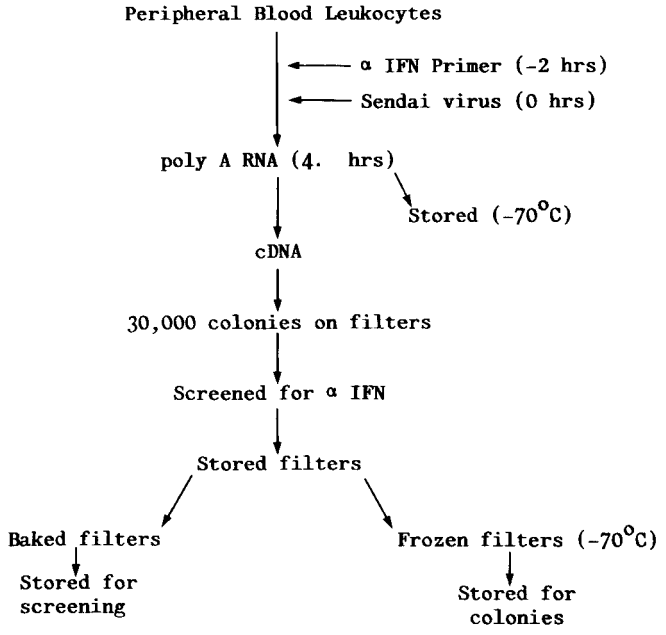


Fig. 1. The preparation and induction of peripheral blood leukocytes (PBLs), the synthesis and screening of cDNA, and the storage of filters were done as previously reported [17].

Figure 3. Duplicate filters, one containing transformed bacterial colonies and the other containing replica colonies baked at 80°C for screening the IFN-α genes, were stored at −70°C [17].

PBL poly(A)$^+$ RNA, isolated 4 hr after induction by Sendai virus, was subsequently removed after more than 12 mo of storage at −70°C and tested for hybridization by oligonucleotide probes complementary to the mRNA for several cytokines, including TNF cloned from leukemia cell line HL-60 [11,12]. No meaningful signals were observed for IFN-γ, IL-2, and lymphotoxin, whereas strong hybridization with the TNF probe was observed. Subsequently, the filters stored at −70°C which contained the cDNA library made with the 4-hr poly(A)$^+$ RNA were screened for TNF cDNA. Five positive clones were obtained after screening approximately 30,000 clones with the 17-base TNF probe [15–17]. The largest cDNA insert, pBR322-TNF1, was identical in sequence to the mature TNF obtained from leukemia cell lines HL-60 [11,12] and U-937 [13] which were induced with phorbol esters and endotoxin [17]. Screening of this same cDNA library with corresponding oligonucleotide probes also yielded IFN-β cDNA and IL-1β cDNA clones.

The TNF cDNA was ligated with an expression vector containing a P_L promoter, a gene for the heat-sensitive repressor, and a consensus ribosomal binding site as indicated in Figure 2. The resulting expression vector pUC9-PL1-TNF1 was transformed into *E. coli* and the 17,300-dalton TNF was induced after a shift in temperature from 30°C to 42°C. Up to 3 × 10^6 U/ml of cytotoxic activity was obtained when cultures were grown for 3 hr after the shift from 30°C to 42°C at

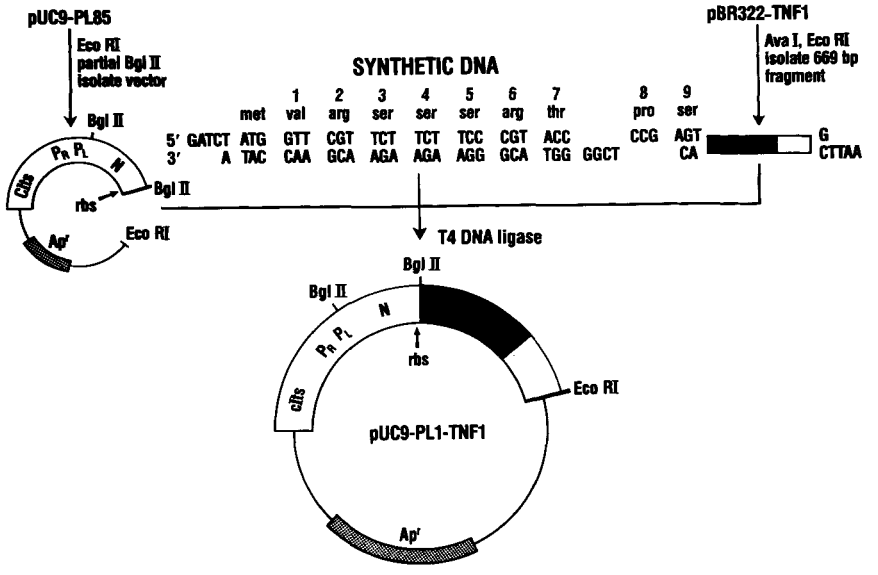
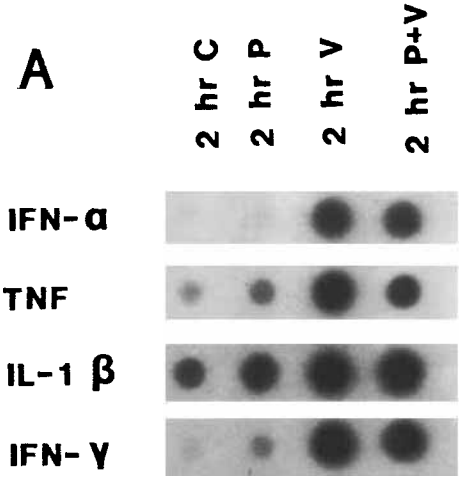


Fig. 2. Construction of a human TNF cDNA plasmid for expression in *E. coli*. An AvaI-EcoRI fragment containing the TNF cDNA was linked to an expression vector containing the bacteriophage P_L promoter, a gene for a temperature-sensitive repressor (cIts), and a consensus ribosome-binding site (rbs) by two complementary synthetic oligonucleotides. Black and white bars of the pBR322-TNF1 fragment represent portions of the structural gene and 3′-untranslated region, respectively. Regions surrounding the junction points were confirmed to be correct by nucleotide sequencing.



B

	20 hr C	20 hr P	20 hr V	20 hr P+V	
IFN-α	<4	29	42,000	34,000	(units/ml)
TNF	11	<1	552	147	
IL-1β	1	7.5	93	100	
IFN-γ	2	2	19	21	

Fig. 3. Lymphokine activity in cultured PBLs under various induction conditions. PBLs were collected from 40 healthy individuals and then processed and cultured as previously described [17]. At −2 hr, some cell cultures were primed (P) with 100 U/ml of leukocyte IFN (10^6 U/mg), and at 0 hr, cultures were either not treated (C) or were induced with 200 hemagglutination U/ml of Sendai virus (V). **A**: Dot blots of total RNA isolated at 2 hr were hybridized with ^{32}P-RNA probes (2×10^8 cpm/μg) synthesized from cDNAs of IFN-αD, TNF, IL-1β, or IFN-γ by using SP6 RNA polymerase or T7 RNA polymerase as described [17]. Exposure times (−80°C, two screens) were 1 hr (IL-1β, TNF, and IFN-α) and 64 hr (IFN-γ). **B**: Assays of lymphokine activities. Twenty hours after virus infection, cell cultures were centrifuged and supernatants were stored at −70°C and then assayed for lymphokine activity. IFN-α, IL-1β, and IFN-γ activities were determined from radioimmune assay kits obtained from Bethesda Research Laboratories, Cistron, and Centocor, respectively. TNF activity was measured (triplicate assays) in cytotoxic units by using L_{929} cells and standardized against an internal purified TNF [17].

OD_{600} = 2.5 [17]. One unit is the amount required to give 50% cytotoxicity in the standard assay utilizing L_{929} cells [19].

Induction of IFN-α, IFN-γ, TNF, and IL-1β.

We examined the induction of various cytokines in populations of mixed human PBLs in response to Sendai virus infection. We compared the levels of their expression to that of IFN-α, whose induction is well characterized in this system [3]. All lymphokines we have examined (IFN-α, TNF, IL-1β, and IFN-γ) are induced to varying levels in virus-infected PBLs, as determined by RNA blot hybridizations and measurements of the proteins present in culture supernatants (Fig. 3). Since the RNA hybridization probes for the four genes were of equal specific activities and approximately equal lengths, the hybridization signals obtained for the RNA samples under the indicated culture conditions could be compared directly. As shown in Figure 3A for the virus-infected cultures, IL-1β mRNA is approximately twofold higher than TNF or IFN-α mRNA, whereas IFN-γ mRNA is 50–100-fold lower. Previously, we established that TNF and IFN-α mRNAs typically represent about 0.11% of the total virus-induced PBL mRNA [17]. In a cDNA library prepared from PBLs harvested 4 hr after Sendai virus infection, we identified TNF clones as 0.05% and IL-1β clones as 0.3% of the total. These results indicate that IL-1β is transcribed at high levels in PBL cultures, although the activity present in the culture supernatants is relatively low. IL-1β apparently lacks a leader peptide, as determined by cDNA sequence analysis, and recent studies indicate that IL-1β may be primarily a membrane-bound protein [20].

Although IFN-γ induction by PBLs treated with Sendai virus is low, previous work claimed IFN-γ expression was attributed solely to the mitogenic or antigenic stimulation of select T-cell populationsd. IFN-γ mRNA in phytohemagglutinin-stimulated lymphocytes was detected at low levels 3 hr post induction and peaked at 15 hr [21] as compared to IFN-α and TNF mRNA levels, which peaked 4 and 2 hr postinfection, respectively, in PBLs [17]. We have also observed low but reproducible levels of IFN-α mRNA in induced PBLs (Fig. 3A) that peaked 3–4 hr after virus infection and was barely detectable at 20 hr (unpublished results). IFN-γ induction in a similar system has been previously reported but this response was attributed to a mixed leukocyte reaction and presumably was not virus mediated [22]. However, we detected IFN-γ mRNA at only extremely low levels in uninfected PBLs as compared to virus-induced PBLs (Fig. 3A), with the concomitant levels of secreted IFN-γ appearing in culture supernatants. PBL cultures treated with lipopolysaccharide (LPS) (10 μg/ml) also produced low levels four-fold lower than virus-induced PBLs) of IFN-γ mRNA and secreted protein (unpublished results). IFN-γ mRNA was also detected from PBLs cultured in the presence of IFN-α alone (Fig. 3A).

We addressed the effect of pretreating mixed PBLs with human IFN-α (i.e., priming) prior to the addition of Sendai virus. An enhancement of IFN production has been observed by other investigators in certain primed, induced cells or cell lines as compared to unprimed, induced cells (see ref. 3 for a review). No enhancement of IFN-α mRNA or IFN-α production was observed in primed vs. unprimed virus-induced PBLs (Fig. 3, unpublished results). This observation holds for both PBLs primed with crude IFN (10^6 U/mg) or highly purified IFN (2×10^8 U/mg). No general pattern of either increased or decreased induction was observed for primed

and induced PBLs, as compared to only induced PBLs, for the levels of mRNA, and secreted proteins of TNF, IFN-γ, or IL-1β.

Potentiation of TNF Cytotoxicity by IFN-α and IFN-γ

Recombinant human TNF produced at the fermentation facility at Wadley Institutes was purified to a specific activity of 1×10^7 U/mg and tested for cytotoxic properties. Recombinant TNF was added in different concentrations and incubated for 48 hr with different tumor cell lines, and the amount of TNF required to cause 50% cytotoxicity was determined by a dye uptake test (neutral red) using a Titertek multiscan reader [19]. As indicated in Table I, there was a range of TNF concentrations required to effect 50% cell kill depending on the cell lines tested. For example, the human breast tumor cell line BT-20 required 250 U/ml TNF for 50% kill whereas the human colon tumor cell line SK-CO-1 required 75,000 U/ml TNF for 50% kill. In fact, tumor cell lines of similar origin required different levels of TNF for 50% kill such as human breast tumor cell line SK-BR-3, which required 62,500 U/ml TNF in contrast to the 250 U/ml of TNF required for the BT-20 breast cell line.

Also indicated in Table I is the reduction in the amount of TNF required for 50% cell kill when IFN-α and IFN-γ were present. Pretreatment with IFN-α and IFN-γ for 24 hr prior to the addition of TNF resulted in 2–200-fold potentiation for various cell lines. Treatment of the cell lines in Table I with either IFN-α or IFN-γ at the concentrations used in the potentiation studies described above resulted in no apparent cytotoxic effects. The degree of potentiation varied with different cell lines. Although melanoma cell line G-361 was not potentiated by IFN-α, melanoma cell

TABLE I. Potentiation of TNF Cytotoxicity by IFN-(α, γ)

Human cell line[b,c,d]	TNF (U/ml) required to kill 50% of cells		
	TNF	TNF + IFN-α	TNF + IFN-γ
BT-20 (breast)	250	180 (1.3)[a]	30 (8.3)
HT-144 (melanoma)	312	40 (7.8)	12 (26.0)
ME-180 (cervix)	1,000	90 (48.6)	5 (200.0)
A-549 (lung)	30,000	30,000 (0)	6,700 (4.5)
SK-OV-3 (ovary)	50,000	8,750 (5.8)	4,350 (11.3)
BRO (melanoma)	19,000	1,500 (12.7)	1,200 (15.8)
SK-CO-1 (colon)	75,000	50,000 (1.5)	9,000 (8.0)
$MLCL_1$ (melanoma)	9,000	2,400 (3.8)	1,200 (7.5)
SK-BR-3 (breast)	62,500	1,875 (33.0)	13,000 (5.0)
734B (breast)	45,000	600 (75.0)	10,000 (4.5)
G-361 (melanoma)	20,000	20,000 (0)	7,700 (2.6)
Colon 205 (colon)	15,000	2,500 (6.0)	10,000 (1.5)

[a]Potentiation index $= \frac{\text{TNF (U/ml) causing 50\% cytotoxicity in the untreated cells}}{\text{TNF (U/ml) causing 50\% cytotoxicity in the treated cells.}}$

[b]Cells were grown in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco) and 50 g/ml of gentamycin (Shering Corp, Kennelworth, NJ).

[c]1–2 $\times 10^4$ cells were seeded per well in a 96-well plate (Costar, Cambridge, MA) and incubated 24 hr at 37°C. For potentiation studies, 5,000 U/ml of IFN-α (natural IFN-α isolated from human PBLs induced with Sendai virus; 1–5 $\times 10^6$ U/mg specific activity) or 500 U/ml IFN-γ (Cellular Products, Buffalo, NY; 5×10^6 U/mg specific activity) were present during the 24-hr incubation. After 24 hr, TNF was added for 48 hr and cell cytotoxicity was determined by dye uptake test (neutral red) by using a Titertek reader [19].

[d]Cell lines were obtained from American Type Culture Collection (Rockville, MD).

lines $MLCL_1$ and BRO were potentiated 3.8- and 12.7-fold respectively as indicated in Table I. Although it is difficult to conclude whether tumor cell lines of a specific tumor origin are resistant to TNF cytotoxicity or potentiation by IFN, it is possible to conclude that TNF cytotoxicity is potentiated by IFN-α and -γ with various degrees of efficiency depending on the cell lines employed. What in vivo significance these results represent in terms of tumor origin remains to be established since the cell lines have probably been altered since the cell lines were established. Nevertheless, potentiation of TNF cytotoxic activity by IFN-α and IFN-γ which has been observed by other investigators [23] could have significance in vivo and in combination therapy involving TNF and IFN.

Animal Testing of Recombinant TNF

Purified recombinant TNF (1×10^7 U/mg) was tested for cytotoxic activity against sarcoma 180 tumors in BALB/c mice as indicated in Figure 4. In BALB/c nu/+ mice, both doses of recombinant TNF (2.4×10^4 and 6×10^3 U/mouse) caused

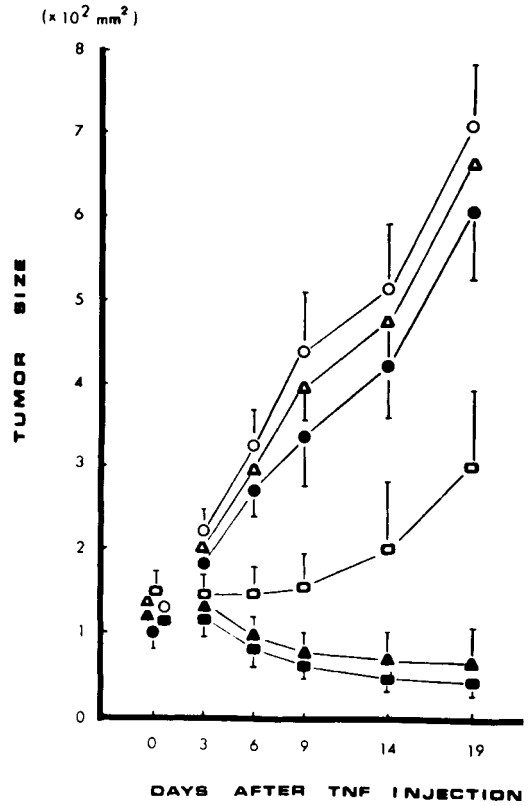


Fig. 4. Effect of TNF on sarcoma-180 transplanted into BALB/c nu/nu or nu/+ mice. Sarcoma-180 (1×10^7 cells) were inoculated s.c. 7 days prior to the administration of TNF. TNF (6×10^3 U/mouse ▲ nu/+, △ nu/nu, or 2.4×10^4 U/mouse ■ nu/+, □ nu/nu) was given as a single i.v. injection on day 0. Control mice received saline (● nu/+, ○ nu/nu). The results represent the mean value of six to eight animals and standard error. The tumor size of TNF treated nu/+ mice differs significantly after day 6 from nu/+ controls ($P < .001$). The tumor size of TNF-treated (24,000 U/mouse) nu/nu mice differs significantly after day 6 from TNF-treated (6,000 U/mouse) and control nu/nu mice ($P < .02$).

regression of sarcoma 180. However, in nu/nu mice, the lower dose of recombinant TNF (6×10^3 U/mouse) showed no effect. These data suggested that the T-cell-mediated immune response may be implicated in the antitumor effect of TNF. To test the acute toxicity of recombinant TNF, BALB/c mice were given purified recombinant TNF (1×10^7 U/mg specific activity) intravenously at different doses in 0.1-ml sterile phosphate-buffered saline containing human serum albumin (1 mg/ml). The control mice were injected with only phosphate-buffered saline and human albumin. The LD_{50} for recombinant TNF was determined to be 6×10^7 units of TNF/kg. The results of autopsies performed on BALB/c mice containing different concentrations of recombinant TNF are indicated in Table II. The cause of death at the LD_{50} appeared to be respiratory failure, which is consistent with interstitial lymphohistiocytic infiltrate presence in the lung at low TNF concentrations and pneumonitis at high concentrations, as indicated in Table II. Doses of 6×10^7 and 3×10^7 U/kg depressed the value of glucose and alkaline phosphatase and elevated blood urea nitrogen (BUN) and serum glutamate pyruvate transaminase (SGPT) as indicated in Table III. All values returned to normal after 72 hr for surviving animals. The therapeutic dose of TNF in sarcoma 180 bearing BALB/c mice was determined to be 3×10^5 U/kg [24]. These results indicate that TNF has a wide therapeutic index.

Clinical Trials of Recombinant TNF

Phase I clinical trials of purified recombinant TNF (1×10^7 U/mg) produced at the Wadley Institutes were initiated on September 25, 1985. Phase I clinical trials are primarily concerned with determining the side effects and tolerated dose of a drug. TNF was administered intravenous (I.V.) at escalated doses on alternate days by a modified Fibonacci scheme as indicated in Table IV. Eighteen patients having the malignancies indicated in Table V participated in the phase I studies. The highest doses received ranged from 3,000 U/kg to 250,000 U/kg. Also presented in Table V is the total dose received by patients having various forms of cancer.

The side effects for recombinant TNF are indicated in Table VI. Several of the side effects, such as chills, fever, and malaise, have been found for other cytokines, such as IFN-α [4]. At high doses of 250,000 U/kg, chills, fever, severe weakness, nausea, vomiting, diarrhea, and temporary neutropenia caused the dose to be lowered to more tolerable levels. The phase I clinical trials indicate that purified recombinant TNF can be administered I.V. three times per week up to 150,000 U/kg with tolerable side effects [25]. Patients had chills and fever within 10–15 min of injection with the fever ranging from 37.3 to 39.4°C. At least four patients who had been treated for over 4 wk showed a rise in triglycerides from 88.5 to 168.5 mg/dl. The half-life (T-1/2) of recombinant TNF was biphasic at 150,000 U/kg. The first phase T-1/2 was approximately 2 min and the second phase was 31 min following TNF injection.

Phase II clinical trials of recombinant TNF are in progress. Phase II trials are primarily concerned with determining drug effectiveness. The phase II trials of TNF involve a dose of 150,000 U/kg delivered I.V. three times per week. Twenty-three patients have participated in the phase II trials since its initiation. In addition to the clinical trials of recombinant TNF alone, clinical trials involving 90,000 U/kg of TNF delivered I.V. three times per week in combination with IFN-α are in progress.

DISCUSSION

Described is an organized effort employing gene cloning techniques and a comprehensive cancer center to analyze the immune network and develop immune

TABLE II. Autopsy Examination of BALB/c Mice 24 Hr After Single I.V. TNF Injection

Dose (U/kg)	Heart	Lungs	Liver	Spleen	Thymus	Lymph node	Kidney	G.I. tract
LD_{50} dead animal	Massive congestion	Focal pneumonitis	Subcapsular necrosis	Follicular hyperplasia	Lymphoid hyperplasia	Not identified	Severe congestion & proteinaceous material in tubules	Lymphoid aggregates in stomach
6×10^7 (LD_{50})	None	Focal pneumonitis	Vacuolization of hepatocytes	Follicular hyperplasia	Lymphoid hyperplasia	Follicular hyperplasia	Lymphohistiocytic infiltrate	Lymphoid hyperplasia of small intestine
3×10^7	None	Interstitial lymphohistiocytic infiltrate	Peripheral lymphohistiocytic infiltrate	Follicular hyperplasia	Lymphoid hyperplasia	None	Minimal interstitial lymphohistiocytic infiltrate	None
1.8×10^7	None	Interstitial lymphohistiocytic infiltrate	Peripheral lymphohistiocytic infiltrate	Follicular hyperplasia	Lymphoid hyperplasia	Follicular hyperplasia	Minimal interstitial lymphohistiocytic infiltrate	None
9×10^6	None	Interstitial lymphohistiocytic infiltrate	Peripheral lymphohistiocytic infiltrate	Follicular hyperplasia	Lymphoid hyperplasia	Follicular hyperplasia	Minimal interstitial lymphohistiocytic infiltrate	None
3×10^6	None	None	None	None	None	None	None	None

TABLE III. Acute Toxicity Study of TNF*

Dose (U/kg)	BUN (mg/dl)	Glucose (mg/dl)	Alk. Phos. (I.U./liter)	SGPT (I.U./liter)
6×10^7	110.0 ± 12.9	46.5 ± 1.7	42.0 ± 8.5	252.0 ± 53.4
3×10^7	80.7 ± 18.0	87.4 ± 21.5	54.2 ± 9.3	166.2 ± 69.1
1.8×10^7	25.5 ± 2.0	133.6 ± 7.4	59.4 ± 17.7	119.0 ± 4.0
9×10^6	32.7 ± 3.2	130.0 ± 5.4	77.2 ± 17.3	163.0 ± 24.9
3×10^6	30.4 ± 6.2	114.0 ± 9.7	64.2 ± 13.3	112.0 ± 30.3
Control	32.0 ± 5.6	98.6 ± 1.1	88.5 ± 27.4	86.0 ± 38.6

*Animals were injected intravenously with different TNF doses in 0.1 ml sterile phosphate saline containing human serum albumin (1 mg/ml). Each dose of TNF represents results of three BALB/c female mice. Determinations were made on animals 24 hr after TNF injection. Blood urea nitrogen (BUN), glucose, alkaline phosphatase (Alk. Phos.), and serum glutamate pyruvate transaminase (SGPT) levels were determined on an Abbott VP analyzer by the clinical chemistry laboratory of Granville C. Morton Hospital at Wadley Institutes.

TABLE IV. TNF Dose Escalation Regimen

Day	TNF (U/kg)[a]
1	1,000
3	2,000
5	3,000
7	5,000
9	8,000
11	13,000
13	21,000
15	34,000
17	55,000
19	89,000
21	144,000
23	234,000

[a]Based on a 70-kg human.

factors for therapeutic utility. Since several cytokines such as tumor necrosis factor, lymphotoxin [11], IFN-α, and IFN-β contain regions of homology in their gene and protein structures, the construction of cDNA libraries for proven cytokine genes may also permit the isolation of new but related cytokine cDNAs by using known cDNAs or related oligonucleotide probes for cDNA screening. As indicated above, we isolated TNF cDNA from a human PBL cDNA library, in contrast to tumor cell lines employed by others [11–13]. These results are significant because the PBL cDNA library was constructed primarily for IFN-α cDNA isolation, but the bacterial colonies were stored for future screening opportunities. At the time there was no reason to expect TNF cDNA to be present in this library, but the availability, 1 yr after the establishment of the PBL cDNA library, of the induced PBL RNA- and cDNA-containing colonies permitted rapid testing for the presence of TNF mRNA and eventually the isolation of a TNF cDNA clone [15–17]. We were able to isolate and engineer for expression in *E. coli* the recombinant TNF in only 5 wk. As indicated, we have also isolated several IFN-α clones including IFN-αD, which is consistent with previous studies [6] as well as IFN-β and IL-1β. Several potentially related but not identical cytokine cDNA clones from this cDNA library are under investigation.

Analysis of the induction of the various cytokine mRNAs indicated that the various cytokines tested, TNF, IFN-α, IL-1β, and IFN-γ, all respond to induction by

TABLE V. TNF Phase I Clinical Trial

Case no.	Diagnosis	Highest U/kg	Highest dose	Total dose
01	Chondrosarcoma	90,000	5,040,000	37,572,000
02	Ca. prostate	90,000	20,000,000	257,530,000
03	Melanoma	90,000	6,390,000	382,350,000
04	Ca. lung	3,000	210,000	420,000
05	Ca. lung	90,000	5,400,000	49,940,000
06	Ca. rectum	250,000	17,500,000	106,460,000
07	Ca. lung	55,000	3,850,000	9,960,000
08	A.G.L.	150,000	13,500,000	222,815,000
09	Ca. breast	55,000	3,410,000	12,215,000
10	Ca. breast	175,000	8,400,000	66,046,000
11	Osteosarcoma	175,000	9,950,000	100,500,000
12	Astrocytoma	200,000	10,000,000	94,075,000
13	Ca. colon	200,000	18,000,000	128,082,000
14	Ca. colon	5,000	363,000	363,000
15	Ca. breast	175,000	8,100,000	67,716,000
16	Ca. lung	175,000	12,775,000	255,500,000
17	A.M.L.	150,000	6,450,000	11,906,000
18	Ca. colon	175,000	7,390,000	123,959,000

TABLE VI. TNF Phase I — Side Effects

Effect	No. of patients	Beginning at TNF dose (units/kg)
Chills	17	1,000
Fever	18	1,000
Malaise	14	1,000
Fatigue	12	1,000
Anorexia	11	1,000
Nausea	10	1,000
Vomiting	9	1,000
Headache	7	3,000
Joint pain	3	8,000
Lower back pain	3	2,000
Diaphoresis	2	3,000
Shaking without chills	2	150,000
Flushing	1	21,000
Anxiety	1	90,000
Itching, no rash	2	34,000
Pallor	2	175,000
Shortness of breath	1	3,000
	1	
Dry mouth	1	1,000
Diarrhea	1	250,000

Sendai virus and that TNF and IFN-α mRNA are both induced at high levels (0.11% of total virus-induced PBL mRNA) [17]. These results contrast with TNF mRNA levels estimated from previous work for HL-60 cells of 0.0035% [11] and 0.002% [12] and for U-937 cells of 0.01% [13]. In addition, the inducers utilized in the previous studies were phorbol esters and endotoxin, not Sendai virus. Hence the availability and screening of the PBL cDNA library not only resulted in the isolation

of useful cytokine cDNAs but also resulted in new insights into TNF induction and its relationship to other cytokines.

As indicated above and in previous studies [17] the level of TNF activity in induced PBL culture supernatants was about 200-fold lower than IFN-α activity. These results are in contrast to high levels of TNF and IFN-α mRNA levels. Previously we proposed that TNF mRNA may be less efficiently translated than IFN-α mRNA based on mixing studies [17]. Possibly related to these results is the potentiation of TNF activity by low levels of IFN-α and IFN-γ as indicated in Table II and by other investigators [23]. Perhaps low levels of TNF are sufficient for in vivo utility if IFN-α or -γ is present. The coexistence of TNF and IFN-α can be effected by induction by viruses such as Sendai virus. Whether TNF mRNA is subjected to translational regulation by factors such as other cytokines remains to be delineated.

Further evaluation of the in vivo function of TNF is necessary. TNF has been shown to contain a plethora of activities, such as cytotoxicity [2]; modulation of HLA-A, B antigens [26]; induction of IFN-β_2 [27]; activation of phagocytosis [28]; and induction of cachexia-related symptoms [29]. Delineation of cytokine function has involved analysis of cytokines on cells in culture, injection into animals, and human clinical trials as described in these studies and cited references. Some studies have involved the surveillance of cytokine mRNA and activities in response to other cytokines or inducers [17,30]. Clearly, exogenous delivery of cytokines has questionable relevance to the natural in vivo function of these proteins although such studies permit the evaluation of their therapeutic uses. One approach which has not been employed and which could be fruitful in generating insight into cytokine in vivo function would be to establish transgenic animals containing cytokine genes which are inducible to high levels. Such a system could permit the modulation of cytokine levels under controlled conditions and the evaluation of their effects under more natural conditions.

Since the animal studies indicated that recombinant TNF had a high therapeutic index it was with considerable interest that the clinical trials were initiated. As indicated in Table V, a range of doses were given 18 patients in the phase 1 clinical trials. Based on these studies 150,000 U/kg was determined to be the tolerated dose. These studies also indicated that the half-life of TNF was short and biphasic (2 min and 31 min T-1/2). The short half-life certainly may bear on its clinical effectiveness. Phase II clinical trials utilizing 150,000 U/kg are in progress. Preliminary phase II studies do not appear encouraging for TNF delivered I.V., but there is considerable interest in our trials involving intralesional TNF injections and TNF plus IFN-α.

The major issues for TNF utility appear to be high-dose toxicity and short half-life. During the clinical trials, a rapid reduction of circulating granulocytes was consistently observed in patients after TNF injection. Further study in vitro indicated that TNF rapidly binds to high-affinity receptors on granulocytes and induces cell adherence [31]. A parallel relationship was observed between TNF-induced granulocyte adherence and TNF-increased expression of surface adhesive proteins (Mac-1, p150,95) (unpublished data). The data suggested that TNF induced granulocyte adhesion by enhancing the expression of surface adhesive proteins. Enhanced granulocyte activities including phagocytic, antibody-dependent cell cytotoxicity and killing activity to parasites have also been reported [28,32]. The finding that TNF regulates granulocyte function is of particular interest, since this action may represent the role of TNF against infectious disease.

One of the limiting factors in the use of TNF as an antitumor drug is its toxicity at high doses. It has been shown that cyclooxygenase inhibitors, indomethacin or ibuprofen, can prevent the toxic effects of TNF in animals [33]. Combined administration of TNF with cyclooxygenase inhibitors may allow safer administration of high doses of TNF to cancer patients.

Further approaches to improving TNF delivery involve intralesional delivery of TNF; clinical trials involving the combination of TNF plus IFN-α which relate to the potentiation data described above; generation of TNF analogs which are presently under analysis; and combination of TNF with other cytokines. Further analysis of the immune network and testing of immune factors as described in these studies should result in an improved understanding of the immune system and development of new agents which may be useful for antiviral and anticancer therapies.

ACKNOWLEDGMENTS

We thank Sal Camparini, Bradley Hepner and Jerry Sanders for human leukocyte preparation; Dr. Karen Pennington for cytotoxicity assays; Saundra Davis, Paul John, and KaNan Vickroy for technical assistance; Dr. Steven J. Sandler, Dr. Rajinder Sidhu, and Cheryl Hendrix for development of pUC9-PL85; and Dr. S.J. Ahmed for assistance with clinical trial data. We thank Ms. Mickie Cox for the preparation of this manuscript. Some of this work was performed in the Oree Meadows Perryman Laboratory. This work was supported by the Meadows Foundation, the Samuel Roberts Noble Foundation, the Mary Kay Foundation, the Haggarty Foundation, and by a National Institutes of Health grant to A.P.B.

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