In vivo protective effect of tumour necrosis factor α against experimental infection with herpes simplex virus type 1

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C57BL/6 mice, which differ genetically from other strains by their resistance to herpes simplex virus type 1 (HSV-1) infection, were inoculated intraperitoneally with different doses of tumour necrosis factor α (TNF-α). Mice pretreated with 100 ng, or even 10 ng, of TNF-α showed prolonged survival compared to control mice that were infected with 10^7 p.f.u. of HSV-1. Significant protection was observed in mice injected 4 or 8 h prior to or after HSV-1 inoculation, respectively. Protection was also observed when mice which differed at their H-2 locus were treated with TNF-α after infection with HSV-1. Interferon could not be detected in the sera of mice at different time points after infection with HSV-1 or injection of TNF-α and there was no enhanced interferon titre in mice treated with both TNF-α and HSV-1, suggesting some interferon-independent protection. However, mice treated with TNF-α showed a marked activation of natural killer (NK) cells compared to untreated control mice or mice that were treated with HSV-1 alone. To test whether enhanced NK cell activity is responsible for TNF-α-induced protection, mice were injected with the NK cell-specific antibody anti-asialo Gm-1. In this experimental protocol the survival rate was almost unaffected, indicating that the observed protection was not due to activation of NK cells and that TNF-α is involved in the regulation of antiviral mechanisms other than the activation of interferons. Although additional production of interferon induced by TNF-α cannot be excluded, an antiviral effect of TNF-α on the course of HSV-1 infection may be postulated from our data.

Introduction

Carswell et al. (1975) reported the isolation of tumour necrosis factor (TNF), which was originally defined as a serum factor from Bacillus Calmette-Guérin- and LPS-treated animals. (Mannel et al., 1980). TNF-α, mainly produced by cells of the monocyte lineage (Satomi et al., 1981), is induced by the necrosis of certain tumors and is toxic for tumor cell lines (Sugarman & Aggarwal, 1985). Since TNF-α has been molecularly cloned and recombinant products are available, experiments on the biological functions of the protein have been performed (Kornbluth & Edgington, 1986; Voth et al., 1988, 1990). We have previously shown that cloned human TNF-α is able to induce natural killer (NK) cell activity in the peritoneal cavity of mice (Voth et al., 1988). We also demonstrated that activated NK cell activity was not due to interferon (IFN) production, which represents one of the most potent NK cell inducers, because injection of TNF-α failed to elevate 2',5'-oligoadenylate synthetase (2'-5'OAS) levels in peripheral blood mononuclear cells (PBMC) (Voth et al., 1988). 2'-5'OAS is the most characteristic enzyme for the indication of induction of IFN. Mestan et al. (1986) have reported another capacity of TNF-α that has so far been attributed to IFN, namely in vitro antiviral effects. We therefore studied whether TNF-α might have in vivo antiviral capacities that are mediated by the induction of NK cells. NK cells are characterized by their ability to lyse susceptible tumor cells or virus-infected cells without previous sensitization (Herberman & Ortaldo, 1982). Resistance of mice against primary infection with herpes simplex virus type 1 (HSV-1) is genetically determined (Lopez 1975). This genetic restriction is paralleled in mice by their capacity to produce IFN (Zawatzky et al., 1981). On the other hand, IFN is one of the most effective inducers of NK cell activity. We therefore set up a series of experiments in order to evaluate antiviral activity of TNF-α in mice in vivo and to discriminate between IFN- and TNF-α-mediated mechanisms that may underlie the observed protection.

Methods

Mice. The system used in our studies has been described previously (Voth et al., 1988). Briefly, C57BL/6, DBA/2 and BALB/c mice at 10 to
12 weeks of age were infected intraperitoneally (i.p.) with various doses of HSV-1 strain Wal, subsequently referred to as HSV. Pure recombinant human TNF-α was diluted as recently described and injected i.p. in different doses at various times after viral infection. As described elsewhere (Andervont, 1929), mice in this model die from a viral encephalitis 8 to 10 days after infection and this can be proven by determination of viral titres in the brain (data not shown). Mice surviving for 2 weeks will survive for many months with sequelae and can be considered to have survived the infection. Thus, in our experiments dead mice were recorded daily, starting on the fourth day after infection until day 20 when the experiments were terminated. All experiments were repeated three times; all experimental groups consisted of 10 mice each.

Reagents. Cloned TNF-α was kindly provided by Fa. Knoll. The factor was diluted suitably in Hanks’ solution and the final dose was injected in a volume of 0·2 ml (i.p.). Anti-asialo antibodies were obtained from Paesel. The stock solution contained 1 mg protein in 1 ml double-distilled water. Stock solution (5 ml) was diluted (final concentration 1:50) further in 200 ml Hanks’ solution and injected i.p. 1 day before and 1 day after TNF-α inoculation.

Cytotoxicity test. YAC-1 cells were kept in permanent culture in our laboratory. P815 cells were kindly provided by Dr D. Männel (German Cancer Research Center, Heidelberg, Germany). All tumour cells were washed three times in RPMI 1640 supplemented with 10% foetal calf serum before the test. Peritoneal exudate cells (PEC) of pretreated mice were washed out at various times after treatment, adjusted to a concentration of 1 × 10^6 cells/ml and used as effector cells. Target cells (1 × 10^6/ml) were labelled with 100 μCi Na_2[^31]CrO_4 (specific activity 50 mCi/μCi; Behringwerke) for 1 h at 37 °C. Subsequently, effector cells and target cells were adjusted to various ratios and incubated for 4 h using YAC-1 cells and for 18 h using P815 cells. Spontaneous ^[31]Cr release was determined by incubating targets without effectors; total chromium release was measured in cells to which Triton X-100 had been added. After harvesting, radioactivity in 100 ml of the supernatant was measured using a gamma counter. Cytotoxicity was calculated by the following formula: specific lysis % = [(test release - low control release)/(High control release - low control release)] × 100.

All tests were done in triplicate and the means were calculated.

Interferon assay. Peritoneal fluid was rendered cell-free by centrifugation and virus was inactivated by dialysis against acid buffer (pH 2). The one-step plaque reduction assay using mouse L-929 cells and vesicular stomatitis virus for IFN determination has been described (Haveli & Vilcek, 1972).

Virus titration. The virus titre in peritoneal fluid was determined by plaque titration on RITA cells (monkey kidney cells) in log₁₀ steps.

Statistical evaluation. Results were expressed as the mean and the standard error of the mean (x ± S.E.) and the statistical significance (n.s., not significant; P < 0·01 and P < 0·001) was analysed by the Kolmogorov Smirnov test (Sachs, 1984). The statistical analysis was performed on a Macintosh SE/30 computer (Apple Computer) using Stat-View software (Abacus Concepts).

Results

Kinetics and dose dependence of the protection of HSV-1-infected mice by pretreatment with TNF-α

Ten C57BL/6 mice were treated i.p. with 100 ng recombinant TNF-α and infected 8 h later with 10^7 p.f.u. HSV-1 at the same local site. Control mice were treated with 10^7 p.f.u. HSV-1 i.p. HSV-1 alone. Dead mice were recorded daily starting on the fourth day after infection until day 20 when the experiment was terminated. As can be seen in Fig. 1, in the TNF-α treated group, 80% of the mice survived. To determine whether the dose could be reduced further, mice were inoculated with 10 ng TNF-α 8 h prior to infection with HSV-1. As can be seen in Fig. 1, in this experimental situation 60% of the mice survived. However, all mice of the control group died within 2 weeks after infection.

Post-infection protective effect of TNF-α

Owing to the potential therapeutic use of TNF-α, we injected 100 ng of TNF-α 4, 8 and 24 h after infection with 10^7 p.f.u. HSV-1 i.p. into C57BL/6 mice. From Fig. 2 it can be seen that mice treated with TNF-α as early as 4 h post-infection showed a survival rate of 80%. However, a 60% survival rate was obtained when the mice were treated with TNF-α 8 h after infection. Mice that received TNF-α 24 h after HSV-1 infection were only slightly protected; 20% of mice in this group survived (Fig. 2).

Genetic restriction of TNF-α-mediated protection

Since it is known that strains of mice genetically differ in their susceptibility to HSV-1, we infected DBA/2 and BALB/c mice with HSV-1 at a concentration that kills 50% of the mice. Again, the experimental group was treated with 100 ng of TNF-α 4 h after infection. As can be seen in Fig. 3, TNF-α treatment resulted in significant protection in these strains; in untreated HSV-1-infected
Effect of TNF-α on HSV-1 infection

Effect of TNF-α on HSV-1 infection in C57BL/6 mice. (a) ○, Mice treated with 100 ng TNF-α 4 h after infection with 10⁷ p.f.u. HSV-1 i.p.; (b) ○, TNF-α treatment 8 h after HSV-1 infection i.p.; (c) △, TNF-α treatment 24 h after HSV-1 infection i.p.; (d) ●, mice infected with 10⁷ p.f.u. HSV-1 alone. (a: b, n.s.; a: c, P < 0.001; a: d, P < 0.001; b: d, P < 0.001; c: d, P < 0.001).

Fig. 3. Genetic restriction of TNF-α-mediated resistance against HSV-1 infection. (a) ○, BALB/c mice infected with 2 x 10³ p.f.u. HSV-1 i.p. and treated with 100 ng TNF-α 4 h after infection; (b) △, BALB/c mice infected with 2 x 10³ p.f.u. HSV-1 i.p.; (c) ○, DBA/2 mice infected with 2 x 10² p.f.u. HSV-1 i.p., and treated with 100 ng TNF-α 4 h after infection; (d) ●, DBA/2 mice infected with 2 x 10² p.f.u. HSV-1. (a: b, P < 0.001; c: d, P > 0.001).

Table 1. Correlation between survival rate and presence of NK cell activity in TNF-α-treated or untreated HSV-1-infected C57BL/6 mice

<table>
<thead>
<tr>
<th>Strain of mouse</th>
<th>No. of mice</th>
<th>Dose of HSV-1 (p.f.u.)</th>
<th>Treatment 8 h after infection†</th>
<th>Specific lysis (% ± s.e.)‡</th>
<th>Survival (%) after 3 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>10</td>
<td>10⁷</td>
<td>None</td>
<td>20 ± 12</td>
<td>0</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>10</td>
<td>10⁷</td>
<td>100 ng TNF-α</td>
<td>60 ± 24</td>
<td>80</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>10</td>
<td>10⁷</td>
<td>Anti-asialo antibodies</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>10</td>
<td>10⁷</td>
<td>100 ng TNF-α and Antiasialo antibodies</td>
<td>0</td>
<td>70</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>10</td>
<td>0</td>
<td>100 ng TNF-α</td>
<td>40 ± 23</td>
<td>100</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>10</td>
<td>0</td>
<td>None</td>
<td>5 ± 4</td>
<td>100</td>
</tr>
</tbody>
</table>

* In all experiments the effector:target ratio was 10:1 and results are the mean of three experiments.
† All mice injected i.p. with either 10⁷ p.f.u. HSV-1, 100 ng TNF-α, anti-asialo antibodies or a combination of the compound.
‡ PEC were collected 24 h after the i.p. injection for NK cell activity determination.

DBA/2 mice (2 x 10² p.f.u.) 20% of the mice survived whereas in the TNF-α-treated group 80% of the mice survived. Similar results were obtained when BALB/c mice were treated with TNF-α; whereas 70% died due to injection of 10³ p.f.u. HSV-1, 90% of the TNF-α-treated mice survived.

Involvement of NK cells in TNF-α-mediated resistance

TNF-α is a potent inducer of NK cell activity in the peritoneal cavity of mice. Since NK cells are known to be involved in the elimination of virus-infected cells (Herberman & Ortaldo, 1982), we determined whether TNF-α-mediated resistance was due to induction of NK cells. We therefore measured NK cell activity in the peritoneal cavity of mice that were infected with either HSV-1 or treated with TNF-α alone. Another group of mice was treated with antibodies that eliminate NK cell activity. The survival rate was compared in both study groups. As indicated in Table 1, determination of NK cell activity in the peritoneal cavity of C57BL/6 mice infected with 10⁷ p.f.u. HSV-1 revealed that in 20% of NK cells, activity was present 24 h after infection at an effector:target cell ratio of 10:1. However, mice that had been treated with 100 ng TNF-α 4 h after infection produced NK cell activity of 60%. In the study group that had been treated with anti-asialo antibodies, no NK cell activity was noted. Untreated mice had little or no NK cell activity in the peritoneal cavity (Table 1). However, when the survival rate was measured there was no difference between NK cell-depleted mice and those mice which showed functional NK cell activity (Table 1).
measurements. no IFN titres or titres lower than 3 IU/ml (data not shown). p.f.u.) or both compounds. Mice injected i.p. with TNF-α alone showed no increased survival when TNF-α is injected i.p. 4 h after infection and is absent when TNF-α is injected 24 h after HSV-1 infection. One might argue that the strong time dependence of TNF-α-mediated protection may limit the value of TNF-α as an antiviral agent in vivo. However, the fact that doses as low as 100 ng are enough to achieve protection must be considered. Such doses do not cause any side-effects and further experiments are planned using several TNF-α injections starting from day 1 or 2 after infection with the aim of generating a therapy concept.

We have previously identified TNF-α as an inducer of NK cells in antiviral resistance is still not clear. Data in a model of murine cytomegalovirus infection show a correlation between high levels of NK cell activity and resistance (Bancroft et al., 1981). Data from Schindler et al. (1981) on experiments in a mouse hepatitis system fail to detect such a correlation. In our model, TNF-α-induced NK cell activity cannot account for the observed resistance because mice with depleted NK cell population show similar survival rates compared to NK cell-competent mice. Second, if TNF-α does not act as an inducer of IFN or by subsequent NK cell activation, one could speculate that TNF-α either produces some direct antiviral capacity or IFN is produced at very low doses that escape our detection but, when acting synergistically with TNF-α, are very effective. Such a synergistic blockade by human IFN-γ and TNF-α on the replication of HSV has recently been demonstrated in vitro (Feduchi et al., 1989). In our experimental situation on the other hand, IFN was induced by the injection of HSV-1; however, we did not observe additional IFN activity which could have produced the observed protection following TNF-α injection. Up to now, IFN, and in particular IFN-α/β, at doses ranging around 100 to 1000 units is believed to play a major role in host defence against infection with HSV-1 in mice (Zawatzky et al., 1982). Our data provide evidence that TNF-α might exhibit additional antiviral activity which may be regulated by mechanisms other than the induction of IFN. Although such an IFN-independent mechanism can only be proven by IFN depletion in mice, an experiment that is impossible to do at present, we were able to show that TNF-α does not induce substantial amounts of IFN. Further experiments using DNA technology in order to detect transcriptional activation of IFN gene products are needed to clarify the role of IFNs in TNF-α-mediated resistance to HSV-1 infection or whether TNF-α alone exhibits an antiviral capacity.

**Tables**

**Table 2. Serum IFN titres in C57BL/6 mice after i.p. injection of TNF-α and infection with HSV-1**

<table>
<thead>
<tr>
<th>No. of mice/time (h) after blood collection</th>
<th>Inducer: HSV-1</th>
<th>Inducer: HSV-1 + TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expt. 1</td>
<td>Expt. 2</td>
</tr>
<tr>
<td>10/4</td>
<td>386 ± 43</td>
<td>478 ± 56</td>
</tr>
<tr>
<td>10/8</td>
<td>1116 ± 412</td>
<td>1268 ± 231</td>
</tr>
<tr>
<td>10/24</td>
<td>96 ± 23</td>
<td>109 ± 34</td>
</tr>
</tbody>
</table>

* All mice were injected i.p. with either TNF-α (100 ng), HSV-1 (10^7 p.f.u.) or both compounds. Mice injected i.p. with TNF-α alone showed no IFN titres or titres lower than 3 IU/ml (data not shown).

† Titres represent mean values of at least three separate measurements.

‡ IFN titres are mean ± s.e.

From these results we conclude that induction of NK cell activity plays no major role in the TNF-α-mediated antiviral effect.

**Induction of IFN by TNF-α in the peritoneal cavity of C57BL/6 mice**

In order to investigate whether TNF-α-mediated protection is due to additional induction of IFN, IFN-α titres were determined in the peritoneal cavity of mice at different time points after infection. As can be seen in Table 2, there was no additional IFN-α in the peritoneal cavity of mice at any time after injection of TNF-α in combination with HSV-1.

**Discussion**

The results presented here indicate that cloned human TNF-α is involved in the protection of mice against experimental infection with HSV-1. Our data suggest that TNF-α mediates some in vivo antiviral activity following i.p. HSV-1 infection. We have demonstrated that DBA/2 and BALB/c mice, as well as C57BL/6 mice, show increased survival when TNF-α is injected i.p. 4 h and 8 h after infection with HSV-1. However, TNF-α-mediated protection is low when TNF-α is given more than 8 h after infection and is absent when TNF-α is injected 24 h after HSV-1 infection. One might argue that the strong time dependence of TNF-α-mediated protection may limit the value of TNF-α as an antiviral agent in vivo. However, the fact that doses as low as 100 ng are enough to achieve protection must be considered. Such doses do not cause any side-effects and further experiments are planned using several TNF-α

**References**


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