Human tumor necrosis factor produced by human B-cell lines: Synergistic cytotoxic interaction with human interferon

(lymphokine/antitumor factor/cancer therapy)

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ABSTRACT   Human cell lines of hematopoietic origin were tested for production of tumor necrosis factor (TNF). B-cell lines transformed by Epstein–Barr virus release a factor (referred to as hTNF) that is cytotoxic for mouse L cells sensitive to mouse TNF but not for L cells resistant to mouse TNF. Exposure to 4β-phorbol 12β-myristate 13α-acetate augmented production of hTNF. hTNF activity was not found in supernatants of cell lines of T-cell, monocytic, or promyelocytic origin. Partially purified hTNF has a molecular weight of approximately 70,000, has no interferon activity, is acid labile, is destroyed by heating at 70°C for 1 hr, induces cross-resistance to mouse TNF in vitro, and causes hemorrhagic necrosis of Meth A mouse sarcoma in the standard in vivo mouse TNF assay. Tests with a panel of 23 human cancer cell lines showed that hTNF is cytotoxic for 7 cell lines, cytostatic for 5, and has no effect on 11. Comparative studies with human α, β, and γ interferons indicated that sensitivity to hTNF and interferon can be distinguished. Combined treatment with hTNF and α or γ interferon resulted in a synergistic cytotoxic effect.

Tumor necrosis factor (TNF) was recognized by Carswell et al. (1) during a study of the antitumor activity of serum from mice infected with bacillus Calmette–Guérin (BCG) and subsequently injected with endotoxin. Serum from these mice contains a factor that induces hemorrhagic necrosis of certain mouse sarcomas in vivo and has cytotoxic/cytostatic effects on mouse and human tumor cells in vitro (1–5). Serum from mice singly treated with BCG or endotoxin did not have these properties. Other agents, such as Corynebacterium parvum or zymosan, that cause hyperplasia of the reticuloendothelial system and increase sensitivity to endotoxin lethality, can substitute for BCG in priming for TNF release. Endotoxin, however, appears to be unique in its ability to elicit TNF release (1, 4–6). By using these methods for TNF production, factors with TNF-like activities have also been found in rats (1) and rabbits (1, 7, 8).

Biochemical characterization has shown that mouse serum TNF exists in at least two forms: a 150,000 M_r form (4, 9) and a 40,000–60,000 M_r form (3–5). TNF in rabbit serum has been reported to have a molecular weight of 39,000 (10) and 67,000 (11). Our studies have indicated that both in vivo and in vitro activities of mouse TNF appear to be a property of the same molecule. The cellular source of TNF in the mouse was initially assumed to be the macrophage, because the agents used to prime for TNF production cause massive hyperplasia of macrophages in liver and spleen (1). From studies of macrophage-rich cell populations in vitro, Matthews (12) and Mannel et al. (13) reached a similar conclusion with regard to the source of mouse and rabbit TNF. Direct evidence that macrophages are at least one cell type in the mouse capable of producing TNF comes from stud-

ies with cloned lines of mouse histiocytomas (ref. 13; unpublished data). These cells constitutively produce low levels of TNF that are greatly increased after exposure to endotoxin.

In the present study we have examined the capacity of human cell lines of hematopoietic origin to produce a TNF-like factor.

MATERIALS AND METHODS

Cell Lines. See Tables 1 and 2. LuKII cells were obtained from W. Stewart (14). B-cell lines transformed by Epstein–Barr virus were derived from patients with melanoma (15). These and other cell lines were from our cell bank or from the collection of Jørgen E. Fogh or Peter Ralph of Sloan–Kettering Institute.

In Vitro TNF Assay. TNF-sensitive and -resistant L-M cells were derived from clone 929 mouse L cells (American Type Culture Collection). TNF activity was assayed by adding equal volumes of serially diluted samples to wells (24-well Costar plates) seeded 2–3 hr previously with 5×10^4 trypsinized L cells and determining cell death at 48 hr by phase-contrast microscopy (1).

In Vivo TNF Assay. (BALB/c $\times$ C57BL/6)F_1 female mice were injected intradermally with 5×10^5 Meth A BALB/c sarcoma cells. After 7 days (tumor size approximately 7 mm average diameter), mice received a single intravenous injection of the TNF preparation. After 24 hr, tumor hemorrhagic necrosis was scored according to ref. 1.

Interferon (IFN) Assays. Human IFN activity was measured by inhibition of the cytopathic effect of vesicular stomatitis virus on WISH or GM2767 cells (16) and compared against an international standard in the case of IFN-α and the laboratory standard in the case of IFN-γ.

Mouse TNF. A standard lot of partially purified mouse serum TNF with a specific activity of 2×10^4 units of TNF per mg of protein was used in these studies. (A unit of TNF is defined as the amount of protein causing killing of 50% of the L cells in the standard in vitro TNF assay.) This preparation of mouse TNF lacked IFN activity.

Source of IFNs. Human IFNs were obtained from the following sources: Recombinant IFN-α (Hoffmann–La Roche), 2–4×10^8 units/mg of protein; leukocyte-derived IFN-α (prepared for the Sloan–Kettering Institute by Kocher Laboratory, Berne, Switzerland), 0.64×10^6 units/mg of protein; leukocyte-derived IFN-γ, 1×10^7 units/mg of protein; fibroblast-derived IFN-β (Roswell Park Memorial Inst.), 1×10^7 units/ mg of protein.

Abbreviations: TNF, tumor necrosis factor; hTNF, human TNF; BCG, bacillus Calmette–Guérin; PMA, 4β-phorbol 12β-myristate 13α-acetate; IFN, interferon.
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Screening of Human Cells of Hematopoietic Origin for TNF Production. Cells were cultured at 5×10^5 per ml in RPMI 1640 medium containing 8% fetal calf serum with or without 4β-phorbol 12β-myristate 13α-acetate (PMA) (Sigma) at 10 ng/ml. After incubation for 48 hr at 37°C, the cells were collected by centrifugation and resuspended at 1×10^6 per ml in medium without PMA for an additional 48 hr. Culture supernatants were freed of cells by centrifugation and frozen at -20°C before screening 1/2 diluted supernatants for TNF activity by the L cell assay.

Preparation and Concentration of hTNF from LuKII Cells. LuKII cells were cultured at 5×10^5 per ml in RPMI 1640 medium containing 5% fetal calf serum and PMA at 10 ng/ml and incubated for 48 hr at 37°C. At 48 hr, cells were collected by centrifugation and resuspended at 8–10×10^5 per ml in serum-free R-ITS PREMIX (insulin, transferrin, selenium serum substitute, Collaborative Research, Waltham, MA) and 2 nM ethanolamine and incubated an additional 48 hr. Supernatants were spun free of cells and frozen at -20°C. LuKII supernatants were concentrated by Amicon stirred cells (PM 10 membrane) and applied to a DEAE-Sephadex A-50 column (40×2.6 cm) equilibrated with 0.05 M Tris·HCl/0.15 M NaCl buffer, pH 7.3. Fractions (5 ml) were eluted with the same buffer and hTNF-active fractions were pooled and concentrated by Amicon cells. Molecular weight was estimated by Sephacryl S-200 column chromatography.

Effect of hTNF and IFN on Human Cell Lines. Cells were trypsinized, rinsed twice, plated at 4–5×10^4 per well (24-well Costar plates) in minimal essential medium containing 8% fetal calf serum and incubated at 37°C. After 16–24 hr, the medium was aspirated and 1-ml dilutions containing DEAE-fractionated hTNF, IFN, or hTNF and IFN in medium were added. Total cell counts (viable and nonviable) were determined at 3, 5, and 7 days by phase-contrast microscopy. Cultures were re-fed on day 5 with fresh medium containing the same concentrations of hTNF and IFN.

RESULTS

TNF Production by Human Cell Lines of Hematopoietic Origin. Supernatants from the cell lines listed in Table 1 were screened for TNF production by using the L cell assay system developed for the detection of mouse TNF (1, 6). Supernatants were tested on TNF-sensitive L cells [designated $L_{(S)}$] and on the TNF-resistant L cell line [designated $L_{(R)}$]. The $L_{(R)}$ line was developed by repeated passage of sensitive L cells in medium containing mouse TNF. Table 1 shows that cell lines of B-cell origin produce a factor with TNF-like activity—e.g., cytotoxic for $L_{(S)}$ but not $L_{(R)}$—and that PMA is necessary for efficient TNF production. No TNF activity was detected in the supernatants of six T-cell or three monocytic cell lines (with or without PMA stimulation). The LuKII line consistently produced high levels of TNF after PMA induction and was therefore selected as the source for subsequent studies of TNF production and characterization.

Fractionation and Characterization of Human TNF (hTNF). Concentrated samples of LuKII supernatants were fractionated on DEAE-Sephadex A-50 columns and individual fractions were assayed on $L_{(S)}$ and $L_{(R)}$ cells. TNF activity was detected in the initial flow-through fractions, and peak fractions were pooled and concentrated. The specific activity of these preparations was 2–5×10^4 units/mg of protein (a 25-fold increase over the initial supernatants), and these preparations were used for characterization. The molecular weight of hTNF was estimated to be approximately 70,000. hTNF activity was destroyed by a 12-hr exposure to low or high pH: 90% of the activity was lost at

Table 1. Screening of human cell lines of hematopoietic origin for TNF production

| | Cell viability (%) after exposure to culture supernatants | | | |
| | $L_{(S)}$ cells | | $L_{(R)}$ cells | |
Cell line	No PMA	PMA	No PMA	PMA
B cell				
BE*	47	20	100	100
BI*	57	43	100	98
CL*	95	37	100	97
CW*	28	8	100	100
DE*	45	18	100	100
DM*	18	11	100	100
DS*	88	38	100	100
EJ*	27	16	100	100
EL*	70	16	100	100
EQ*	15	11	100	100
ER*	47	22	100	100
FC*	19	8	100	100
FD*	44	16	97	100
FG*	80	30	100	97
ARH-77	68	12	100	100
DAUDI	97	97	NT	NT
LuKII	20	13	100	100
RPMI 1788	78	35	100	100
RPMI 8226	96	84	100	86
RPMI 8866	32	10	100	98
SK-LY-16	98	100	100	100
SK-LY-18	99	100	100	98
ARA-10	77	39	100	97
BALL-1	100	100	100	100
T cell				
CCRF-CEM	100	100	100	100
P-12	95	94	99	98
MOLT-4	100	100	99	96
T-45	100	100	100	100
HPB-ALL	100	97	100	100
TALL-1	96	94	96	96
Monocyte				
J-111	100	100	99	98
THP	96	80	99	99
U-937	100	100	99	99
Promyelocyte				
HL-60	96	97	99	98

NT, not tested.
* Epstein–Barr virus-transformed cell line derived from peripheral blood.

pH 2.0 and 45% of the activity was lost at pH 10. Activity was stable over a pH range of 6 to 8. With regard to heat stability, hTNF was destroyed at 70°C for 60 min but was stable at 56°C for 60 min. Activity was preserved over long periods of storage at -78 or -20°C. IFN assays demonstrated the presence of 100 units of IFN-α per ml in the unfractionated supernatant from PMA-stimulated LuKII cells. No IFN activity was detected in hTNF preparations after DEAE-Sephadex chromatography. To investigate further the relationship of mouse TNF to hTNF, L cells were made resistant to hTNF by repeated passage in hTNF-containing medium. hTNF-resistant cells showed complete cross-resistance to mouse TNF. DEAE-fractionated hTNF was assayed by the standard *in vivo* mouse TNF assay (1) and found to cause necrosis of Meth A tumors. Three hundred micrograms of the hTNF preparations produced $+ + +$ necrosis in 1/5 mice and $+ +$ in 4/5 mice; 150 μg produced $+ +$ necrosis in 5/7 mice and $+$ in 2/7 mice; 75 μg produced $+ +$ necrosis in 1/5 mice and $+$ in 4/5 mice. No necrosis was observed in control mice after injection of comparable DEAE-Sephadex
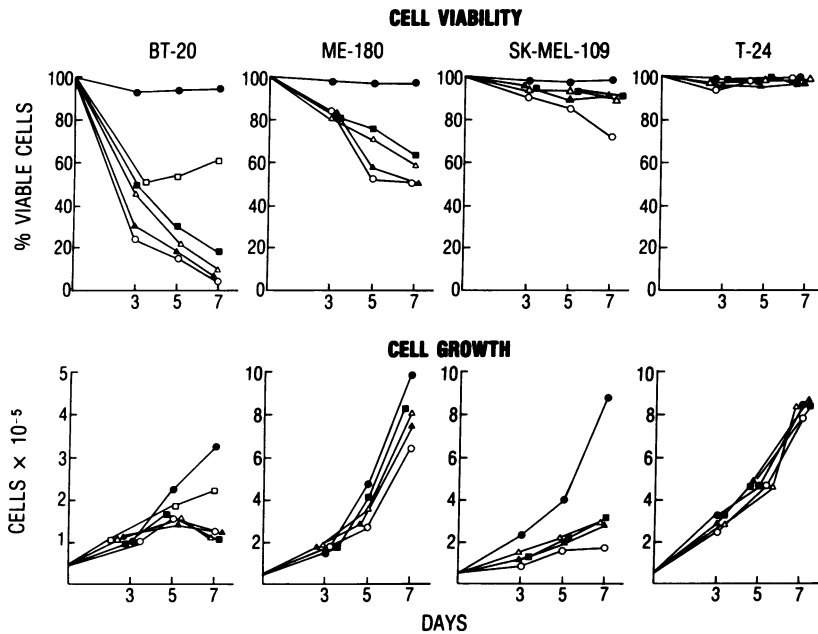
CELL VIABILITY

CELL GROWTH

FIG. 1. Effect of hTNF on four human tumor cell lines. hTNF units/ml added to cultures: ●, control cultures; □, 32 units (BT-20); ■, 64 units (ME-180, SK-MEL-109) or 126 units (BT-20, T-24); △, 129 units (ME-180, SK-MEL-109) or 253 units (BT-20, T-24); ▲, 258 units (ME-180, SK-MEL-109) or 505 units (BT-20, T-24); ○, 645 units (ME-180, SK-MEL-109) or 1,263 units (BT-20, T-24).

fractions of culture medium alone. *Limulus* assays indicated no difference in the amount of endotoxin in TNF-active and TNF-inactive (control) fractions.

Cytotoxic or Cytostatic Effect of hTNF on Human Tumor Cell Lines. Fig. 1 illustrates the effect of hTNF on four cell lines: BT-20 breast cancer line (cytotoxic effect), ME-180 cervical cancer line (cytotoxic effect), SK-MEL-109 melanoma line (cytostatic effect), and T-24 bladder cancer line (no effect). Table 2 summarizes the results of tests with 23 tumor cell lines and 4 cultures of normal cells. With a 35% or greater reduction in cell viability or cell number at 7 days as the criterion of either cytotoxicity or cytostasis, hTNF has a cytotoxic effect on 7 cell lines, a cytostatic effect on 5 cell lines, and no effect on 15 lines. Three of four cell lines of breast cancer origin were sensitive to the cytotoxic effect of hTNF, whereas the predominant effect of hTNF on melanoma cell lines was cytostasis. None of the four cultures derived from normal tissues was hTNF sensitive.

Table 2. Effect of hTNF on human cell lines

Cytotoxic effect	No effect
SK-MG-4 (astrocytoma)	T-24 (bladder cancer)
MCF-7 (breast cancer)	5637 (bladder cancer)
BT-20 (breast cancer)	MDA-MB-361 (breast cancer)
SK-BR-3 (breast cancer)	S-48 (colon cancer)
ME-180 (cervix cancer)	SK-LC-4 (lung cancer)
SK-CO-1 (colon cancer)	SK-LC-6 (lung cancer)
RPMI 7931 (melanoma)	SK-LC-12 (lung cancer)
	SK-MEL-19 (melanoma)
Cytostatic effect	SK-UT-1 (uterus cancer)
SK-LU-1 (lung cancer)	SAOS-2 (osteogenic sarcoma)
RPMI 4445 (melanoma)	U20S (osteogenic sarcoma)
SK-MEL-29 (melanoma)	WI-38 (fetal lung)
SK-MEL-109 (melanoma)	MY (normal kidney epithelium)
SK-OV-3 (ovary cancer)	F-136-35-56 (fetal lung)
	F-136-35-56 (fetal skin)

CELL VIABILITY

CELL GROWTH

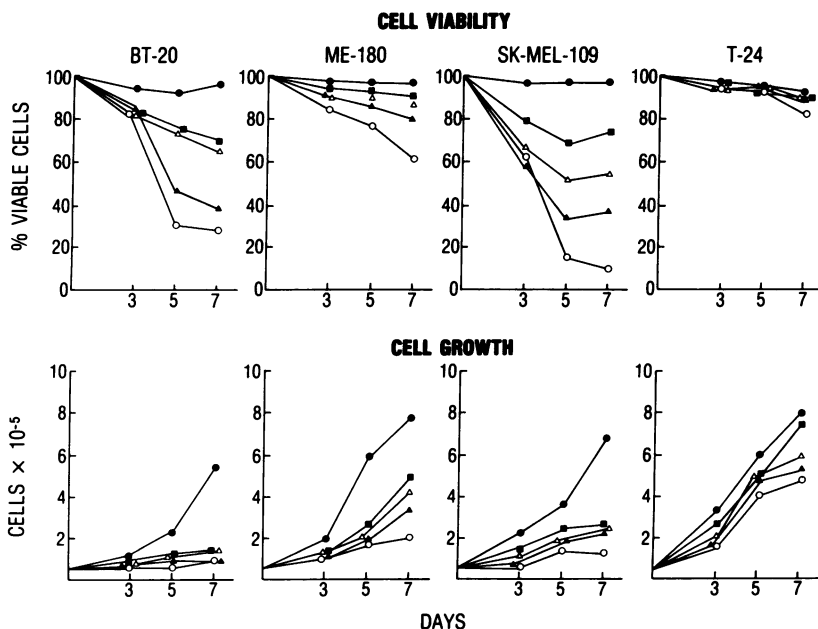FIG. 2. Effect of recombinant IFN-α on four human tumor cell lines. IFN units/ml added to cultures: ●, control cultures; ■, 781 units; △, 3,125 units; ▲, 12,500 units; ○, 50,000 units.
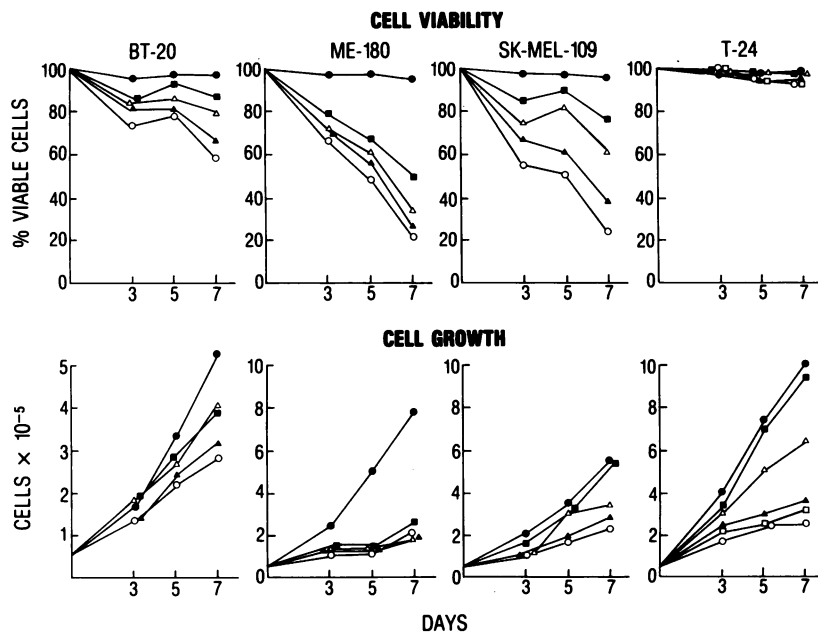
FIG. 3.   Effect of IFN-γ on four human tumor cell lines. IFN units/ml added to cultures: ●, control cultures; ■, 7.8 units; △, 31.25 units; ▲, 125 units; □, 500 units; ○, 2,000 units.

Effect of Human IFNs on Human Tumor Cell Lines—Synergistic Action of TNF and Interferon. Recombinant IFN-α and natural IFNs α, β, or γ were tested on a panel of human cell lines. Figs. 2 and 3 show the response of BT-20, ME-180, SK-MEL-109, and T-24 to recombinant IFN-α and natural IFN-γ and Table 3 summarizes the results of tests with the various IFN preparations at 7 days. In general, the number of antiviral units of IFN required to cause a 35% or greater cytotoxic effect was higher with IFN-α than with IFN-β or -γ. None of the IFN preparations showed cytotoxicity or cytostasis on $L_{(S)}$ cells, indicating absence of TNF-like activity. Fig. 4 shows the influence of combined treatment with hTNF and recombinant IFN-α or natural IFN-γ. Synergistic effects of hTNF and IFN are clearly evident in tests with BT-20, ME-180, and SK-MEL-109, in which the combined cytotoxic effect is greater than that seen with hTNF or IFN alone. Similar results were obtained with mouse TNF and human IFNs α and γ. By using the approach suggested by Clarke (17), the combined cytotoxic effect of IFN and hTNF was clearly shown to be synergistic rather than additive.

DISCUSSION

The finding that human cell lines of B-cell origin release a TNF-like molecule after stimulation with PMA suggests that normal B cells have this capacity as well. Whether other human cell types produce TNF remains to be determined, but the fact that cloned lines of mouse histiocytoma do so after endotoxin stimulation indicates the need for further studies of this cell type in humans. The identification of the cytotoxic factor released by the human B-cell lines as the human homolog of mouse TNF

rests on its differential reactivity with TNF-sensitive and TNF-resistant mouse L cells and the similar pattern of response (cytotoxic/cytostatic/no effect) of the human cell panel shown in Table 1 to the human B-cell factor and to mouse TNF (unpublished data). Further facts relating mouse TNF and hTNF are (*i*) L cells made resistant to hTNF show a cross-resistance to mouse TNF, and (*ii*) hTNF preparations cause hemorrhagic necrosis of Meth A sarcoma, the standard *in vivo* TNF assay. Although TNF appears to lack species specificity with regard to its antitumor effects, we have found that hTNF has a higher specific activity on human cells as compared to mouse cells. Other features that are shared by mouse and human TNF are acid lability, relative resistance to heat, and augmented toxicity in the presence of metabolic inhibitors such as actinomycin.

Sensitivity to TNF is not an uncommon trait of human cancer cell lines. The normal cell types that have been studied to date have not shown sensitivity to mouse TNF (18) or hTNF, but these tests must be expanded before the apparent tumor specificity of TNF can be assessed. The response of TNF-sensitive tumor cells can be classified either as cytotoxicity or cytostasis. Whether this represents a continuum in response (with cytotoxicity indicating higher sensitivity) or a qualitatively different response is unclear. In studies on the influence of TNF on the cell cycle in sensitive L cells, the most evident effects are an initial accumulation of cells in G2, followed by lysis of cells in telophase (Z. Darzynkiewicz, personal communication). Similar studies with human cell lines showing a predominant cytotoxic or cytostatic effect may clarify the relationship between these two TNF responses.

Neither mouse nor human partially purified TNF has de-

Table 3.   Cytotoxic and cytostatic effects of human IFNs on five human cell lines

| | Number of anti-viral units causing 35% cytotoxicity or cytostasis at day 7 | | | | | | | | | |
| | BT-20 | | ME-180 | | SK-MEL-109 | | T-24 | | WI-38 | |
IFN	CT	CS	CT	CS	CT	CS	CT	CS	CT	CS
Natural IFN-α	270	25	6,500	110	1,250	26	>50,000	1,900	>50,000	230
Recombinant IFN-α	3,125	<781	44,000	<781	1,400	<781	>50,000	16,000	>50,000	>50,000
Natural IFN-β	<49	<49	260	<49	<49	<49	>12,500	<49	>25,000	160
Natural IFN-γ	130	72	<7.8	<7.8	22	27	>2,000	25	>2,000	>2,000

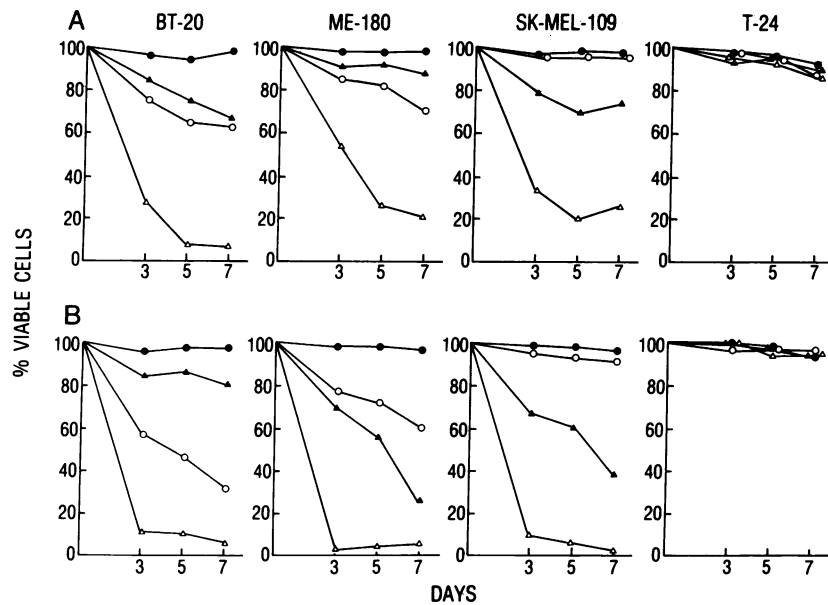CT, cytotoxic; CS, cytostatic.

FIG. 4. Effect of hTNF and IFNs on four human tumor cell lines. (*A*) Recombinant IFN-α. hTNF units/ml and IFN units/ml added to cultures: ●, control cultures; ○, hTNF alone at 4 units (BT-20), 16 units (ME-180), or 33 units (SK-MEL-109, T-24); ▲, IFN alone at 781 units (SK-MEL-109, T-24) or 3,125 units (BT-20, ME-180); △, hTNF and IFN together, in the amounts given above for each cell line. (*B*) Natural IFN-γ. hTNF units/ml and IFN units/ml added to cultures: ●, control cultures; ○, hTNF alone at 25 units (BT-20), 50 units (ME-180), 100 units (SK-MEL-109), or 200 units (T-24); ▲, IFN alone at 31.25 units (BT-20) or 125 units (ME-180, SK-MEL-109, T-24); △, hTNF and IFN together, in the amounts given above for each cell line.

tectable IFN activity, and the various human IFNs lack detectable TNF activity. Thus, TNF and IFN represent two classes of antitumor substances produced by cells of the hematopoietic system and, in the case of IFN-β, by other cell types as well. There are a number of other lymphokines that have been described, but these appear to act predominantly by regulating immune responses rather than having a direct cytotoxic or cytostatic effect on cells. Lymphotoxin is an exception in this regard (19, 20), and further studies on the reactivity of lymphotoxin with human cancer cells and its relationship to TNF are required.

In view of the strong anticellular activity of IFNs on a broad range of cancer cells *in vitro*, as shown in this study and in studies by others (21–24), it is surprising that the clinical effects of IFN have not been more evident. The *in vitro* synergistic antitumor action of TNF and IFN observed in the present study suggests that combined treatment with both factors might result in better clinical responses and may also provide clues with regard to requirements for optimal antitumor effects of interferon *in vivo*. If other factors, such as TNF, are necessary for maximal IFN action, deficiencies of these factors in the tumor or in the patient could account for the relatively modest antitumor effects of IFNs observed to date.

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