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IN VIVO ANTI-TUMOUR ACTIVITY OF RECOMBINANT HUMAN AND MURINE TNF, ALONE AND IN COMBINATION WITH MURINE IFN-γ, ON A SYNGENEIC MURINE MELANOMA

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TNF, a protein released by induced macrophages, is believed to mediate, at least in part, the tumoritoxic effects of activated macrophages. *In vitro*, it has cytotoxic effects on transformed cells but not on normal cells, and *in vivo* it causes necrosis of tumours. Recently, both human and murine TNF became available as pure recombinant proteins. Subsequent work confirmed its *in vitro* cytotoxic activity, selective for transformed cells, and revealed other, non-cytotoxic effects on some normal cells. *In vitro*, the B16BL6 melanoma cells, syngeneic with C57BL6 mice, are resistant to the cytotoxic effects of rTNF but become sensitive when they are also treated with rIFN-γ. We report that established, s.c. B16BL6 tumours *in vivo* can be induced to necrotize and regress by a combined systemic treatment with rTNF and murine rIFN-γ. Although TNF is not species-specific *in vitro*, the effects of treatment with human and murine rTNF *in vivo* are different: with murine rTNF, the synergism with rIFN-γ is relatively less clear, the addition of IFN-γ is not necessary to induce regression, toxicity is more pronounced and additional mechanisms of tumoritoxicity could be involved. Relapses are frequent but complete cures have been observed. These results give further evidence in favour of a potential clinical use of TNF in combination therapy, e.g. with IFN-γ. However, there is still a need to develop better regimens, especially for consolidation, and to continue research in order to understand and limit the toxicity, which could be mediated by the activating effects of TNF on some normal cell types.

TNF is a protein released by induced macrophages. Originally detected in the serum of BCG-infected mice injected with endotoxin (LPS) (Carswell *et al.*, 1975; Old, 1985), it causes necrosis of tumours *in vivo* and has cytotoxic effects *in vitro* on a series of human and murine transformed cells but not on normal cells (Carswell *et al.*, 1975; Old, 1985; Helson *et al.*, 1975; Ruff and Gifford, 1981; Williamson *et al.*, 1983; Haranaka *et al.*, 1984).

Recently, the cDNA genes encoding human TNF (Pennica *et al.*, 1984; Marmenout *et al.*, 1985; Shirai *et al.*, 1985; Wang *et al.*, 1985) and murine TNF (Fransen *et al.*, 1985; Pennica *et al.*, 1985) have both been cloned and expressed in *E. coli*. The recombinant proteins were purified to homogeneity. Subsequent research confirmed the selective cytotoxic effects *in vitro* on tumour cells previously seen with the natural TNF (Fransen *et al.*, 1986b; Sugarman *et al.*, 1985).

It is now realized that TNF also has a number of non-cytotoxic effects on normal cells. It exerts a mitogenic effect on both mouse and human untransformed fibroblasts (Fiers *et al.*, 1986; Vilcek *et al.*, 1986) and, in the case of a precursor to a cytotoxic T-cell (Erard *et al.*, 1984), can replace the specific action of IL-1 (Fiers *et al.*, 1986). At least in human systems TNF, in synergism with IFN-γ, activates neutrophils *in vitro* (Shalaby *et al.*, 1985). TNF is also identical to cachectin (Torti *et al.*, 1985; Beutler *et al.*, 1985*a*) and could be a mediator of other effects caused by endotoxin (Beutler *et al.*, 1985*b*). Furthermore, TNF directly or indirectly influences gene expression in untransformed target cells. For example, it enhances the synthesis of class-I HLA-antigens in vascular endothelial cells and in dermal fibroblasts (Collins *et al.*, 1986) and induces another surface antigen on endothelial cells (Pober *et al.*, 1986). This latter effect is another property that TNF shares with IL-1.

TNF binds to specific receptors on the membrane of the target cells (Rubin *et al.*, 1985), and is subsequently internalized and degraded (Baglioni *et al.*, 1985). The presence or absence of TNF receptors, however, is not correlated with sensitivity or resistance to the cytolytic effects of TNF (Tsujimoto *et al.*, 1985; Ruggiero *et al.*, 1986).

In contrast to IFN-γ, TNF shows little species-specificity. Murine and human TNF are 79% homologous at the protein level and only the murine natural TNF is a glycoprotein (Marmenout *et al.*, 1985). The *in vitro* activities of murine and human rTNF were compared, and only a very limited degree of species-specificity was found: some cells required a lower concentration of the homologous rTNF to attain a similar degree of lysis. Also, for some cell lines the synergism with IFN-γ was more pronounced in treatments with the homologous rTNF (Fransen *et al.*, 1986*a*).

The synergistic activity of IFN-γ and TNF was expected from the synergism between IFN and a similar factor produced by B-lymphocytes (Williamson *et al.*, 1983) and was shown *in vitro* for human tumour cells (Fransen *et al.*, 1986*b*) and murine tumour cells (Fransen *et al.*, 1986*a*).

We have now investigated whether the *in vitro* findings regarding the cytotoxic activity, the synergism with IFN-γ and the species-specificity of TNF are also valid for the *in vivo* situation.

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Abbreviations: TNF, tumour necrosis factor; rTNF, recombinant tumour necrosis factor; rIFN-γ, recombinant interferon gamma; LPS, lipopolysaccharide; IL-1, interleukin 1; i.p., intraperitoneal; p.l., paralesional; PBS, phosphate-buffered saline; TSI, tumour size index; CHO-cells, Chinese hamster ovary cells; s.c., subcutaneous (ly); TNS, tumor necrosis serum.

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As a syngeneic tumour model we chose the B16BL6 melanoma (Hart and Fidler, 1980), derived from a spontaneous melanoma and syngeneic with C57BL6 mice. *In vitro,* this tumour is not sensitive to the cytotoxic activity of TNF unless treatment is combined with murine rIFN-γ (Fransen *et al.*, 1986*a*).

MATERIAL AND METHODS

Cytokines

Murine and human rTNF, produced by *E. coli* containing appropriate plasmids (Fransen *et al.*, 1985; Marmenout *et al.*, 1985) were purified to apparent homogeneity. Human rTNF has a specific activity of 2×10^7 U/mg and the preparation contained less than 1 ng endotoxin/mg protein. Murine TNF has a specific activity of 5×10^7 U/mg and the preparation contained less than 40 ng endotoxin/mg protein. TNF aliquots were stored at −70°C, then thawed and diluted in endotoxin-free PBS immediately before injection.

Glycosylated murine rIFN-γ was produced in CHO (Chinese hamster ovary) cells and partially purified on a CPG10 column (Serva, Heidelberg, FRG). It was stored in the desorption buffer containing 50% glycerol and 1.5M NaCl in PBS. The specific activity was around 10^5 U/mg protein and endotoxin contamination was 1–10 ng endotoxin/mg protein. Murine IFN-γ was titrated on L929 cells challenged with VSV (vesicular stomatitis virus). IFN-γ activity is expressed in laboratory units. Endotoxin levels were measured by the LAL-assay (Limulus amoebocyte lysate assay).

Animals

C57BL/6J mice were obtained from IFFA-CREDO (Saint-Germain sur l'Arbresle, France). Male mice, about 8 weeks old at inoculation, were used throughout. All the mice in a single experiment were received as one group from the breeder.

Tumour

B16BL6 tumour cells were a gift from Dr. M. Mareel by courtesy of Dr. I. Fidler (Hart and Fidler, 1980). Cells were expanded *in vitro*. At the day of inoculation (day 0), cells were suspended by a short EDTA treatment and washed once in complete medium and twice in PBS. Five million cells in 0.1 ml of PBS were injected s.c. in the backs of the mice just in front of the hind limb, causing the appearance of an s.c. tumour nodule at the inoculation site in all animals between days 8 and 10. All the mice used in a single experiment were inoculated on the same day and with cells of the same suspension. A few days later they were allocated at random to groups before tumour nodules appeared.

Treatment and monitoring

Mice were treated with cytokines and/or controls at the days and according to the regimens indicated below. Two routes of administration were used: the intraperitoneal (i.p.) way as a systemic administration route and the paralesional (p.l.) way as a local one. By p.l. injection we understand an s.c. injection near the site of the tumour but outside the tumour nodule. I.p. injections were made in a total volume of 0.5 ml while p.l. injections had a total volume of 0.1 ml.

Tumour size was assessed every 2 or 3 days and expressed as tumour size index (TSI), calculated as the product of the largest diameters at right angles to each other (Balkwill *et al.*, 1985).

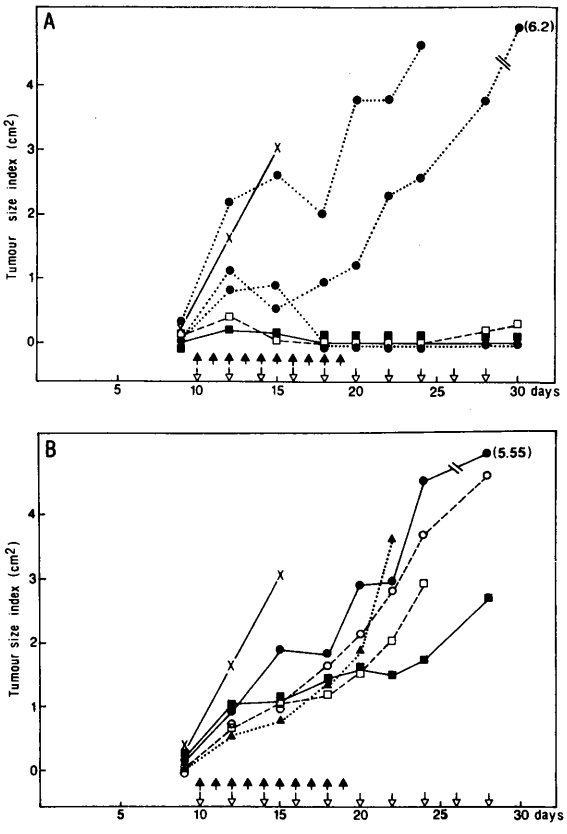


FIGURE 1 – Effects of treatments with human rTNF and/or murine rIFN-γ on tumour growth. Tumour-size index (cm × cm) is plotted against time (days post-inoculation). The control group was the same for Figure 1*a* and 1*b* and consisted of mice treated with daily injections of PBS. All mice received 10 injections, starting on day 10, except for 2 mice of the control group that died on day 17 and 2 mice of the group treated with a combination of rTNF i.p. and rIFN-γ i.p. on alternate days, that died on days 22 and 28, respectively. (▲) or (▽): days of injection as indicated at the bottom of the Figure (closed symbols refer to daily injections, open symbols refer to alternate-day treatments).

(*a*) Results of p.l. treatments: x——x, control group (PBS); ●·····●, 5 μg rTNF (3 individual mice are plotted) daily; ■——■, 5 μg rTNF + 10^3 U IFN-γ, daily; □- - -□, 5 μg rTNF + 10^3 U IFN-γ, alternate days.

(*b*) Results of i.p. treatments: x——x, control group (PBS); ▲·····▲, 10^3 U IFN-γ, daily; ●——●, 5 μg rTNF, daily; ○- - -○, 5 μg rTNF, alternate days; ■——■, 5 μg rTNF + 10^3 U IFN-γ, daily; □- - -□, 5 μg rTNF + 10^3 U IFN-γ, alternate days. (When 1 out of 3 mice survived, the results were not further plotted.)

RESULTS

Treatment of B16BL6 melanoma with human rTNF and murine rIFN-γ

In this study, summarized in Figure 1, 10 groups of 3 mice each were treated. Treatment started at day 10

post-inoculation and comprised 10 injections of 5 μg (10^5 U) human rTNF and/or 10^3 U murine rIFN-γ by different administration routes and regimens (see legend to Fig. 1).

Mice treated with p.l. injections of rTNF in combination with rIFN-γ (Fig. 1*a*) showed regression of the tumour and apparent remission (6/6 mice). Two mice had a relapse, while the other 4 died from wound problems (peritoneal perforation) during or following rejection of the scab that had resulted from tumour necrosis. Mice treated with p.l. injections of 5 μg human rTNF alone gave varying responses: one had complete remission (no relapse during a 6-month follow-up period), one had growth retardation and one showed no response.

Mice treated with human rTNF and/or murine rIFN-γ i.p. (Fig. 1*b*) showed tumour-growth retardation but no necrosis or regression. Daily injections of the combination gave the strongest tumour-growth retardation.

Comparison of effects of human rTNF and murine rTNF alone and in combination with murine rIFN-γ

Groups of 6 mice each were treated with 5×10^5 U (25 μg) human rTNF or 5×10^5 U (10 μg) murine rTNF and/or 5×10^3 U murine rIFN-γ. Treatment, started on day 9, consisted of daily injections lasting until day 42 except in the case of those mice whose tumour had completely regressed (Fig. 2). Figure 2*a* and *b* gives the mean values for the groups treated with human rTNF (Fig. 2*a*) and murine rTNF (Fig. 2*b*), respectively. As a common reference the means for the PBS-treated and murine rIFN-γ-treated controls are plotted on both Figures. The variability within the groups can be assessed from Figure 2*c*–2*k*, in which the results for individual mice are plotted, together with the time of death and the exact periods of treatment.

I.p. administration of human rTNF and murine rIFN-γ in combination gave better results than the administration of either alone (Fig. 2*a*). The augmentation of the dose—compared with the experiment reported above—had both positive and negative effects. In the group that received the combination (Fig 2*k*), 4/6 mice showed not only tumour-growth retardation but even an apparently total necrosis. On the other hand, the toxicity of treatment caused the death of 4/6 mice by day 25. The two mice that showed an apparently total necrosis and survived the treatment developed a relapse at the margin of the necrotized area soon after the treatment was stopped (within 4–6 days). The i.p. administration of either human rTNF alone (Fig. 2*i*) or murine rIFN-γ alone (Fig. 2*d*) resulted at most in a aslight growth retardation, but in no case regression or total necrosis (0/6 mice). A statistically meaningful evaluation of these growth retardations is impossible due to the aggressive growth of the tumour in the control animals. However, even assuming that the growth retardation is real, this is not the "clinically significant" tumour-destroying effect which is being sought. Nevertheless, when each of the groups treated with human rTNF i.p. alone, murine rIFN-γ alone or PBS, is compared with the group treated with the combination of human rTNF i.p. and murine rIFN-γ i.p., the observed difference in the number of mice that show an apparently total necrosis is significant ($p<0.025$ as assessed by the Chi-square test).

Local (p.l.) treatment of the tumour with 5×10^5 U (25 μg) human rTNF combined with 5×10^3 U murine IFN-γ i.p. (Fig. 2*g*) resulted in apparently total necrosis (6/6 mice), which was already obvious after only 2 injections. Treatment was continued for a few days (see Fig. 2*g* for details) and was then stopped. All mice had relapses. P.l. injections of 5×10^5 U (25 μg) human rTNF (Fig. 2*e*) alone showed growth retardation and even temporary regression or partial (central) necrosis in 5 out of 6 mice, but in no case a total necrosis ($p<0.005$ for differences in total necrosis between these two groups).

I.p. administration of 5×10^5 U (10 μg) murine rTNF alone (Fig. 2*j*) caused tumour-growth retardation in 6/6 mice until the 13th day of treatment. Subsequently, all tumours necrotized ($p<0.005$ for total necrosis vs. control). Four of 6 mice died shortly after the necrosis had become total or nearly total; two of these had a perforated peritoneum. Both surviving mice showed marginal relapses a few days after the treatment had been stopped.

Tumours borne by mice receiving a treatment consisting of 5×10^5 U (10 μg) murine rTNF p.l. and 5×10^3 U murine rIFN-γ i.p. (Fig. 2*h*) continued to grow until day 3 of treatment, then regressed and necrotized between days 9 and 13 of treatment. Two of 6 mice died during tumour necrosis but before this had become total, while one mouse died from a perforated peritoneum. Of the 3 remaining mice, one developed a metastasis in the inguinal lymph node while the two others are alive and well after a follow-up period of more than 6 months ($p<0.025$ *vs.* control for total necrosis).

The response to p.l. treatment with 5×10^5 U (10 μg) murine rTNF without IFN-γ (Fig. 2*f*) was more variable. Four of 6 mice showed regression and apparently total necrosis, but sooner or later all had a relapse. One mouse died after the onset of necrosis but before necrosis had become total, while another did not show a clear necrosis.

The fact that the clear tumour-destroying effect in some groups was not followed by a corresponding increase in survival or complete cures will be discussed below.

DISCUSSION

The results reported here clearly show an *in vivo* anti-tumour effect of both murine and human rTNF administered alone or in combination with IFN-γ. It should be emphasized that the B16BL6 tumour is insensitive *in vitro* to the effect of TNF unless murine IFN-γ is also added. To achieve these results we had to work out a treatment regimen that is quite different from the single-shot cures described for the highly sensitive MethA sarcomas (Haranaka *et al.*, 1984).

As we expected, the difference between p.l. and systemic treatments seen in the first experiments vanished when we increased the dose. This strongly suggests that the difference is due to pharmacokinetic rather than intrinsic factors. Daily injections were superior to regimens with longer intervals.

The results described were obtained with extra-tumoral administration of TNF. This might be important in view of the possible use of TNF in the treatment of

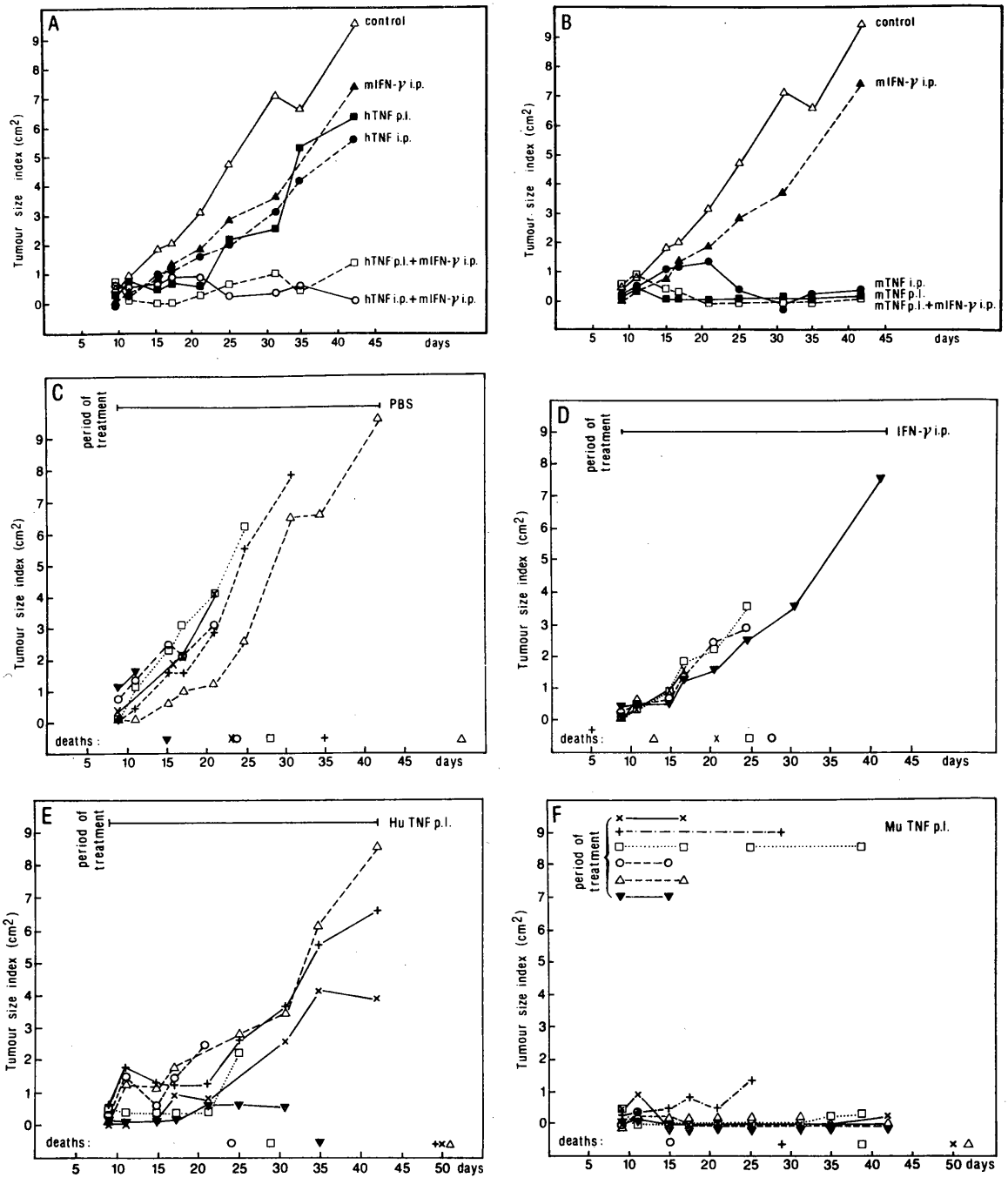
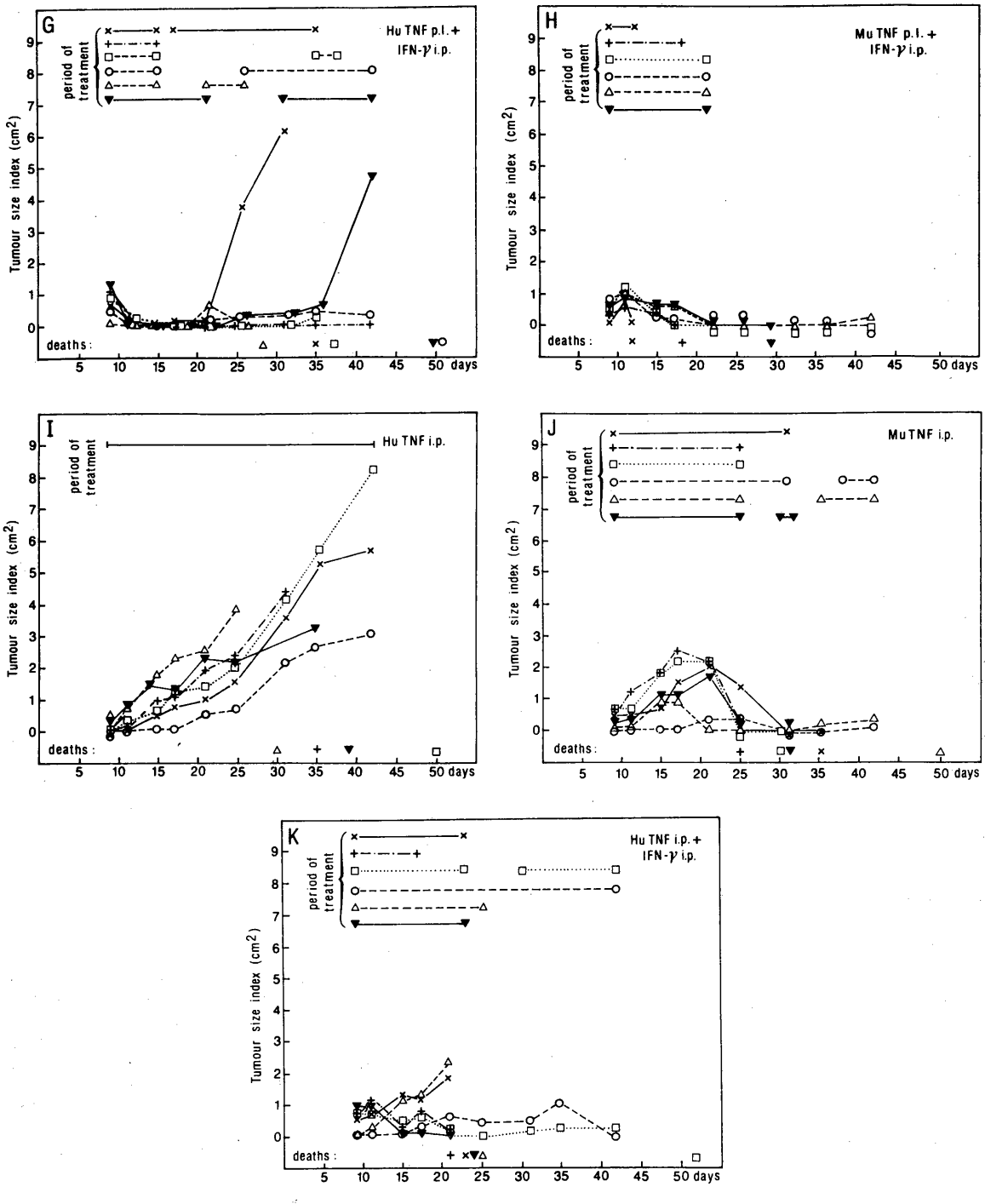


FIGURE 2 – Comparison of effects of treatment with human or murine rTNF, alone and in combination with murine rIFN-γ. Tumour-size index (cm × cm) is plotted against time (days post-inoculation). Results in Figure 2*a* and 2*b* are means of 6 mice or of the surviving mice. As a reference, PBS-treated controls and mice treated with murine rIFN-γ alone are plotted in both *a* and *b*. Treatment (daily injections) started on day 9 and lasted until day 42 except for mice in which the tumour had apparently regressed completely. In the latter cases, treatment was arrested a few days later, and restarted when relapse became obvious. Panels *c-k* show the variance within the groups. Tumour-size index is plotted against time for individual mice and the exact periods of treatment are indicated at the top of the figure. Time of death of individual mice are indicated by symbols at the bottom of the figure. Time of death of mice that were alive at day 55 are mentioned below.

(*a*) Results with human rTNF: △——△, control group (PBS) (*c*); ▲- - -▲, 5 × 10^3 U murine rIFN-γ i.p. (*d*); ■——■, 5 × 10^5 U (25 μg) human rTNF p.l. (*e*); □- - -□, 5 × 10^5 U (25 μg) human rTNF p.l. + 5 × 10^3 U murine rIFN-γ i.p. (*g*); ●- - -●, 5 × 10^5 U (25 μg) human rTNF i.p. (*i*); ○——○, 5 × 10^5 U (25 μg) human rTNF i.p. + 5 × 10^3 U murine rIFN-γ i.p. (*k*).

(*b*) Results with murine rTNF: △——△, control group (PBS) (*c*); ▲- - -▲, 5 × 10^3 U murine rIFN-γ i.p. (*d*); ■——■, 5 × 10^5 U (10 μg) murine rTNF p.l. (*f*); □- - -□, 5 × 10^5 U (10 μg) murine rTNF p.l. + 5 × 10^3 U murine rIFN-γ i.p. (*h*); ●——●, 5 × 10^5 U (10 μg) murine rTNF i.p. (*j*). (*c*) - (*k*) x——x; +-·-+; □·····□; ○- - -○; △- - -△; ▼——▼, TSI for individual mice.

Days of death of mice that were alive on day 55: (*d*) 56; (▼) (*f*): 70; (▼) (*g*): 77(+); (*h*): one mouse died on day 59 (△) while the other two were still alive on day 198 (□,○); (*i*): 56 (x); 70(○); (*j*): 56(○); (*k*): 70(○).



disseminated disease, since results obtained with intratumoral administration of TNF cannot always be repeated with systemic administration of the drug (Haranaka *et al.*, 1984; Balkwill *et al.*, 1986). Whether the difference between intra- and extra-tumoral administration of the drug is also due to pharmacokinetic factors is not very clear, since augmentation of the systemic dose does not result in an improvement of the response (Balkwill *et al.*, 1986).

The fact that we saw some response when we gave human rTNF alone—a treatment that has no effect at all *in vitro*—can be explained in two ways. Firstly, it is conceivable that the endogenous level of IFN-γ, or of another factor that acts synergistically with TNF, is near the threshold level of such a cooperation. An alternative explanation is that indirect mechanisms of action of TNF are at work: in the human system, an activation of neutrophil functions (Shalaby *et al.*, 1985) and effects on endothelial cells (Pober *et al.*, 1986) have been demonstrated *in vitro*. Both could be involved in the anti-tumour activity.

It is clear, however, that in the case of human rTNF the addition of exogenous rIFN-γ is of major importance to the effectiveness of the treatment. This is in

accordance with the *in vitro* data but does not prove the importance of direct cytotoxic mechanisms, since indirect mechanisms could also be enhanced by IFN-γ (Shalaby *et al.*, 1985). The fact, however, that human rIFN-γ acted synergistically with TNF in a heterotransplanted human tumour model in nude mice (Balkwill *et al.*, 1986) (a system in which, due to the stringent species-specificity of interferons, human rIFN-γ has no influence on indirect mechanisms) strongly suggests that at least part of the synergistic anti-tumour activity of rIFN-γ is due to direct effects on the tumour.

The synergism is less clear with murine rTNF, where exogenous IFN-γ does not seem to affect the induction of regression. This absence of synergism in the induction of necrosis was also seen in another experiment, in which we used murine rTNF and murine rIFN-γ (results not shown). However, it is quite possible that the addition of IFN-γ could be important in reducing relapse frequencies. Another difference between human and murine rTNF is the slower induction of regression by murine rTNF. These results do not correspond with the *in vitro* data. *In vitro*, synergism is more pronounced with the homologous rTNF, and there is only a slight quantitative difference in effectiveness, in that more units of the heterologous rTNF are required to obtain the same degree of cytolysis (Fransen *et al.*, 1986*a*). Again, the two alternatives mentioned above can explain this difference: murine TNF could be a stronger inducer for endogenous cooperative factors in the mouse, or murine TNF could better elicit indirect anti-tumour mechanisms in the mouse.

The contrast between the difference in the effects of murine and human rTNF observed in the animal on the one hand, and the absence of such a difference regarding the direct cytotoxicity of the two rTNFs observed *in vitro* on the other hand, would suggest that the effects induced by TNF on normal cells are the result of the triggering of receptors or receptor-coupled mechanisms different from those responsible for direct cytotoxicity to malignant cells. Until now, it has been generally accepted that TNF has no species-specificity. This may be true for some but probably not all *in vitro* assays and is certainly not correct *in vivo*. For this reason, great care must be taken in extrapolating or comparing *in vitro* observations or observations with TNS to an *in vivo* situation with rTNF. Indeed, while most of the earlier work was done with murine or rabbit TNS, contemporary research is mainly performed with recombinant human TNF.

The two major obstacles to a more successful outcome of TNF anti-tumour therapy are relapses and toxicity. Relapses, which are frequent and generally occur soon after arrest of treatment, may possibly be prevented by the development of regimens for consolidation treatment. Therapeutic toxicity, which often has a fatal outcome, may be attributed to three factors: irrelevant, model-related problems (perforation of the peritoneum during rejection of the scab), necrosis-related problems (tumour-lysis syndrome) and also more direct TNF-induced toxicity. Assessing the relative contribution of each component, and hence the TNF-inherent toxicity, is not easy because of the lack of a good control group. Indeed, it is known that endotoxin and TNF are more toxic in tumour-bearing animals (Berendt *et al.*, 1980; Old, 1985). The only valid control would consist of C57BL6 mice bearing a tumour showing no response at all to the combination of IFN-γ and TNF. In this respect, when human rTNF is given alone (Fig. 2*a*), lethality is no higher than in the PBS-treated controls, even after correction for tumour size. However, part of the lethality is due to TNF-inherent toxicity probably caused by TNF effects on normal cells, *e.g.* endothelial cells. Our current research is directed towards the understanding and selective inhibition of these effects. Healthy mice treated with TNF showed weight loss, anorexia, hypothermia, malaise and scab formation upon repeated injections of TNF at the same site. These effects tend to be more pronounced with murine than with human rTNF and seem to be mouse-strain-dependent (data not shown). Murine IFN-γ was more toxic than we expected. However, to ascertain this toxicity is due to IFN-γ itself or to contaminants in the preparation, we must await further purification. That the beneficial effects of the murine rIFN-γ are not due to contaminants is almost certain, since a similar synergism was observed on human tumour cells with pure human rIFN-γ, both *in vitro* (Fransen *et al.*, 1986*a*) and *in vivo* (Balkwill *et al.*, 1986).

Our results provide further evidence for the considerable clinical potential of rTNF in tumour therapy, especially in combination therapy, *e.g.* with IFN-γ. Nevertheless, better regimens and/or adjuncts must be developed, especially for consolidation treatment, and research to limit the toxicity must continue.

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