

## Serum TNF- $\alpha$ levels in melanoma-bearing and healthy mice

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To determine differences in tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) persistency after TNF- $\alpha$  therapy, serum levels were monitored in tumor bearing and healthy mice. Melanoma B-16-bearing mice were treated peritumorally and healthy mice subcutaneously with recombinant human TNF- $\alpha$ , lacking 1-3 amino acids from N-terminal part (TNF- $\alpha$ Nv3). In healthy mice the peak TNF- $\alpha$  serum levels were detected one hour after application, while in tumor-bearing mice two hours after application. Also TNF- $\alpha$  elimination from serum of tumor-bearing mice was slower compared to healthy mice. The data suggest that TNF- $\alpha$ Nv3 pharmacokinetic mechanisms in tumor-bearing and healthy mice are different.

**Key words:** tumor necrosis factor; pharmacokinetics; melanoma, experimental; mice

### Introduction

Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is a monocyte/macrophage derived protein, originally identified by its ability to induce haemorrhagic necrosis of some tumors *in vivo*<sup>1</sup> and cytotoxicity against certain tumor cells *in vitro*.<sup>2</sup> The results of clinical trials with recombinant human TNF- $\alpha$  indicate that the side effects of TNF- $\alpha$  treatment can be severe.<sup>3,4</sup> For rational dose regimen, which could diminish the side effects, it is important to know how TNF- $\alpha$  is absorbed from the site of application, distributed through the body, and its activity preserved.

Despite the fact that TNF- $\alpha$  activity is at least partially species specific, animal models were often used to study pharmacokinetics of human TNF- $\alpha$ . However in all previous pharmacokinetic studies in mice,<sup>5</sup> rats,<sup>6,7</sup> rabbits and monkeys,<sup>8</sup> human recombinant TNF- $\alpha$  was applied to healthy animals.

Therefore the aim of our study was to compare absorption and distribution of human TNF- $\alpha$  in tumor-bearing and healthy mice. Besides antitumor effect, serum TNF- $\alpha$  levels were measured after local TNF- $\alpha$  application. Human recombinant TNF- $\alpha$ , lacking 1-3 amino acids from N-terminal part (TNF- $\alpha$ Nv3), was applied peritumorally in melanoma B-16 bearing mice and subcutaneously in healthy mice.

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## Materials and methods

### Animals

Inbred C57Bl/6 mice were purchased from Rudjer Bošković Institute, Zagreb, Croatia. Animals were maintained in constant room temperature ( $24^{\circ}\text{C}$ ) at a natural day/night cycle in a conventional mouse colony. Mice in good condition, without signs of fungal or other infections, 8-10 weeks old, were used in the experiments.

### Tumors

Melanoma B-16 was maintained in C57Bl/6 mice by serial transplantation. Tumor cells from the fourth isotransplantat generation were prepared for the described experiments by gentle mechanical disaggregation. Solid subcutaneous tumors, dorsolaterally in animals, were initiated by injection of  $5 \times 10^5$  viable melanoma cells. The viability of the cells was determined by trypan blue dye exclusion test.<sup>9</sup>

### Tumor necrosis factor

Recombinant human tumor necrosis factor- $\alpha$  analog, lacking 1-3 amino acids from N-terminal end (TNF- $\alpha$ Nv3), was used (ZIMET, Jena, Germany). Specific activity was  $2.2 \times 10^7$  U/mg, tested on L929 cells in the presence of actinomycin-D. Other properties of TNF- $\alpha$ Nv3 were described before.<sup>10</sup> TNF- $\alpha$ Nv3 was diluted in PBS (pH 7.4) before use.

### Treatment

TNF- $\alpha$ Nv3 was administered subcutaneously in tumor free and peritumorally in tumor-bearing mice.<sup>9</sup> The treatment was started when the tumors reached  $30-40 \text{ mm}^3$  in volume. TNF- $\alpha$ Nv3 application dose was  $2 \times 10^5$  U per animal, which gave  $9 \times 10^3 \pm 1039$  U/g of animal.

### Tumor measurement

Tumor growth was followed by measuring three mutually orthogonal tumor diameters with a Vernier calliper. Tumor volumes were calculated by formula  $\pi/6 \cdot a \cdot b \cdot c$  (a, b, c are tumor diameters). From the measurements, arithmetic means (AM) and standard errors of the means (SE) were calculated for each experimental group with minimum of 10 mice. Growth delay of the tumors was calculated from individual tumor growth, by determining the time required for tumor to reach  $150 \text{ mm}^3$  of volume, and subtracting the relevant time in the control group.<sup>9,11</sup>

### Blood sample collection and handling

One day before TNF- $\alpha$ Nv3 application blood was collected from all animals in the experiment for monitoring the serum TNF- $\alpha$  level. After the application of TNF- $\alpha$ Nv3 blood samples were taken from four animals at the times indicated in the figures. Blood was collected from the orbital sinus of the animals, immediately centrifuged (3000 rpm/min) for 10 min at  $4^{\circ}\text{C}$  and sterilised by filtration ( $0.22 \mu\text{m}$  cellulose acetate filter, Costar). Samples were stored at  $-70^{\circ}\text{C}$  for TNF- $\alpha$  assay.

### Cells and culture conditions

L929 murine transformed fibroblasts were obtained from Istituto Zooprofilattico Sperimentale, Brescia, Italy. Cells were cultured in Eagle's minimal essential medium (EMEM) with 10% fetal calf serum (FCS). Incubation was carried out at  $37^{\circ}\text{C}$  in a humidified 5% CO<sub>2</sub> incubator.

### Determination of TNF- $\alpha$ serum concentrations

a) *L929 bioassay.* Cytotoxicity of TNF- $\alpha$  was determined on L929 cells<sup>12</sup> in the presence of actinomycin D. The viable cells density was determined 20 h after TNF- $\alpha$ Nv3 treatment.

Culture medium was removed and the cells, fixed with glutaraldehyde, were stained with crystal violet (0.5 % in 20 % methanol). Bound dye was eluted with 0.1 ml of 1% sodium dodecyl sulfate. The optical density was measured at 540 nm using microplate photometer CLS962 (Cambridge Life Sciences plc). Cytotoxic activity expressed as TNF- $\alpha$  U/ml was defined as the reciprocal of the dilution which gives 50% cell killing. The detection limit of the assay was 40 U/ml of serum. Cytotoxic activity for one sample was calculated as AM of six independent measurements. AM of three TNF- $\alpha$ Nv3 activities in sera prepared from three mice in test group, are presented in figures.

*b) TNF- $\alpha$  ELISA.* Immunologically active TNF- $\alpha$  was determined by a "sandwich" enzyme immunoassay (ELISA) specific for human TNF- $\alpha$  (Du Pont). The detection limit was 100 pg/ml of serum. TNF- $\alpha$ Nv3 value for one sample was calculated as AM of two independent measurements. AM of three TNF- $\alpha$ Nv3 values in sera prepared from three mice in test group, are presented in the results.

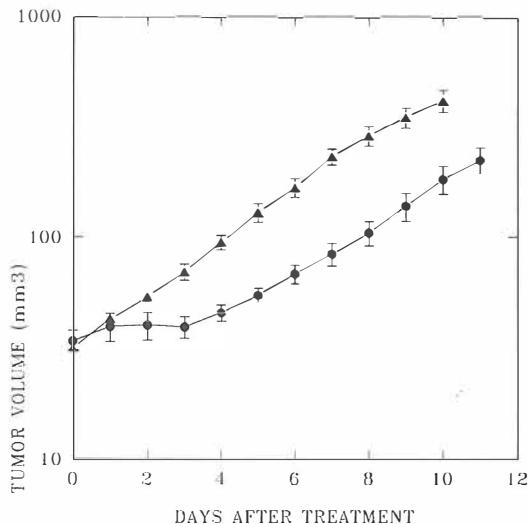
#### Statistical evaluation

The results were evaluated by Student's unpaired probability test and correlation coefficient. All statistical procedures were computed using CSS Programme Stat Soft. Levels of less than 0.05 were taken as indicating significant differences.

## Results

### Antitumor effect of TNF- $\alpha$ Nv3

Melanoma B-16-bearing mice were treated peritumorally with  $2 \times 10^5$  U TNF- $\alpha$ Nv3, when tumors reached average tumor volume  $34.4 \pm 3.9 \text{ mm}^3$ . Tumor growth delay after TNF- $\alpha$ Nv3 treatment ( $3.8 \pm 0.4$  days) was significant ( $P < 0.05$ ), compared to tumor growth in control animals, which were treated with physiological saline (Figure 1). No side effects were observed after peritumoral treatment with TNF- $\alpha$ Nv3.



**Figure 1.** Antitumor effect of TNF- $\alpha$ Nv3 on subcutaneous melanoma B-16 after peritumoral (●) treatment with TNF- $\alpha$ Nv3 ( $2 \times 10^5$  U/mouse); controls (▲).

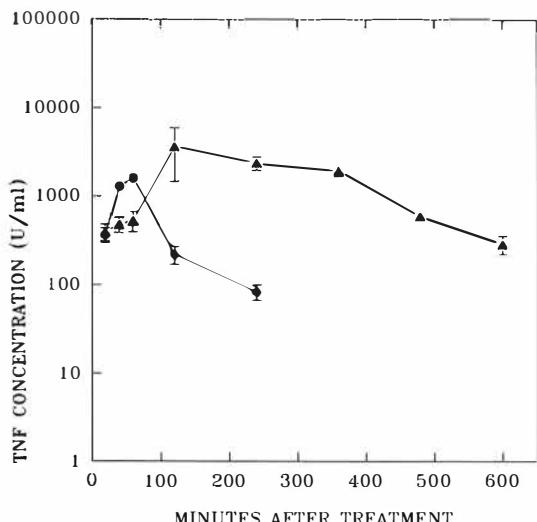
### Endogenous TNF- $\alpha$ levels in mice before application of TNF- $\alpha$ Nv3

Endogenous serum TNF- $\alpha$  levels were measured with L929-cell assay method, in sera collected one day before the TNF- $\alpha$ Nv3 application from tumor-bearing and healthy animals. In all serum samples tested (15 from healthy and 24 from tumor bearing animals), endogenous TNF- $\alpha$  levels were below the reliable detection limit (40 U/ml) of the method.

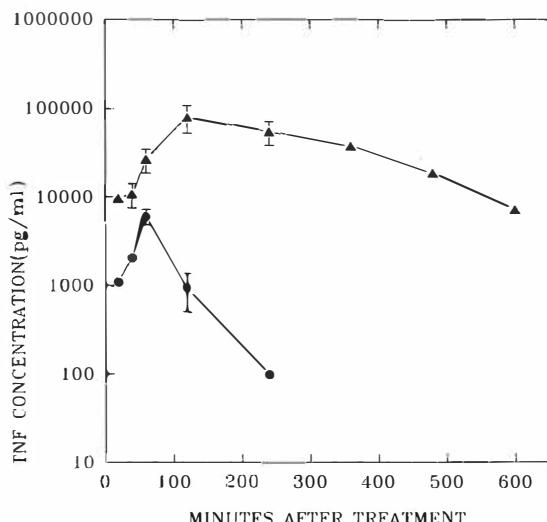
The same serum samples were tested also on ELISA to test the method for possible interference of other immunological substances. Results were negative for all serum samples. This confirms that no component in the murine serum, prepared as described, interferes with our ELISA assay system.

### Serum levels of TNF- $\alpha$ Nv3 after peritumoral application

Serum TNF- $\alpha$  concentrations in B-16-melanoma bearing mice were compared to concentrations of TNF- $\alpha$  in healthy mice without tumors. Tumor-bearing mice were treated peritu-



**Figure 2.** Mean  $\pm$  SE serum levels time curves of cytotoxically active TNF- $\alpha$ Nv3 after peritumoral application ( $2 \times 10^5$  U/mouse) in B-16-bearing ( $\blacktriangle$ ) and healthy mice ( $\bullet$ ).



**Figure 3.** Mean  $\pm$  SE serum levels time curves of TNF- $\alpha$ Nv3 antigen after peritumoral application ( $2 \times 10^5$  U/mouse) in B-16-bearing ( $\blacktriangle$ ) and healthy mice ( $\bullet$ ).

morally, while healthy mice subcutaneously with  $2 \times 10^5$  U TNF- $\alpha$ Nv3. In the same serum samples, TNF- $\alpha$  cytotoxic activity (Figure 2) and immunologically active TNF- $\alpha$  molecules (Figure 3) were determined. Delayed TNF- $\alpha$  serum peak levels were observed in tumor-bearing mice (120 min.) compared to healthy mice (60 min.). Also, TNF- $\alpha$  serum concentrations were higher in tumor-bearing mice. The highest TNF- $\alpha$  serum level in tumor-bearing mice was  $3.6 \times 10^3$  U/ml (L929 assay) or  $8 \times 10^4$  pg/ml (ELISA), and  $1.6 \times 10^3$  U/ml or  $6 \times 10^3$  pg/ml in healthy mice ( $P < 0.05$  in both assays). High TNF- $\alpha$  serum levels persisted in tumor-bearing animals, compared to quick clearance from healthy mice. Ten hours after TNF- $\alpha$ Nv3 application its level in tumor bearing animals was  $2.8 \times 10^2$  U/ml or  $7 \times 10^3$  pg/ml, while in healthy mice already after four hours dropped to 83 U/ml or  $1 \times 10^2$  pg/ml.

## Discussion

The *in vivo* antitumor effects of murine TNF- $\alpha$ <sup>13</sup> and human TNF- $\alpha$ <sup>14</sup> have been reported on

different murine tumors transplanted in syngeneic mice. In our previous study antitumor effects of TNF- $\alpha$  analog TNF- $\alpha$ Nv3 after peritumoral application was established on SA1 sarcoma and B-16 melanoma tumors.<sup>9,11</sup> In this study the potent antitumor effect of TNF- $\alpha$ Nv3 is reconfirmed on B-16 melanoma tumors.

The results of TNF- $\alpha$  serum level measurement show that TNF- $\alpha$ Nv3 pharmacokinetic mechanisms in tumor-bearing and healthy mice are different. TNF- $\alpha$  concentration-time profiles, presented in Figures 2 and 3 indicate, that TNF- $\alpha$  in serum of B-16-bearing mice persists longer than in serum of healthy mice.

TNF- $\alpha$ Nv3 was measured in sera cytotoxically and immunologically in tumor-bearing and healthy mice after peritumoral or subcutaneous application, respectively. Relationship between results obtained by the two assays shows almost perfect positive correlation ( $R = 0.88$  for healthy and  $R = 0.98$  for tumor-bearing mice). Besides, it was established that no component from sera interfered with ELISA assay used. Although TNF- $\alpha$  levels were often found to be increased in sera of cancer patients<sup>15</sup> we didn't detect endogenous TNF- $\alpha$  before TNF- $\alpha$ Nv3

application neither in sera from healthy nor in sera from tumor-bearing mice. Therefore we believe that all TNF- $\alpha$  activity detected in serum originates from TNF- $\alpha$ Nv3 applied.

The influence of B-16 tumor on the persistence of TNF- $\alpha$  activity in serum is documented for the first time. If other tumors also affect the persistence of TNF- $\alpha$  serum concentration in a similar way, the pharmacokinetic parameters from healthy animals can not be extrapolated to tumor-bearing animals; consequently for the prediction of human therapy only pharmacokinetic data obtained by tumor-bearing animal models can be used.

In conclusion, this study demonstrates that peritumoral treatment with TNF- $\alpha$  analog, lacking first three amino acids from N-terminal part, has antitumor effect on B-16 tumors. Comparison of TNF- $\alpha$  serum concentration-time profiles in tumor-bearing and healthy mice, indicate that the absorption, distribution and/or metabolism of TNF-Nv3 in B-16 tumor-bearing mice are significantly different from those in healthy mice. The mechanism for mentioned differences between tumor-bearing and healthy mice remains to be clarified in further studies.

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