Therapeutic Effect of Anti–Tumor Necrosis Factor-α Antibody on the Murine Model of Viral Myocarditis Induced by Encephalomyocarditis Virus

Takehiro Yamada, MD; Akira Matsumori, MD; Shigetake Sasayama, MD

Background Tumor necrosis factor-α (TNF-α) has been reported to have an antiviral effect in vitro; however, its in vivo effect remains to be clarified.

Methods and Results To investigate the role of TNF-α in viral myocarditis using a murine model induced by encephalomyocarditis virus (EMCV), we evaluated (1) plasma TNF-α levels by enzyme-linked immunosorbent assay (ELISA), (2) the effect of recombinant human TNF-α for its possible antiviral effect in vivo, and (3) the effect of anti-murine TNF-α monoclonal antibody (mAb) in vivo. Four-week-old DBA/2 mice were inoculated intraperitoneally with EMCV (day 0). Mice were injected intravenously daily with 1 μg of TNF-α or 2×10⁶ units of anti-TNF-α mAb starting on day −1, day 0, or day 1 until day 2 (TNF-α study) or day 4 (anti-TNF-α mAb study). A portion of the mice were killed on day 5 (protocol 1); their hearts were removed, and plaque assays were performed to demonstrate the myocardial virus content. The remaining mice were killed on day 14 (protocol 2); myocardial lesions were examined histopathologically in terms of severity, and their survival rates were determined. Plasma TNF-α concentration was elevated in the blood of infected mice compared with uninfected mice 3, 5, and 7 days after virus inoculation. The myocardial virus content was higher in the TNF-α-treated group than in the control group. Histopathological analysis revealed that myocardial necrosis and cellular infiltration were more prominent in the TNF-α group than in the control group. The anti-TNF-α mAb improved survival and myocardial lesions when its treatment was started 1 day before virus inoculation. However, it showed no therapeutic effect when administered simultaneously with the inoculation or on day 1.

Conclusions TNF-α may play an important role in the very early stage of the immune response, and anti-TNF-α mAb may prevent the early pathway of acute viral myocarditis.

Key Words • myocarditis • viruses • antibodies

Tumor necrosis factor-α (TNF-α), a cytokine mainly released by activated macrophages, was initially reported as an antitumor agent.¹ Since that time, knowledge about the biological effects of TNF-α has significantly increased.² Many of the activities attributed to TNF-α overlap with other cytokines such as interleukin-1 (IL-1), interferons (IFNs), and interleukin-6 (IL-6).³ TNF-α has been recognized as a common mediator of the immune system. However, contrary to the data in vitro, little is known about the in vivo effects of TNF-α. Whether the effects of TNF-α are beneficial or deleterious to the host may depend on the circumstances involved, the amount of production, and whether it is endogenously produced or exogenously administered.⁴ In some instances, in vitro activity has been shown to contradict its activity in vivo. For instance, TNF-α inhibited endothelial cell growth in vitro but it was angiogenic in vivo.⁵ Recently, an in vitro antiviral effect of TNF-α has been reported in selected cell lines⁶-⁸; however, whether TNF-α is antiviral in vivo has not been established.⁹,¹⁰ Moreover, TNF-α is identical to cachectin,¹¹ which causes shock and eventual death in mice.¹²,¹³ Therefore, the doses, methods of administration, and protocols should be considered carefully in predicting its in vivo effect. In this study, we investigated a role of TNF-α in viral infection using an experimental model of murine viral myocarditis induced by encephalomyocarditis virus (EMCV), against which a novel antiviral effect of TNF-α was reported in vitro.⁶ Massive inflammation occurs in the heart several days after viral infection, with severe myocardial necrosis and mononuclear cellular infiltration leading to congestive heart failure.¹⁴,¹⁵ We first determined plasma TNF-α levels in EMCV-infected mice and then investigated the effects of a nonlethal dose of recombinant human TNF-α and anti-TNF-α monoclonal antibody (mAb) in this model of acute viral myocarditis.

Methods

Tumor Necrosis Factor-α

Recombinant human TNF-α produced in Escherichia coli by recombinant DNA techniques and purified to homogeneity (≥99% on the basis of SDS-PAGE analysis) as described previously¹⁶ was used (provided by Suntory Institute for Biochemical Research, Osaka, Japan). The specific activity of TNF-α was about 4.8×10⁵ units/mg of protein, based on its cytotoxic activity in L929 cells.

Mortality in Mice After Treatment With TNF-α

Because a high dose of TNF-α is lethal to mice, various doses were injected intravenously to normal DBA/2 mice to elucidate the acute toxic effect of TNF-α. The mice were observed for 48 hours after the treatment.
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Virus and Cell
The M (myocarditrophic) variant of EMCV was used. Virus stock was prepared as described previously and stored at -70°C until it was diluted for use. Mice were inoculated intraperitoneally with 0.1 mL of EMCV diluted in Eagle's minimum essential medium (MEM) to a concentration of 10 plaque forming units (pfu)/mL. Viral titers in the heart were determined by plaque formation on Fl (human amion) cells. Cells were suspended to a concentration of 2 x 10^9/mL in Eagle's MEM with 10% fetal calf serum (FCS) plus 10 μg/mL penicillin G and streptomycin in six-well plastic plates and were allowed to grow for 2 days at 37°C in 5% CO2.

Virus Inoculation and Treatment of Mice
A total of 270 4-week-old DBA/2 male mice were obtained from the Shizuoka Agricultural Cooperative Association (Shizuoka, Japan). The day of virus inoculation was defined as day 0 in the following studies. Mice were observed daily, and when found dead, necropsies were performed. Surviving mice were killed on day 5 (protocol 1) or on day 14 (protocol 2).

Plasma TNF-α Determination
Thirty mice were inoculated with 10 pfu/0.1 mL of EMCV on day 0. Mice were killed on days 1, 3, 5, and 7. Blood was obtained, and plasma TNF-α levels were determined using enzyme-linked immunosorbent assay (ELISA).

A solid-phase sandwich ELISA kit for murine TNF-α was used (Otsuka Pharmaceutical Co, Tokushima, Japan). Ninety-six-well microtissue plates were coated with 100 μL of anti-TNF-α monoclonal antibody per well. Between subsequent steps in the assay, the coated plates were washed three times in washing buffer. After 30 minutes of preincubation at 37°C in wash buffer, 100 μL of either standard TNF-α control solution, or serum from the mice was added for 2 hours at 37°C. Goat polyclonal anti-murine TNF-α antibody was then added for 90 minutes followed by horseradish peroxidase-conjugated donkey anti-goat immunoglobulin antibody for 1-hour incubation at room temperature, whereby 100 μL of diluted o-phenylenediamine was added. The enzyme reaction was stopped with 100 μL of 1 mol/L sulfuric acid, and the absorbance was measured at 492 nm using a microtissue plate photometer.

TNF-α Study
Each mouse in the TNF-α-treated groups was injected intravenously with 1 μg of TNF-α diluted in 0.1 mL of PBS. A total of 120 mice were inoculated. Mice were injected intravenously daily with 1 μg/0.1 mL of TNF-α started on day -1 (TNF -1D group: protocol 1, n=10; protocol 2, n=20), or once in the heart of virus infection (TNF ±0D group: protocol 1, n=10; protocol 2, n=20). After 30 minutes of preincubation at 37°C in wash buffer, 100 μL of either standard TNF-α control solution, or serum from the mice was added for 2 hours at 37°C. Goat polyclonal anti-murine TNF-α antibody was then added for 90 minutes followed by horseradish peroxidase-conjugated donkey anti-goat immunoglobulin antibody for 1-hour incubation at room temperature, whereby 100 μL of diluted o-phenylenediamine was added. The enzyme reaction was stopped with 100 μL of 1 mol/L sulfuric acid, and the absorbance was measured at 492 nm using a microtissue plate photometer.

Anti-TNF-α mAb Study
One hundred fifteen mice were inoculated in the same way as in the TNF-α study. Mice were injected daily with 1 μg (2 x 10^9 units) IV diluted in 0.1 mL PBS of anti-mouse TNF-α mAb (Genzyme) started on day -1 (AB -1D group: protocol 1, n=10; protocol 2, n=18), on day 0 (AB ±0D group: protocol 1, n=10; protocol 2, n=18), and on day +1 (AB +1D group: protocol 1, n=10; protocol 2, n=16) until day 4. Infected control mice were injected daily with 0.1 mL IV saline started on day -1 until day 4 (saline group: protocol 1, n=10; protocol 2, n=18). Five mice were treated with anti-TNF-α mAb alone daily from day -1 until day 4 as an uninfected group (protocol 2, n=5). Mice were killed, and virus titers and histopathological lesions were defined in the same way as in the TNF-α study.

Virus Titers of Murine Hearts
For infectivity assay, hearts of infected mice were removed aseptically on day 5, and longitudinal halves were weighed and homogenized in 2 mL of Eagle's MEM. After centrifugation at 1500g for 15 minutes at 4°C, 0.1 mL of supernatant was inoculated into FL cell monolayers for 60 minutes at 37°C in 5% CO2. Cells were then overlaid with 3 mL of medium containing 4% FCS and 1% methylcellulose. After 2 days of incubation at 37°C in a humidified atmosphere containing 5% CO2, cells were fixed with acetic acid and methanol (in a ratio of 1:3) and stained with crystal violet (1%), and plaques were counted with an inverted microscope. The myocardial virus titer was expressed as log_{10} pfu/mg.

Histopathology
The other halves of the hearts on day 14 were fixed in 10% formalin and embedded in paraffin. Several sections were stained with hematoxylin and eosin and scored (0 to +4) by a blinded, skilled observer for myocardial necrosis and cellular infiltration. The mean value was cited. The scores were 0 (none), no myocardial lesion; 1+, lesions involving <25% of the myocardium; 2+, lesions involving 25% to 50% of the myocardium; 3+, lesions involving 50% to 75% of the myocardium; 4+, lesions involving >75% of the myocardium. Representative photomicrographs are shown in Fig 1.

Statistical Analysis
Survival of mice was analyzed by the Kaplan-Meier method. Statistical comparisons of plasma TNF-α levels, BW, HW, BW/HW ratio, and histopathological scores were performed by one-way ANOVA. Differences were considered statistically significant at P<.05. Results are expressed as mean±SEM.

Results
Mortality After TNF-α Injection
Intravenous injection of 100 or 10 μg of TNF-α was lethal for normal DBA/2 mice 48 hours after treatment. Their mortality was 100% (10 of 10 mice) and 58% (11 of 19), respectively. However, 1 or 2 μg of TNF-α was not lethal for mice; mortality in each was 0% (0 of 10 each). Therefore, we chose the dose of 1 μg to be injected per mouse in the TNF-α study.

Plasma TNF-α Levels in Murine Viral Myocarditis
As shown in Fig 2, plasma TNF-α levels were elevated significantly (P<.05) in the blood of infected mice on day 3 (24.2±1.6 pg/mL), day 5 (37.8±2.1 pg/mL), and day 7 (25.7±1.0 pg/mL) in comparison with that of uninfected mice (20.7±1.3 pg/mL).

Effect of TNF-α on Murine Viral Myocarditis
The survival rates of each infected group on day 14 were 47% in the saline group, 26% in the TNF -1D group, and 33% in the TNF ±0D and TNF +1D groups (Fig 3). There were no significant differences in survival rates among the TNF-α-treated groups; however, a significant decrease was found when the survival rates
of total mice of three TNF groups were compared with
that of the saline group (47% versus 31%, P<.05).
There was significant increase of the HW/BW ratio in
the TNF −1D group in comparison with that of the
saline group (P<.05) (Table 1). The histopathological
scores of necrosis and cellular infiltration in the surviv-
ing mice on day 14 were significantly higher in the TNF
−1D group in comparison with those of the saline group
(P<.05). The myocardial virus contents on day 5 of the
TNF −1D group (4.6±0.5 log_{10} pfu/mg, n=5; range, 4.2
to 4.8) and the TNF ±0D group (4.4±0.7 log_{10} pfu/mg,
n=6; range, 3.8 to 5.1) were significantly higher than
those of the saline group (3.7±0.3 log_{10} pfu/mg, n=9;
range, 3.4 to 4.1) (P<.05).

**Fig 1.** Photomicrographs of myocardial necrosis and cellular infiltration were scored by a blinded observer on a scale of 0 to +4 in
terms of severity. A, Lesions involving <25% of the myocardium (+1); B, lesions involving 25% to 50% of the myocardium (+2); C,
lesions involving 50% to 75% of the myocardium (3+); and D, lesions involving >75% of the myocardium (+4).

**Fig 2.** Bar graph shows time course of plasma tumor necrosis factor-α (TNF-α) level in DBA/2 mice after encephalomyocarditis
virus inoculation. Plasma TNF-α elevated in infected mice on
days 3, 5, and 7 in comparison with that of uninfected mice
(P<.05).

**Fig 3.** Plot shows the effect of tumor necrosis factor-α (TNF-α) on survival in viral myocarditis in DBA/2 mice. Significant de-
crease of the survival rate was found when the total mice of three
TNF-α groups were compared with the saline group (CR, 47% vs
31%, P<.05).
TABLE 1. Effects of Tumor Necrosis Factor-α on Murine Viral Myocarditis

<table>
<thead>
<tr>
<th></th>
<th>Surviving Mice n</th>
<th>Body Weight, g</th>
<th>Heart Weight, g \times 10^{-1}</th>
<th>HW/BW Ratio, \times 10^{-3}</th>
<th>Cardiac Histology Necrosis</th>
<th>Myocardial Virus Titer, log_{10} pfu/mg tissue</th>
</tr>
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<tr>
<td>Uninfected group</td>
<td>5</td>
<td>21.2±1.2</td>
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<td>0.0±0.0</td>
<td>0.0±0.0</td>
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<td>Infected group</td>
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<td></td>
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<tr>
<td>Saline group</td>
<td>19 9</td>
<td>12.4±1.3†</td>
<td>0.93±0.34†</td>
<td>7.6±1.2*</td>
<td>1.5±0.8</td>
<td>1.9±1.0</td>
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<td></td>
</tr>
<tr>
<td>1 day before VI</td>
<td>19 5</td>
<td>13.2±1.2†</td>
<td>1.05±0.43*</td>
<td>8.0±1.3*</td>
<td>2.4±1.0†</td>
<td>2.6±1.2†</td>
</tr>
<tr>
<td>Day of VI</td>
<td>18 6</td>
<td>14.7±1.3†</td>
<td>1.13±0.33*</td>
<td>7.6±1.1*</td>
<td>1.8±0.8</td>
<td>2.0±1.2</td>
</tr>
<tr>
<td>1 day after VI</td>
<td>18 6</td>
<td>14.3±1.4*</td>
<td>1.20±0.20</td>
<td>7.6±1.2*</td>
<td>1.7±0.9</td>
<td>1.9±1.1</td>
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</tbody>
</table>

TNF-α indicates tumor necrosis factor-α; HW/BW, heart weight/body weight; and VI, virus inoculation. *P<.05, †P<.05 vs uninfected group; ‡P<.05 vs saline group. Values are mean±SEM.

Effect of Anti-TNF-α mAb on Murine Viral Myocarditis

The survival rates on day 14 were 38% in the saline group, 67% in the AB -1D group, 38% in the AB ±0D group, and 20% in the AB +1D group (Fig 4). Significant increase of survival was found in the AB -1D group in comparison with that of the saline group (P<.05). Mean BW and mean HW decreased, and mean HW/BW ratio increased in the infected groups on day 14; they were significantly different from those in uninfected normal mice (Table 2). There was significant decrease of the HW/BW ratio in the AB -1D group in comparison with that of the saline group (P<.01). The histopathological scores of necrosis and cellular infiltration in the surviving mice on day 14 were significantly improved in the AB -1D group when they were compared with the saline group (P<.05).

Discussion

Elevated levels of plasma TNF-α have been reported in patients with infectious diseases7-20; however, the pathophysiological role is not well understood. In this study, plasma TNF-α concentration was elevated in the blood of EMCV-infected mice on days 3, 5, and 7 in comparison with that of uninfected mice. In this acute phase of viral myocarditis, viremia is predominant, and mice develop severe congestive heart failure.14,15 The cells that possibly produce TNF-α may be activated macrophages and lymphocytes infiltrating in the myocardium. In autopsy specimens of human myocarditis, mononuclear cells have been observed when stained with anti-TNF-α antibody.21

Viruses are known to be one of the inducers of TNF-α.22,23 The TNF-α production after injecting endotoxin (LPS) is reported to vary among strains of mice.24 suggesting that TNF-α production is genetically controlled by MHC. It is of interest that TNF-α genes are sited close to the MHC gene25 and TNF-α infusion increases HLA-DQ antigen expression.26 Virus susceptibility also is markedly different among strains of mice.27 B10.A mice, resistant to cossackievirus B3, developed autoimmune myocarditis with viral inoculation after pretreatment with LPS.28 The high TNF-α production induced by LPS is considered to be related to virus susceptibility. Our results demonstrate that the myocardial virus content was higher in the TNF-α-treated group than in the control group. Histopathological evaluation revealed that myocardial necrosis and cellular infiltration were more prominent in the TNF-α group. DBA/2 mice are highly susceptible to EMCV14 and also have high TNF-α-productive activity.24 Therefore, it may be possible that the injected TNF-α caused further susceptibility to EMCV leading to higher mortality and higher virus proliferation in this strain. On the other hand, the susceptibility to toxoplasmic encephalitis29 and radiore sistence30 in mice has been also reported to be related to TNF-α gene induction. TNF-α infusion caused increased susceptibility in the former29 and anti-TNF-α Ab aggravated mortality in the latter.30 These data suggest that TNF-α plays an important role in the primitive host defense or homeostasis against exogenous stress such as viruses, parasites, and radiation.

The antiviral effect of TNF-α in vitro was first reported against EMCV.6-8 The induction of IFN-β, IFN-β, oligo-2',5'-adenylate synthetase (2-5A),32 or NK cell activation33 was initially thought to be the candidate of the antiviral mechanism of TNF-α. TNF-α and IFN induced a common set of proteins34; however, TNF-α mRNA induction is reported to be earlier than interferon mRNA in viral infection,22 and IFN-β mRNA induction by TNF-α is considered to be responsible for some similarities in the actions of TNF-α and IFNs.35 On the other hand, TNF-α strongly stimulates nuclear factor NF-κB in activating the HIV virus.36

Fig 4. Plot shows the effect of anti-tumor necrosis factor-α on survival in viral myocarditis in DBA/2 mice. Significant increase of the survival rate was found in the anti-tumor necrosis factor-α Ab-treated mice compared with the saline group (CR) when the treatment was started 1 day before virus inoculation (67% vs 28%, P<.05).
TABLE 2. Effects of Anti-Tumor Necrosis Factor-α Monoclonal Antibody on Murine Viral Myocarditis

<table>
<thead>
<tr>
<th>Uninfected group</th>
<th>Surviving Mice</th>
<th>Body Weight, g</th>
<th>Heart Weight, HW/BW Ratio, x10^-3</th>
<th>Cardiac Histology Necrosis Infiltration</th>
<th>Myocardial Virus Titer, log10 pfu/mg tissue</th>
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<tr>
<td>10</td>
<td>10</td>
<td>213±1.1</td>
<td>1.23±0.45</td>
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<td>Infected group</td>
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<tr>
<td>Anti-TNF-α Ab</td>
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<tr>
<td>18</td>
<td>7</td>
<td>14.9±1.3†</td>
<td>1.13±0.40*</td>
<td>7.6±1.0</td>
<td>2.2±0.7</td>
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<tr>
<td>Saline group</td>
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<td></td>
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<tr>
<td>18</td>
<td>12</td>
<td>18.3±1.7*</td>
<td>1.18±0.42*</td>
<td>5.9±0.8‡</td>
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<td>1 day before VI</td>
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<td>18</td>
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<td>15.8±1.2†</td>
<td>1.23±0.40</td>
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<td>1.7±0.9</td>
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<td>Day of VI</td>
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<td>18</td>
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<td>14.5±1.4*</td>
<td>1.20±0.40</td>
<td>7.8±1.3‡</td>
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<td>1 day after VI</td>
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<td>14.5±1.4*</td>
<td>1.20±0.40</td>
<td>7.8±1.3‡</td>
<td>2.1±0.8</td>
</tr>
</tbody>
</table>

TNF-α indicates tumor necrosis factor-α. HW/BW, heart weight/body weight; and VI, virus inoculation.

†P<.01, *P<.05 vs uninfected group; ‡P<.05 vs saline group. Values are means±SEM.

There may be different pathways concerning the antiviral effect of TNF-α in which IFN is involved and is not involved as well as a pathway to augment viral replication. Recently, the Fas antigen, a 200-kD human cell surface component, has been reported to be associated with the TNF-α receptor. The anti-Fas antigen mAb showed an antiviral effect on HIV-infected cells, suggesting that TNF-α might demonstrate the antiviral effect by activating the Fas antigen.37

The peripheral mononuclear cells in patients who received TNF-α also showed antiviral activity.38 However, in vivo effects of TNF-α against viral diseases are paradoxical among animals. C57BL/6 mice treated with TNF-α before virus inoculation showed prolonged survival in herpes simplex virus type I infection compared with mice untreated.9 However, TNF-α pretreatment did not show an antiviral effect in lymphocytic choriomeningitis virus–infected mice of the same strain.10 The opposite effect may occur by the kinds of virus, infection routes, and doses when TNF-α is exogenously administered.4 Furthermore, the local production of TNF-α by virus has been reported to be the mechanism of the attenuation of viral replication,39 and the existence of autocrine mechanism of TNF-α is also reported.40 These results may explain the findings that the effect of TNF-α in vitro cannot always be expected in vivo.

Negative inotropic effects of TNF-α on myocardial function cannot be neglected.41,42 Increased severity of myocardial lesions and viral replication in the TNF-α–treated groups in comparison with those of the saline group demonstrated in this study may be due somewhat to these effects. Recently, elevated TNF-α levels in patients with congestive heart failure have been reported,43,44 although it is not determined whether this finding is a primary event that induces cardiac cachexia or a secondary event caused by the hemodynamic state.

The administration of anti–TNF-α (or anticalcinein) Ab or passive immunization of TNF-α prevented septic shock caused by the intravenous infusion of LPS in mice.45,46 The anti–TNF-α Ab showed a protective effect when it was given 2 hours before LPS administration, but it did not when it was given 1 hour before LPS.46 In our study, anti–TNF-α mAb improved survival and showed a protective effect when started 1 day (24 hours) before infection (AB–1D group). However, it did not alter their mortality or prevent myocardial lesions when anti–TNF-α mAb was given later. Anti–TNF-α mAb is reported to be effective in preventing acute cardiac rejection after heart transplantation in rats.47 These data suggest that TNF-α plays a common and essential role in the very early stage of acute viral myocarditis. On the contrary, IFN-α showed an antiviral effect in vivo in our murine model of viral myocarditis even when it was started simultaneously with virus infection but showed no effect when it was started 1 day after that.48 This suggests that IFN-α shows an antiviral effect even when virus replication in the heart has started. The anti–TNF-α Ab also suppresses cell-mediated immunity in vivo. It is of interest to note that anti–TNF-α Ab was effective during the afferent limb or priming limb of immunity, whereas it was ineffective during the efferent limb.49

Conclusions

Exogenously administered TNF-α aggravated EMCV–induced acute myocarditis in DBA/2 mice, although anti-TNF-α mAb improved the same model when its treatment was started 1 day before virus inoculation. Induced TNF-α level in the blood of infected mice 3, 5, and 7 days after the virus inoculation may not act as an antiviral agent similar to IFNs. However, TNF-α may play an important role in the very early stage of the immune system, and anti–TNF-α mAb may prevent the early pathway of acute viral myocarditis.

Acknowledgments

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