

# Human Tumor Xenografts Treated with Recombinant Human Tumor Necrosis Factor Alone or in Combination with Interferons

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## ABSTRACT

We have studied the activity of recombinant human tumor necrosis factor (rHuTNF) on six different human tumor xenografts derived from primary breast and bowel tumors and maintained by passage in nude mice. When 5  $\mu$ g rHuTNF was given daily intratumorally to mice with established (approximately, 0.5 cm) tumors, total tumor regression was observed by 3-4 weeks in three of six xenograft lines. In a further two lines tumor stasis or significant slowing of growth was seen. This anti-tumor action was not accompanied by any consistent macroscopic change in the tumor such as necrosis, but histological examination revealed tumor cell degeneration and a large peritumoral infiltration of host inflammatory cells after 4-7 days therapy. In contrast to these data, little effect was seen when the same dose of rHuTNF was administered i.p. to nude mice bearing these tumors. In only two of six lines was any significant slowing of tumor growth seen. A 5-fold increase in the i.p. dose resulted in improved activity on only one of two xenograft lines tested. Efficacy of the i.p. rHuTNF dose could, however, be enhanced by simultaneous administration of human interferon,  $\alpha$  or  $\gamma$ . No obvious signs of toxicity were observed at all rHuTNF doses administered and weights of control and treated mice at the end of the experiments were comparable.

## INTRODUCTION

TNF<sup>2</sup> is a protein produced by cells of the monocyte series, and possibly natural killer cells, that exerts a cytotoxic or cytostatic activity on some tumor cell lines *in vitro* and has the capacity to induce necrosis of solid transplantable tumors in mice (1, 2). *In vitro* the cytotoxic effects of TNF appear to be selective for transformed cells (3, 4) and can be greatly amplified by metabolic inhibitors such as actinomycin D or IFNs (3, 4, 5).

Like IFNs, TNF probably belongs to a family of molecules with similar properties (1), and although its *in vivo* functions are not yet defined, evidence of various regulatory functions is emerging; for instance, TNF is homologous with cachetin, a macrophage product that suppresses the activation of lipoprotein lipase in cultured adipocytes and is associated *in vivo* with a rise in serum triglycerides and wasting (6); TNF has striking effects on polymorph function (7); TNF can induce class I HLA expression in endothelial cells and fibroblasts (8); and most recently, Degliantoni *et al.* (9) have reported that natural killer cell cytotoxic factor, a soluble factor produced by natural killer cells which might mediate their cytotoxic effects, can be neutralized by an antibody to TNF and that recombinant TNF can mediate natural killer cell cytotoxic factor activity (9).

The *in vivo* activity of TNF on transplantable animal tumors, its selectivity for tumor cells *in vitro*, its synergy with IFNs, and emerging evidence of regulatory functions, have led to great

interest in this factor as an anticancer agent. The molecular cloning of the genes for human and mouse TNF (10, 11, 12) has made such investigations possible.

In order to learn more of the activity of TNF against human cancer we have studied the effects of pure human recombinant TNF both alone and in combination with IFNs on six different human tumor xenograft growing in nude mice. Because of the lack of stringent species specificity of TNF<sup>3</sup> such experiments can provide evidence of both direct cytotoxic effects on the tumors *in vivo* and immunomodulatory activities or deleterious side effects on the host.

## MATERIALS AND METHODS

**Mice.** Female specific pathogen free *nu/nu* mice of mixed genetic background were bred by the Imperial Cancer Research Fund animal breeding unit, Mill Hill, London, United Kingdom. They were housed in negative pressure isolators and used for experiments when 6-9 weeks old.

**Tumors.** The human tumor xenografts were derived from primary untreated human breast or bowel carcinomas implanted s.c. as 1-mm cubes into mice within 4 h of removal from the patient. They were maintained by passage in the nude mice at 6- to 8-week intervals. The histological diagnosis of each of the tumors is shown in Table 1.

**Tumor Necrosis Factor.** Recombinant human TNF (11) was of more than 99% purity and contained endotoxin, less than 40 ng/mg. The specific activity was  $2.5 \times 10^7$  units/mg.

**Interferons.** Recombinant human  $\gamma$ -interferon (Immuneron) was kindly supplied by Biogen S. A. (Geneva, Switzerland). It had a specific activity of  $2 \times 10^7$  units/mg and was greater than 99% pure. Pure human lymphoblastoid interferon (Wellferon) was kindly supplied by Wellcome Research Laboratories (Kent, United Kingdom). It had a specific activity of  $2 \times 10^8$  units/mg. Both IFNs were assayed against their appropriate reference standard in our laboratory before use as described (13).

**Experimental Design.** Six-week-old tumors were minced finely with scissors and 0.05 ml tumor suspension was injected s.c. into a ventral site on each mouse. After 7-21 days when tumors measured approximately 0.5 cm, therapy was started. Each experimental group contained 4 mice each bearing a single tumor. rHuTNF, HuIFN- $\alpha$ (Ly), or rHuIFN- $\gamma$  was diluted to the appropriate concentration with PBS plus BSA, 3 mg/ml (Sigma Chemical Co., Dorset, United Kingdom) and stored in single dose aliquots at  $-70^\circ\text{C}$ . Mice were given injections daily i.p. or i.t. of 0.1 ml of the appropriate TNF dose or PBS/BSA control solution. If tumor regression occurred in the i.t. treated group, therapy continued s.c. at or near the site of the original tumor. rHuIFN- $\gamma$  was injected daily i.p. in a 0.2 ml dose and HuIFN- $\alpha$ (Ly) daily s.c. in a 0.2 ml dose. These results gave maximal circulating serum levels as described (13). Tumors were observed daily for signs of necrosis and measured once weekly with calipers. The tumor size indices shown in Figs. 1-4 are a multiplication of the two largest tumor diameters at right angles to each other.

## RESULTS

**Effects of i.t. or i.p. Administration of rHuTNF Alone.** In the first series of experiments we studied the effect of i.t. adminis-

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<sup>2</sup> The abbreviations used are: TNF, tumor necrosis factor; rHuTNF, recombinant human tumor necrosis factor; i.t., intratumoral; IFN, interferon; rHuIFN- $\gamma$ , recombinant human  $\gamma$ -interferon; HuIFN- $\alpha$ (Ly), human lymphoblastoid cell derived  $\alpha$ -interferon; PBS, phosphate buffered saline; BSA, bovine serum albumin.

Table 1 Origins of the 6 human tumor xenografts

| Tumor code | Original site in patient | Histological diagnosis        | Used between passages |
|------------|--------------------------|-------------------------------|-----------------------|
| 1068       | Breast                   | Mucoid carcinoma              | 41 and 43             |
| NCH        | Breast                   | Infiltrating ductal carcinoma | 8 and 9               |
| NcMc       | Breast                   | Infiltrating ductal carcinoma | 4 and 5               |
| GFW        | Bowel                    | Adenocarcinoma of the cecum   | 8 and 9               |
| GFC        | Bowel                    | Adenocarcinoma of the cecum   | 8 and 9               |
| GF De      | Bowel                    | Adenocarcinoma of the cecum   | 6 and 7               |

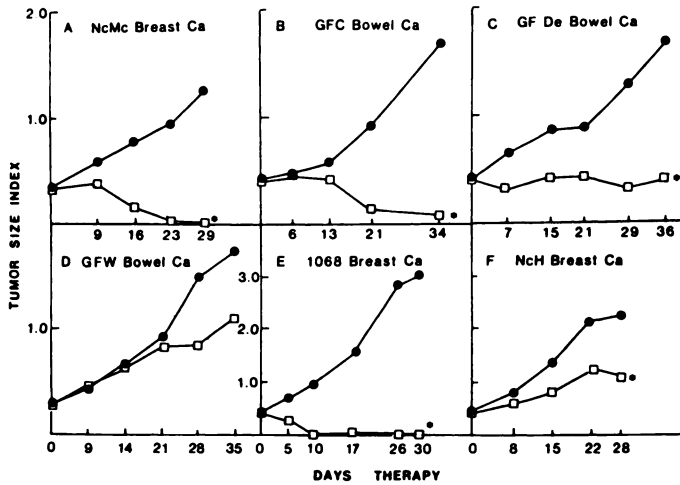


Fig. 1. Effect of i.t. administration of rHuTNF on growth of 6 human tumor xenografts. ●, control solution, i.t. daily in 0.1 ml; □, TNF, 5 μg i.t. daily in 0.1 ml. Each experimental group contained 4 mice; points, mean of data. ★, tumor size index at end of experiment statistically different from control value: P = 0.001 (A); P = 0.0001 (B); P = 0.0009 (C); P = 0.003 (E); P = 0.01 (F) (Student's *t* test). SEs of each group at the end of the experiment were: A, control = 0.10, TNF = 0.022; B, control = 0.67, TNF = 0.088; C, control = 0.11, TNF = 0.15; D, control = 0.25, TNF = 0.27; E, control = 0.38, TNF = 0.01; F, control = 0.25, TNF = 0.18. Ca, carcinoma.

tration of 5 μg rHuTNF daily on the six different human tumor lines (Fig. 1).

In a typical set of results, complete regression of 3/4 of the tumors was seen after 23 days therapy with TNF in the breast tumor xenograft NcMc (Fig. 1A), and similar results were seen with the bowel cancer line GFC (Fig. 1B). The other tumor in each of these groups regressed but was not entirely abrogated. By 26 days all four 1068 breast tumors had disappeared (Fig. 1E). Less striking effects were seen in the other 3 tumor xenografts with tumor stasis observed with GF De (Fig. 1C) (*P* = 0.0009 by Student's *t* test) and a significant slowing of growth with NcH (Fig. 1F) (*P* = 0.01). Some inhibition of the bowel xenograft GFW was also seen but this was not statistically significant. Tumors injected with control PBS/BSA solution grew at the same rate as uninjected controls (data not shown). The tumor sizes within a group did not vary greatly. As an example of this effect SEs of each group are given in the legend to Fig. 1. Careful observation of treated tumors failed to reveal macroscopic signs of necrosis although redness around the tumor and occasionally i.t. hemorrhage was observed. Although over 80 individual tumors were treated in this way only 5 visible necrotic lesions with scabbing and resolution of tumor were observed. In one of the experiments (Fig. 1B), mice whose tumors had regressed completely were observed without therapy for a further 14 weeks without tumor recurrence.

In contrast to the striking antitumoral activity of i.t. administered rHuTNF, the same dose administered i.p. had little effect as shown in Fig. 2. There was no significant difference between control and treated tumors in xenografts NcMc (Fig. 2A), GF De (Fig. 2C), GFW8 (Fig. 2D), and NCH (Fig. 2F);

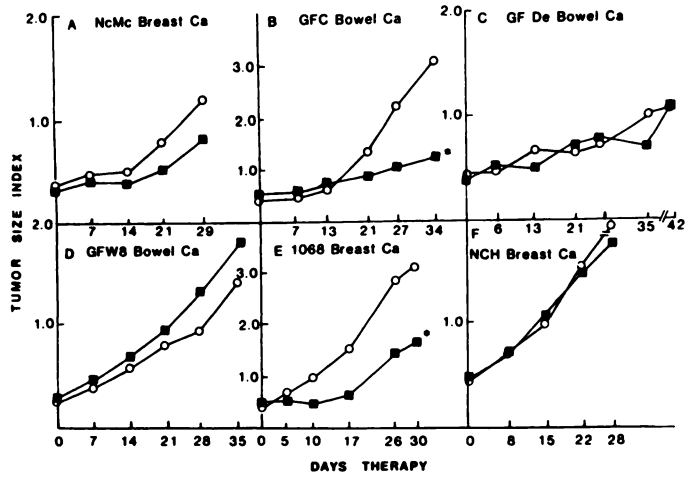


Fig. 2. Effect of i.p. administration of rHuTNF on growth of 6 human tumor xenografts. ○, control solution i.p. daily in 0.1 ml; ■, TNF 5 μg i.p. daily in 0.1 ml. Each experimental group contained 4 mice; points, mean of data. ★, tumor size index at end of experiment statistically different from control at *P* = 0.08 (B) and *P* = 0.03 (E) (Student's *t* test). Ca, carcinoma.

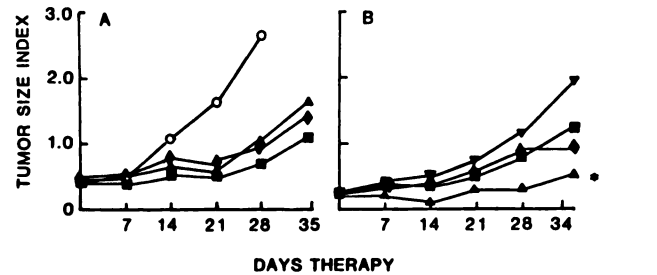


Fig. 3. Dose response curves for i.p. rHuTNF on 1068, and NcMc xenograft. A, 1068. ○, control solution i.p. daily; ■, TNF, 5 μg i.p. daily; ◆, TNF, 10 μg i.p. daily; ▲, TNF, 25 μg i.p. daily. B, NcMc. ○, control solution i.p. daily; ■, TNF, 5 μg i.p. daily; ◆, TNF, 10 μg i.p. daily; ▲, TNF, 25 μg i.p. daily. Each experimental group contained 4 mice; points, mean of data. ★, tumor size index at end of experiment statistically different from control value at *P* = 0.01.

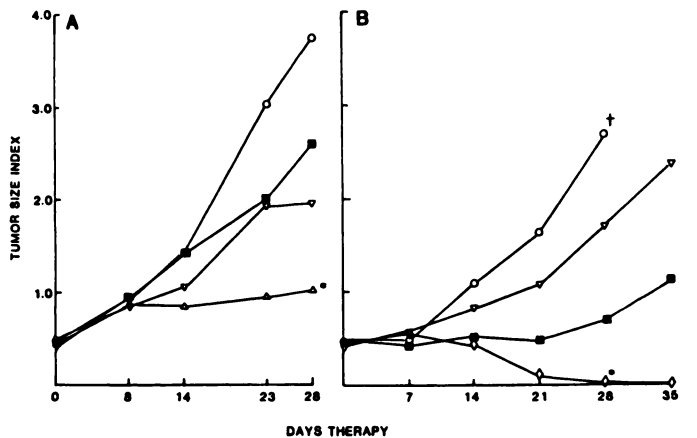


Fig. 4. Effects of combinations of rHuTNF and rHuIFN-γ or IFN-α(Ly) on the 1068 xenograft. A, ○, control solution i.p. daily; ■, TNF, 5 μg i.p. daily; ▽, HuIFN-α(Ly), 1 × 10<sup>6</sup> s.c. daily; △, TNF, 5 μg i.p. + HuIFN-α(Ly) 1 × 10<sup>4</sup> units s.c. daily. B, ○, control solution i.p. daily; ■, TNF, 5 μg i.p. daily; ▽, rIFN-γ, 2 × 10<sup>5</sup> i.p. daily; ◇, rIFN-γ, 2 × 10<sup>5</sup> i.p. daily + TNF, 5 μg i.p. daily. Each experimental group contained 4 mice; points, mean of data. ★, tumor size index at end of experiment statistically different from control value at *P* = 0.02 (A) and *P* = 0.03 (B); +, control mice killed before end of experiment due to ulceration of tumors.

however, in 2 of the lines most sensitive to the i.t. effects of rHuTNF, GFC and 1068, significant slowing of tumor growth was observed (Fig. 2, B and E). In a further series of experiments we tried to increase the therapeutic efficiency of rHuTNF given i.p. by increasing the dose; however, in the 1068 xenograft

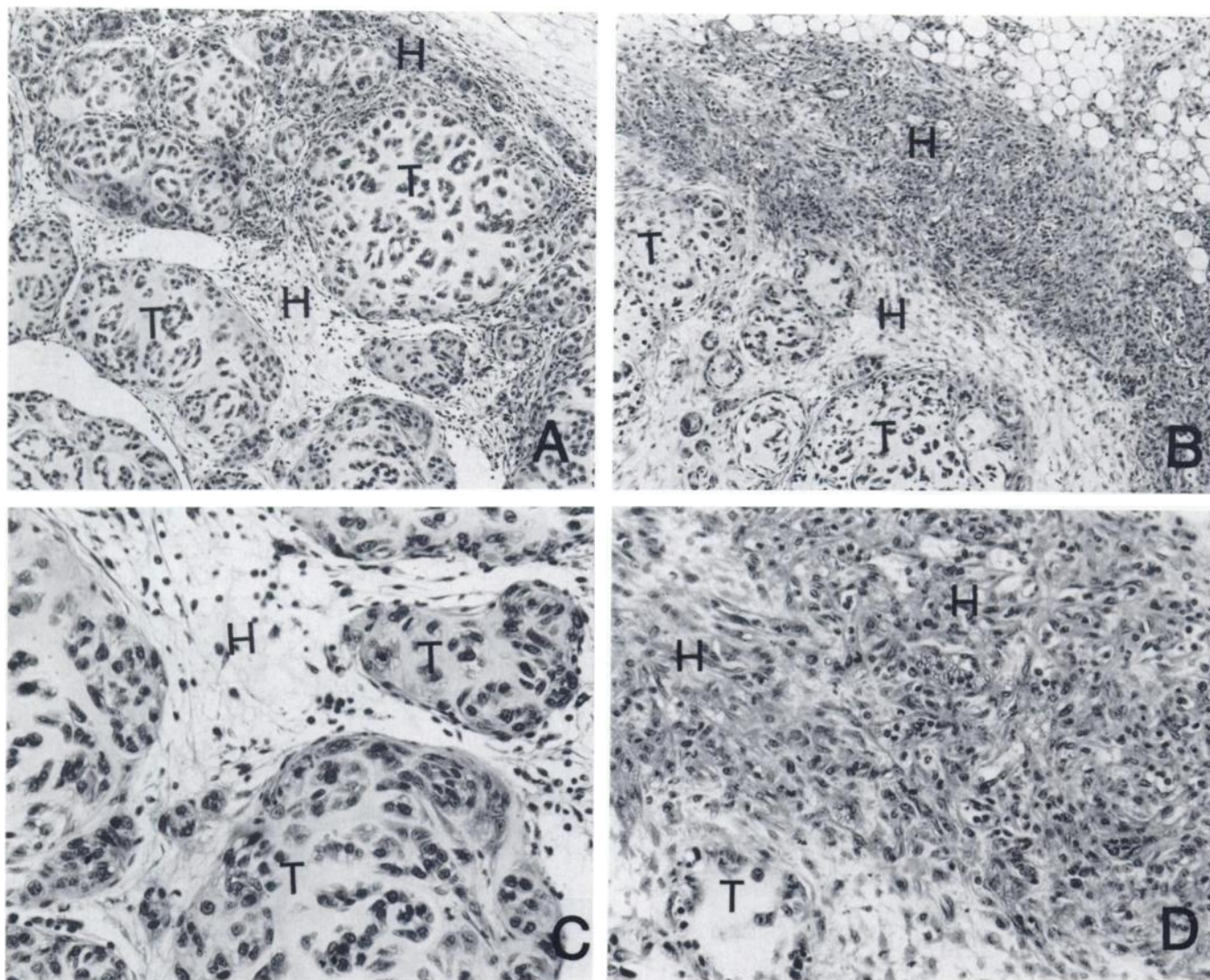


Fig. 5. Histological appearance of control i.t. (A and C) and TNF, 5  $\mu$ g i.t. treated (B and D) 1068 breast cancer tumors after 4 days therapy *in vivo*.  $\times 100$  (A and B).  $\times 300$  (C and D). Tumors were fixed in methacarn (methanol:chloroform:acetic acid, 60:30:10). Hematoxylin and eosin. T, tumor area; H, host stromal reaction to the tumor. Note the thickened peritumoral area in B and D.

which was inhibited by 5  $\mu$ g rHuTNF i.p. daily, up to 5-fold increases in dose produced no greater effect; in fact, the 25- $\mu$ g/day dose was marginally less effective although this difference was not significant (Fig. 3A). In contrast a 5-fold increase in the dose given to the NcMc tumor, resistant to 5  $\mu$ g rHuTNF daily i.p., resulted in a significant slowing of tumor growth and regression of one-fourth of the tumors ( $P = 0.01$ ) (Fig. 3A).

**Effects of rHuTNF in Combination with IFNs.** Recombinant HuTNF given i.p. to mice bearing the 1068 xenograft caused a slowing of tumor growth (Figs. 2E and 3A). We then investigated whether combination of this therapy with human IFN- $\alpha$  or - $\gamma$  would enhance this effect.

As shown in Fig. 4, combination with either IFN resulted in an antitumor effect greater than that seen with each agent alone and this was more marked with rHuIFN- $\gamma$  (Fig. 4B). rHuIFN- $\gamma$  had no significant effect on tumor growth alone but when combined with a tumor growth inhibitory dose of rHuTNF complete regression of all 4 tumors was seen ( $P = 0.03$ ). With HuIFN- $\alpha$ (Ly) combinations, either agent alone slowed tumor growth but the combination was tumor static (Fig. 4A) ( $P = 0.02$ ).

**Side Effects of rHuTNF Therapy.** No obvious side effects

were seen at any dose of rHuTNF given i.p. or i.t. alone or in combination with IFNs. Occasional flaking of the ventral skin was seen, but mice showed no signs of lethargy or wasting. At the end of several experiments mice were weighed once their tumors were removed but no differences in control and treated mice were observed; e.g., in the experiment shown in Fig. 3A the weights of groups of 4 mice at the end of the experiment were as follows: control,  $28.40 \pm 2.42$  (SE) g; TNF, 5  $\mu$ g i.p. daily,  $28.57 \pm 1.38$  g; TNF, 10  $\mu$ g i.p. daily,  $28.91 \pm 3.6$  g; TNF, 25  $\mu$ g i.p. daily,  $28.32 \pm 0.94$  g.

**Histology of Treated Tumors.** We looked at tumors treated with rHuTNF either i.t. or i.p. after 4–7 days therapy. No obvious differences between control and rHuTNF i.p. 5  $\mu$ g daily treated mice were seen even in the 1068 xenograft which was sensitive to this dosage regimen; however, striking changes were seen 4–7 days after the start of therapy in the i.t. treated tumors 1068, NCH, and GFC. As shown in Fig. 5 marked degenerative changes were seen within the tumors which were also surrounded by a prominent inflammatory cell infiltrate containing macrophages, eosinophils, polymorphs, lymphocytes, and small blood vessels. These changes were seen in all 3 tumors.

Biopsies taken at a later date from areas where the tumor had regressed revealed normal skin components.

## DISCUSSION

This study has shown that rHuTNF is an effective antitumor agent for human breast and bowel xenografts growing *s.c.* in nude mice when administered locally. These results confirm that pure TNF can have broad spectrum antitumor activity and are similar to data obtained with recombinant TNF on murine tumor cell lines grown in syngeneic mice (10)<sup>4</sup> and with partially purified serum derived TNF used against murine tumors and human tumor xenograft (2, 14). Systemic administration of rHuTNF was not, however, so successful and this is probably a pharmacokinetic problem. TNF administered by any route disappears completely from the circulation by 3 h and exposure to TNF for 3 h/day is not sufficient to kill sensitive cell lines *in vitro*.<sup>5</sup> Serum derived TNF was more effective systemically (2, 14) but this material may well have contained other cytotoxins and lymphokines that contributed to the therapeutic effect.

Another reason for the failure of systemic therapy is suggested by the experiments of Haranaka *et al.* (14). Using partially purified murine TNF derived from serum of endotoxin treated mice they found that *i.v.* therapy of MethA sarcomas was much less effective in BALB/c *nu/nu* than in BALB/c *nu/+* mice whereas *i.t.* therapy was equally effective in both. Thus a role for host T-cells in response to TNF could be suggested. These findings need repeating with purified recombinant TNF.

One difference between our results and those obtained with cell lines grown *in vivo* is the lack of macroscopic evidence of hemorrhagic necrosis; however, the microscopic appearance of tumors treated *i.t.* with TNF was of interest. In particular the peritumoral cuffing of host inflammatory cells may indicate that a host response to the tumor was underway. It is possible that such a host response would be more marked if recombinant murine TNF was used. Although TNF is not strictly species specific, mouse TNF is approximately 2.5-fold more active on murine cells than human cells<sup>3</sup> and could conceivably have a greater immunomodulatory role; however, in preliminary experiments<sup>6</sup> we have found that recombinant murine TNF has much the same activity as rHuTNF and as yet we have no evidence of a greater immunomodulatory effect of this cytotoxin.

The rHuTNF was not toxic to mice even at the highest (25  $\mu$ g) dose; however, although the side effects of TNF may be more strictly species related, our preliminary studies indicate that, at least at lower doses, murine TNF caused no significant weight loss or gross toxicity.<sup>6</sup>

The effect of systemic TNF was enhanced by simultaneous administration of IFN- $\alpha$  or - $\gamma$  on the 1068 xenograft line. This confirms results obtained *in vitro* with many cell lines (4, 5) and is a promising finding; however, these results are only preliminary and extension to other xenografts is necessary; also, it is important to remember that human IFN has no effect

on the nude mouse host (13) and that in the clinic IFN/TNF combinations might have enhanced toxicity. We can investigate this in the model system by using murine IFN- $\gamma$  in our experiments.

In conclusion, while these and other studies have demonstrated that TNF can have broad spectrum anticancer activity, further experimentation is required for successful application of this interesting agent to human cancer studies.

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<sup>4</sup> W. Fiers, unpublished data.

<sup>5</sup> L. Fransen and W. Fiers, unpublished data.

<sup>6</sup> F. R. Balkwill, unpublished data.