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Effects of tumor necrosis factor-α treatment on mortality in murine cytomegalovirus-infected mice

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Summary

The effects of treatment with recombinant DNA-derived Tumor Necrosis Factor-α (TNF-α) in a murine model of cytomegalovirus infection were investigated. Treatment of 3-week-old Swiss Webster mice with murine TNF-α prior to infection with murine cytomegalovirus (MCMV) had no demonstrable effect on mortality. However, if mice were treated prior to infection with a combination of murine IFN-γ and murine TNF-α, the dose of IFN-γ required to achieve significant reduction in mortality was reduced by a factor >10. In contrast to the beneficial effects of prophylactic TNF-α treatment in combination with IFN-γ, TNF-α treatment of mice after MCMV infection resulted in increased mortality. The increased mortality occurred when non-lethal doses of TNF-α were used and required virus replication. The effects of TNF-α treatment on mortality in MCMV-infected mice were not predicted from cell culture experiments which evaluated the effects of TNF-α treatment on MCMV replication in primary mouse embryo fibroblasts.

Murine cytomegalovirus; Tumor necrosis factor-α; Interferon-γ; Mouse; Mortality

Introduction

Human cytomegalovirus (CMV) is a herpesvirus which causes serious infections in immunologically immature or compromised hosts such as

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neonates, transplant recipients or AIDS patients (Alford and Britt, 1990). Effective therapy for CMV-infected individuals is limited due to the toxic side effects of currently available therapeutic agents such as ganciclovir and foscarnet, and the need to maintain continuous therapy to prevent recurrence (Alford and Britt, 1990; Balfour et al., 1982; Wade et al., 1982; Koretz et al., 1986). Thus, alternate means of prevention or treatment are desirable.

The utility of cytokines to prevent or reduce the severity of cytomegalovirus infections has been investigated by several laboratories. Both type I (α and β) and type II (γ) interferon inhibit replication of CMV in vitro (Rodriguez et al., 1983; Yamamoto et al., 1987), and prophylactic administration of recombinant DNA-derived IFN-α has been reported to reduce the incidence of CMV infections in transplant recipients (Cheeseman et al., 1979; Hirsch et al., 1983). However, no clinical benefit was observed when IFN-α was administered to patients already suffering from CMV infections (Myers et al., 1980; Myers et al., 1983).

In order to evaluate potential clinical applications of cytokines in cytomegalovirus infections we have tested their efficacy in a mouse model of CMV infection. Murine cytomegalovirus (MCMV) infection of weanling mice results in a generalized infection similar to that observed in human neonates and can result in significant mortality when an appropriate virus inoculum is used. We have previously demonstrated reduced mortality in MCMV-infected mice treated prophylactically with murine IFN-γ (Fennie et al., 1988).

In this publication we present results from our investigation of the effects of tumor necrosis factor (TNF-α) when administered alone or in combination with IFN-γ in this murine model of CMV infection. TNF-α exhibits antiviral activity in some cell culture systems, and both in vitro and in vivo experiments indicate synergistic antiviral activity when used in combination with IFN-γ (Wong and Goeddel, 1986; Czarniecki, 1991). Treatment of mice with TNF-α alone prior to MCMV infection had no demonstrable effect on mortality, but TNF-α administered in combination with IFN-γ prior to MCMV infection significantly augmented the ability of IFN-γ to reduce mortality. In contrast, treatment of mice with TNF-α after MCMV infection significantly enhanced mortality.

Materials and Methods

Cytokines

Cloning, expression, and purification of recombinant DNA-derived murine IFN-γ (Gray and Goeddel, 1983; Burton et al., 1984), murine TNF-α (Pennica et al., 1985), and human TNF-α (Pennica et al., 1984; Aggarwal et al., 1985) from *E. coli* have been described. Specific activity of murine IFN-γ was $1-2 \times 10^7$ International units/mg protein as determined by a cytopathic effect inhibition assay using encephalomyocarditis virus and murine L929 cells (Gray and Goeddel, 1983; Czarniecki et al., 1985). Specific activities of murine TNF-α

and human TNF-α were 4×10^7 units/mg protein and 5×10^7 units/mg protein, respectively, as determined in a standard cytotoxicity assay using actinomycin-D-treated murine L-M cells (Kramer and Carver, 1986). Cytokines were diluted on the day of use into pyrogen-free sterile saline for injection (USP) and administered by intraperitoneal injection. For cell culture experiments cytokines were diluted directly into the cell culture medium.

Cells

Primary mouse fibroblasts prepared from 18 day BALB/c embryos were obtained from the tissue culture facility at the University of California at San Francisco. They were propagated at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin sulfate.

Virus

The Smith strain of Murine cytomegalovirus (MCMV) was obtained from Tom Matthews and Elizabeth Frazier-Smith (Syntex Corporation, Palo Alto, CA). Stocks were prepared as 10% (w/v) homogenates of salivary glands in DMEM. Salivary glands were harvested 2 weeks after inoculation of 3-week-old Swiss Webster mice with a sub-lethal amount of MCMV. Virus was stored in aliquots at −80°C and was thawed immediately prior to use. Virus titers of stocks were determined by plaque assay on primary mouse embryo fibroblasts.

In vitro virus yield reduction assay for MCMV

Confluent monolayers of mouse embryo cells were incubated at 37°C in the presence of cytokines for 24 h prior to infection, rinsed with fresh medium and infected with MCMV using a multiplicity of infection of 1 pfu/cell. After adsorption for 1 h at 37°C, virus was removed, cells were rinsed, and fresh medium without cytokines was added. After incubation for 48 h at 37°C, virus was released by freezing and thawing, and the yield of infectious virus was determined by plaque assay on mouse embryo cells.

Plaque assay for MCMV

Samples for quantitation of infectious MCMV were diluted serially in DMEM using 10-fold increments, and duplicate 0.5 ml aliquots of appropriate dilutions were applied to monolayers of mouse embryo cells in 12-well tissue culture plates (Costar). After adsorption for 1 h at 37°C, samples were aspirated and cells were overlaid with DMEM containing 5% fetal calf serum, 0.7% Noble agar, 50 U/ml penicillin, 50 μg/ml streptomycin sulfate, and 1.25 μg/ml amphotericin B. Cells were fixed with 10% (v/v) formalin on day 5 post-infection and stained with crystal violet to visualize plaques.

Mice and infections

21-Day-old female Swiss Webster mice obtained from Simonsen Laboratories were used for all experiments. Unless otherwise indicated each

experimental group was comprised of 20 animals. MCMV diluted in DMEM was given by intraperitoneal injection in a total volume of 0.2 ml per animal. Each inoculum consisted of 2×10^2 to 1×10^5 pfu of MCMV per animal depending on the amount of mortality desired in a specific study.

Statistical analysis

Significant differences in the harmonic mean survival times of different groups of mice were determined by calculating logrank χ^2 values and accounting for surviving mice by the method of Peto and Pike (1973). P values less than 0.05 in a two-tailed analysis were considered significant.

Results

Effects of TNF-α treatment on replication of MCMV in primary mouse embryo fibroblasts

When primary mouse embryo fibroblasts were treated with murine TNF-α for 24 h prior to infection, only minimal inhibition of infectious MCMV production was observed (Fig. 1). This is in contrast to the marked, dose-dependent inhibition of MCMV replication observed when cells are pretreated with murine IFN-γ (Fig. 1). IFN-γ inhibition of MCMV and human CMV has previously been reported (Fennie et al., 1988; Yamamoto et al., 1987). When cells were pretreated simultaneously with equal amounts of murine IFN-γ and

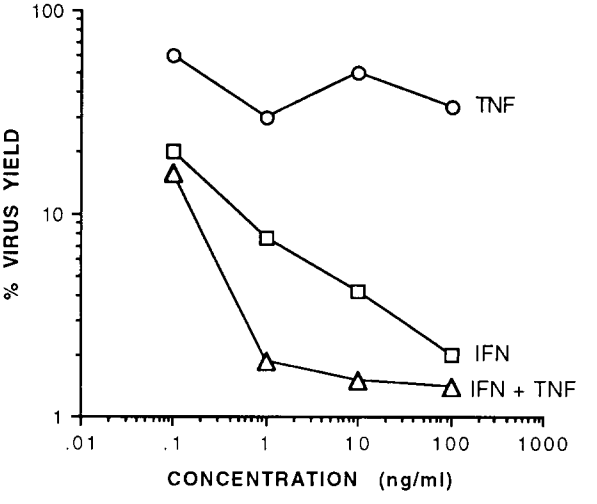

Fig. 1. Effects of murine TNF-α treatment on replication of MCMV in primary mouse embryo cells. Primary mouse embryo cells were treated for 24 h prior to MCMV infection (1 pfu/cell) with murine TNF-α (○), murine IFN-γ (□), or both (△) at the indicated concentrations. Infectious MCMV yields from treated, infected cells were determined by plaque assay and are expressed as the percent of yield from untreated cells.

TNF-α, inhibition of MCMV replication was marginally enhanced compared to cells treated with IFN-γ alone (Fig. 1).

Reduced mortality in mice treated with TNF-α and IFN-γ prior to MCMV infection

We have previously demonstrated prophylactic efficacy of murine IFN-γ in MCMV-infected mice (Fennie et al., 1988). The optimum regimen for administration of murine IFN-γ was determined to be 2 doses administered at 24 h and 4 h prior to MCMV infection. Statistically significant, dose-

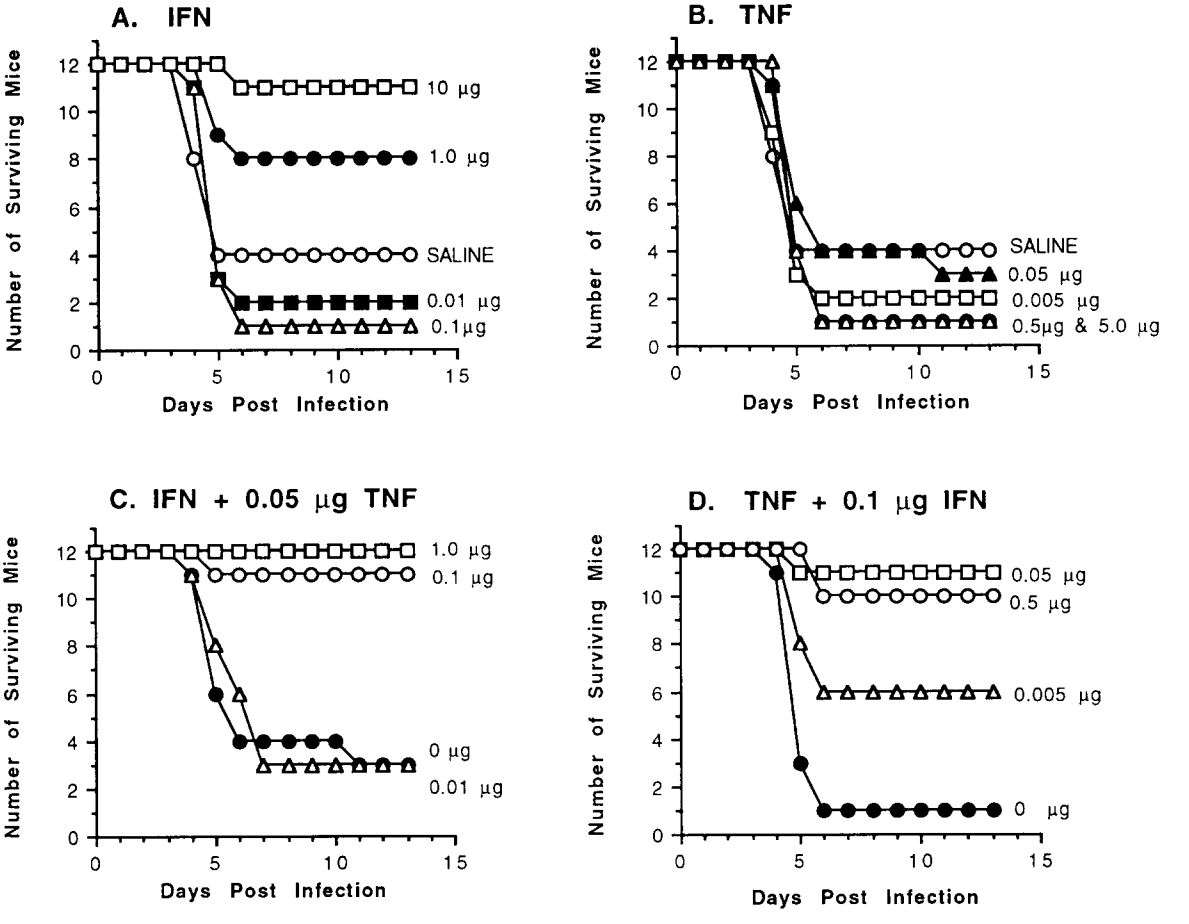

Fig. 2. Effects of prophylactic treatment with TNF-α and IFN-γ on mortality in MCMV-infected mice. Mice were treated with murine TNF-α, murine IFN-γ, or both 24 h and 4 h prior to infection with a lethal inoculum of MCMV. All mice in this experiment were treated and infected in parallel, but results have been plotted on seperate frames for clarity. (A) Dose response of IFN-γ treatment alone. (B) Dose response of TNF-α treatment alone. (C) Dose response of IFN-γ treatment in combination with 0.05 μg TNF-α. (D) Dose response of TNF-α treatment in combination with 0.1 μg IFN-γ.

dependent reductions in mortality are observed when mice are treated with 1 or 10 μg of murine IFN-γ according to this protocol (Fig. 2A). However, treatment of mice with murine TNF-α prior to MCMV infection according to the same protocol did not result in a significant effect on mortality over a wide dose range (Fig. 2B).

Despite the absence of any demonstrable effects of prophylactic TNF-α treatment alone, when low doses of TNF-α (0.05–0.5 μg) were administered in combination with IFN-γ, the dose of IFN-γ required to achieve optimum reduction in mortality was reduced by a factor greater than 10 compared to the dose of IFN-γ required without concomitant TNF-α treatment (Fig. 2C and 2D).

Alternative dosing regimens for combination treatment with TNF-α and IFN-γ were investigated in an independent experiment (Fig. 3). This experiment confirmed that low doses of murine TNF-α (0.14 μg), when administered prior to MCMV infection and simultaneously with a suboptimal dose of murine IFN-γ (0.8 μg) could significantly enhance survival of MCMV-infected mice treated with IFN-γ alone. Dosing with TNF-α for 2 days after IFN-γ treatment (and after MCMV infection), or dosing with TNF-α for 2 days prior to IFN-γ treatment instead of simultaneously with IFN-γ treatment did not significantly enhance survival of MCMV mice compared to those treated with IFN-γ alone.

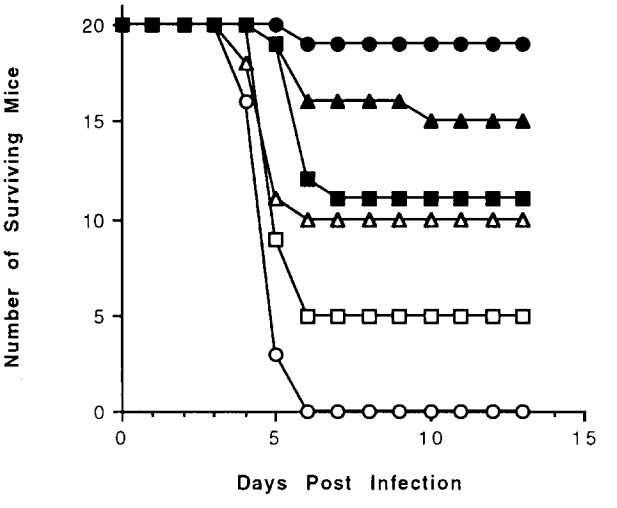

Fig. 3. Optimization of dosing regimen for TNF-α treatment in combination with prophylactic IFN-γ treatment. Mice were treated with murine TNF-α (0.14 μg/animal per dose), murine IFN-γ (0.8 μg/animal per dose) or both according to different schedules. (○) Saline treatment on days −1 and 0 relative to MCMV infection; (□) TNF-α treatment on days −1 and 0; (△) IFN-γ treatment was on days −1 and 0; (●) IFN-γ and TNF-α treatment on days −1 and 0; (▲) IFN-γ treatment on days −1 and 0 and TNF-α treatment on days −3 and −2; (■) IFN-γ treatment on days −1 and 0 and TNF-α treatment on days +1 and +2.

Enhanced mortality in mice treated with non-toxic doses of TNF-α after MCMV infection

Human TNF-α was used for initial studies to investigate the effects of long-term therapeutic administration of TNF-α in MCMV-infected mice. Large amounts of material were required for a study of this nature, and clinical-grade human TNF-α was available in sufficient quantities. Human TNF-α has previously been shown to exhibit activity in murine systems (Kramer and Carver, 19S6; Pennica et al., 1984; Talmadge et al., 1987).

Treatment of mice with high doses of human TNF-α (2 μg/animal per day)

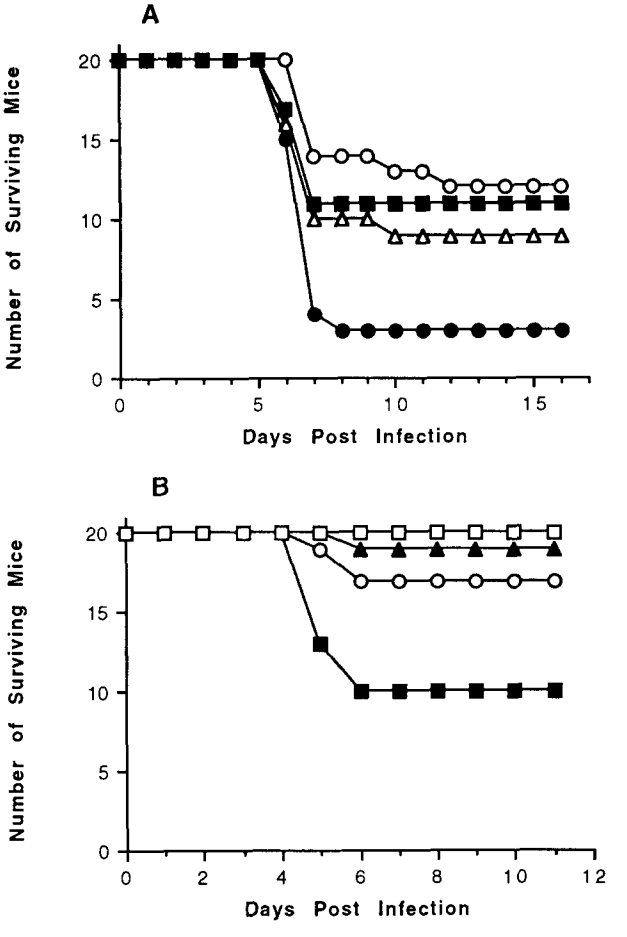

Fig. 4. Effects of human TNF-α treatment on mortality in MCMV-infected mice. (A) Mice were treated daily with human TNF-α for 12 days beginning the day prior to a lethal MCMV challenge: (○) Saline, (■) 0.02 μg TNF-α/animal, (△) 0.2 μg TNF-α/animal, (●) 20 μg TNF-α/animal. (B) Mice were treated daily with human TNF-α for 10 days beginning the day prior to a minimally lethal challenge of MCMV. A mock-infected, TNF-α-treated control group was included in this experiment: (○) Saline, (▲) 0.2 μg TNF-α, (■) 2.0 μg TNF-α, (□) 2.0 μg TNF-α in mock-infected mice.

for 12 days beginning the day prior to MCMV infection resulted in a significant increase in mortality compared to control-treated, MCMV-infected animals (Fig. 4A). Since the doses of TNF-α used in this experiment were well below doses previously shown to be tolerated in adult mice (Talmadge et al., 1987), no control group of TNF-α-treated, uninfected mice was included in this experiment.

In a second experiment, the virus inoculum was reduced to minimize

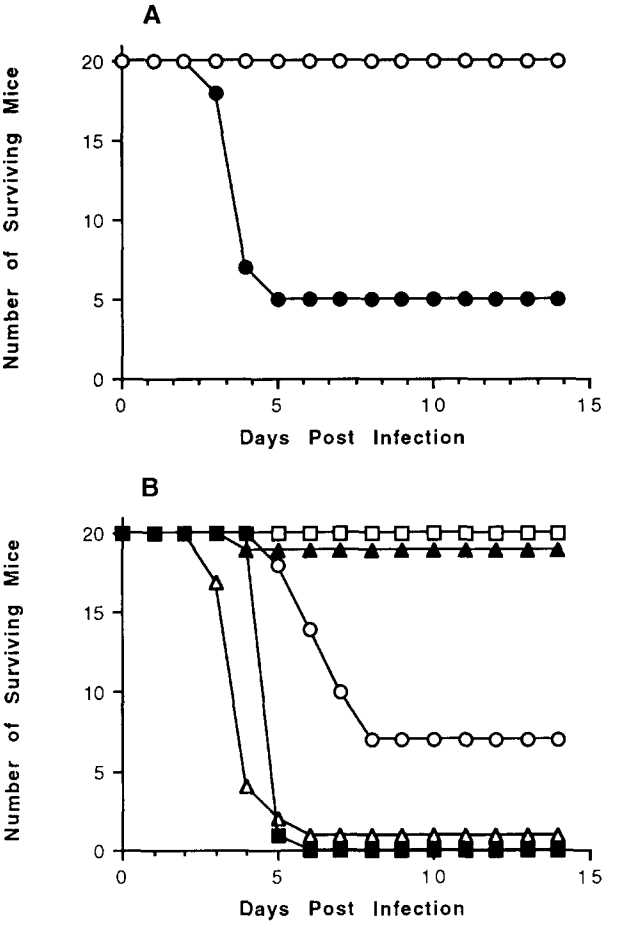

Fig. 5. Effects of daily murine TNF-α treatment on mortality in MCMV-infected mice. (A) Mice were treated daily with murine TNF-α for 8 days beginning the day after infection with a non-lethal challenge of MCMV. No mortality was observed in any groups except that receiving the highest doses of TNF-α (5 μg) and infected with MCMV (○) MCMV-infected mice treated with saline, or 0.5 μg TNF-α, or mock-infected mice treated with 0.5 μg or 5.0 μg TNF-α; (●) MCMV-infected mice treated with 5.0 μg TNF-α. (B) Mice were treated with 5 μg doses of murine TNF-α for 8 days beginning the day after a moderately lethal challenge with MCMV. (○) Saline treatment of MCMV-infected mice; (▲) TNF-α treatment of mock-infected mice, (△) TNF-α treatment of MCMV-infected mice; (□) TNF-α treatment of mice inoculated with heat-inactivated MCMV; (■) TNF-α treatment of mice inoculated with filtered MCMV.

mortality in untreated mice. This experiment confirmed that daily administration of 2 μg of human TNF-α to MCMV-infected mice significantly enhanced mortality compared to control-treated, MCMV-infected mice. A control group of animals mock-infected with vehicle only, exhibited no mortality or obvious signs of morbidity when treated with this same dose of human TNF-α (Fig. 4B).

Similar results were obtained when murine TNF-α was administered to MCMV-infected mice for 8 days beginning the day after infection. Following a non-lethal challenge with MCMV, mortality of 75% was observed in infected mice treated with 5 μg of murine TNF-α. No mortality was observed in infected mice treated with TNF-α doses 0.5 μg per animal per day, or uninfected mice treated with 5 μg TNF-α per day (Fig. 5A). Heat inactivation of the MCMV stock (56°C for 60 min) prior to infection eliminated mortality in TNF-α-treated animals, but filtration of the virus stock through a 0.2 μm filter prior to infection did not significantly affect mortality (Fig. 5B).

Discussion

The effects of TNF-α treatment on mortality in MCMV-infected mice varied remarkably depending on the dose of TNF-α administered, the time of administration relative to infection, and the presence or absence of simultaneous IFN-γ treatment. Prophylactic treatment with TNF-α alone, resulted in no significant effects on mortality in MCMV-infected mice, but TNF-α treatment prior to virus infection and in combination with IFN-γ reduced the dose of IFN-γ required to achieve a significant reduction in mortality by a factor greater than 10. Combination prophylactic therapy with TNF-α and IFN-γ might therefore be useful in clinical situations where exposure to CMV can be predicted and is likely to lead to serious disease. Transplant recipients with no evidence of CMV immunity, or infants receiving transfusions are potential candidates for this type of treatment.

In contrast to the beneficial effects of prophylactic TNF-α treatment when given in combination with IFN-γ, treatment of mice with TNF-α after MCMV infection resulted in increased mortality. TNF-α treatment using doses which are non-toxic in uninfected animals resulted in 75% mortality in one experiment where a nonlethal virus challenge was used. Similar results were obtained in several experiments using both low and high virus inocula. The enhanced mortality was dependent upon virus replication, since TNF-α treatment of mice inoculated with heat-inactivated virus did not result in mortality. Furthermore, filtration of virus stocks prior to infection demonstrated that the TNF-induced toxicity was not the result of contaminating microorganisms in the virus stocks. These results suggest that caution should be exercised in the use of TNF-α for clinical trials. CMV is a ubiquitous herpesvirus which can establish latent or inapparent infections (Alford and Britt, 1990). Administration of high doses of TNF-α, even to patients not exhibiting clinical manifestations of CMV infection, could have adverse

consequences resulting from the interaction of latent CMV and TNF-α.

The mechanism by which TNF-α exerts its effects in MCMV-infected mice is unclear. Previous experiments investigating effects of murine IFN-γ in MCMV-infected mice showed a correlation between a reduction in infectious MCMV yields from cultured mouse embryo fibroblasts and mortality in mice treated with IFN-γ prior to MCMV infection. The correlation was strengthened by the observation that infectious MCMV titers in critical organs of IFN-γ-treated mice were also reduced compared to controls (Fennie et al., 1988). However, no such correlation between cell culture and whole animal experiments was found in the case of TNF-α. TNF-α treatment of cultured mouse embryo fibroblasts had no demonstrable effect on their ability to support MCMV replication, and combination treatment of mouse embryo fibroblasts with IFN-γ and TNF-α resulted in only marginally reduced replication of MCMV compared to treatment with IFN-γ alone.

The discrepancy between results in the cell culture and whole animal systems could be attributed to differences between antiviral activity in cultured mouse embryo fibroblasts and activity in critical target cells responsible for pathology in MCMV-infected mice. Alternatively, the modest reduction in virus titers resulting from combination treatment with IFN-γ and TNF-α at some concentrations may have been adequate to significantly reduce mortality. Mortality in this model is dependent on the amount of virus inoculum (compare mortality of control groups in Figs. 3–5) and even minor effects on virus replication compounded over several replication cycles could significantly effect the pathological consequences of MCMV infection. The prophylactic efficacy of murine IFN-γ and TNF-α combination treatment in mice might also involve the immunomodulatory properties reported for both cytokines (Anderson et al., 1988; Shalaby et al., 1985; Playfair and De Souza, 1987; Shalaby et al., 1988; Gresser et al., 1979; Sonnenfeld et al., 1978).

The mechanisms responsible for enhanced mortality in mice treated with TNF-α after infection with MCMV also remain speculative. Enhanced mortality resulting from treatment with non-toxic doses of TNF-α has been reported in African green monkeys infected with the herpesvirus, simian varicella virus (SVV) (Soike et al., 1989). The TNF-α-induced, dose-dependent mortality in SVV-infected monkeys was accompanied by a dose-dependent increase in SVV viremia, as well as a marked enhancement in the severity of virus induced lesions. These results are consistent with enhanced virus replication resulting from TNF-α treatment. Several demonstrated activities of TNF-α could contribute to enhanced virus replication in TNF-α-treated animals, including intracellular enhancement of virus gene expression by induction of transcription factors in infected cells (Duh et al., 1989; Osborn et al., 1989), or induction of specific virus receptors to facilitate transmission of the virus (Cotran and Pober, 1989; Greve et al., 1989; Staunton et al., 1989). The latter process would be more likely to exert an effect on virus replication in animals, without affecting virus replication in cultured cells. The potential ability of TNF-α to modulate the immunocompetency of MCMV-infected

weanling mice should also be considered as a possible mechanism for the enhanced mortality observed in treated mice.

These experiments emphasize the need for caution in extrapolation of results in cell culture systems to results in whole animal systems. This is especially true when investigating cytokines such as TNF-α which exhibit pleiotropic activities. Dramatic effects on mortality were observed in MCMV-infected mice which would not have been predicted from results obtained in cell culture systems. Furthermore, the effects of TNF-α treatment in MCMV-infected mice could be drastically altered by changing the dosing schedule, or the types of co-treatment. Additional investigation is required to better understand the mechanisms responsible for TNF-α activity in animal systems before practical clinical applications can be developed.

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