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Reduced mortality in murine cytomegalovirus infected mice following prophylactic murine interferon-γ treatment

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Summary

Efficacy of recombinant DNA-derived murine IFN-γ was investigated in a murine model of cytomegalovirus infection. Treatment of 3-week-old Swiss Webster mice with murine IFN-γ prior to infection with murine cytomegalovirus (MCMV) significantly reduced mortality due to MCMV infection. Efficacy was dose-dependent and was observed using either intraperitoneal or intramuscular injection as the route of administration. Two doses, one at 24 h and one at 4 h prior to MCMV infection, were required for optimum efficacy, and doses administered after MCMV infection had no apparent effect. Reduced infectious MCMV titers were observed in critical organs of IFN-γ treated mice and histopathologic lesions induced by MCMV infection were in general less severe and resolved sooner than lesions in untreated mice. Results in this murine model of cytomegalovirus infection suggest that IFN-γ treatment may be useful as prophylactic therapy for human cytomegalovirus infections when a high probability of exposure to the virus exists and consequences of infection may be severe.

Murine cytomegalovirus; Interferon-γ; In vivo efficacy; Mortality; Prophylaxis

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Introduction

Interferons are a class of proteins which have potent antiviral, immunomodulatory, and antiproliferative properties (Stewart, 1979). Pretreatment of cells with IFN in vitro can inhibit replication of a wide variety of subsequently infecting viruses, and in vivo prophylactic efficacy of both type 1 (α and β) and type 2 (γ) IFNs have been demonstrated in several animal models of viral infection (Goeddel et al., 1980; Schellekens et al., 1981; Shalaby et al., 1985a; Fraser-Smith et al., 1984a,b, 1985). Some clinical evidence from studies using IFN-α suggests that prophylactic IFN treatment may be clinically useful in some viral infections where exposure to the virus can be predicted (Cheeseman et al., 1979).

Human cytomegalovirus (CMV) is one example of a virus where exposure leading to serious disease can often be predicted. CMV is a herpesvirus which causes serious infections in immunologically immature or compromised hosts (Ho, 1982). In many cases, the victims are neonates who acquire the virus through a transfusion of contaminated blood, or immunosuppressed transplant recipients who acquire the virus infection from an infected donor organ. Effective therapy for CMV infected individuals is limited due to toxic side effects of currently available chemotherapeutic agents such as nucleoside analogs, and the need to maintain continuous therapy to prevent recurrence (Balfour et al., 1982; Wade et al., 1982; Koretz et al., 1986). Type 1 IFN can inhibit human CMV replication in vitro (Rodriquez et al., 1983), and the severity of murine CMV (MCMV) infections of mice can be reduced using prophylactic treatment with IFN-β (Cruz et al., 1981) or IFN inducing agents (Kern et al., 1978). In addition, prophylactic administration of recombinant DNA derived IFN-α has been shown to reduce the incidence of CMV infections in transplant recipients (Cheeseman et al., 1979; Hirsch et al., 1983). Beneficial effects of therapeutic IFN treatment have not been demonstrated (Myers et al., 1980, 1983).

IFN-γ is a secreted polypeptide product of stimulated T lymphocytes which exhibits antiviral activity like type 1 IFNs, but which has more potent immunomodulatory properties than type 1 IFNs (Sonnenfeld et al., 1978; Gresser et al., 1979). Because of the reported immunosuppressive effects of CMV infection (Ho, 1984; Schooley et al., 1983), as well as the occurrence of serious CMV infections in immuno-compromised individuals, prophylactic therapy using IFN-γ may have even greater efficacy than that observed using IFN-α. In addition, human cells treated with IFN-γ are apparently more resistant to human CMV infection than those treated with IFN-α (Yamamoto et al., 1987). For these reasons we have investigated the effects of prophylactic therapy using recombinant DNA derived murine IFN-γ in a murine model of cytomegalovirus infection.

Murine CMV (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. Our experiments indicate that treatment of weanling mice with murine IFN-γ prior to infection with MCMV can significantly reduce mortality due to MCMV infection, and can reduce the amount of infectious virus present in critical organs of infected mice. In addition,

histopathologic lesions induced by MCMV infection were in general less severe and resolved sooner than lesions in untreated mice. These results suggest that human IFN-γ treatment may be useful as a prophylactic therapy for individuals at risk for CMV infection.

Materials and Methods

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 MCMV was obtained from Tom Mathews 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 sublethal 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 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 and stained with crystal violet to visualize plaques.

Interferon

Cloning, expression and purification of recombinant DNA derived murine IFN-γ from *E. coli* was performed as previously described (Gray and Goeddel, 1983; Burton et al., 1985). Specific activity was approximately 1.3×10^7 IU/mg as determined by a cytopathic effect inhibition assay using encephalomyocarditis virus and murine L929 cells (Gray and Goeddel, 1983; Czarniecki et al., 1985). National Institutes of Health International Reference Standard Gg02-901-533 was used for standardization of assay results. For injection into mice, murine IFN-γ was diluted into pyrogen-free sterile saline for injection (USP). Administration was by intraperitoneal or intramuscular injection. For in vitro experiments murine IFN-γ was diluted directly into the cell culture medium.

In vitro virus yield reduction assay for MCMV

Confluent monolayers of mouse embryo cells were incubated in the presence of INF for 24 h prior to infection, rinsed with fresh medium and infected with MCMV. After adsorption for 1 h at 37°C, virus was removed, cells were rinsed, and fresh medium without INF 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.

Mice and infections

Twenty-one-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 approximately 6×10^4 PFU of MCMV per animal. Mortality with the use of this inoculum of MCMV ranged from 30 to 95%, with a mean mortality of 70%. A single stock preparation of MCMV was used for all experiments.

Assay of infectious MCMV in organs of infected mice

On specified days after infection, organs were harvested from MCVM infected mice and pooled with similar organs from mice in the same group. Homogenates of organs (10% w/v) were prepared in DMEM and infectious MCMV was quantitated by plaque assay on mouse embryo cells.

Statistical analysis

Significant differences in the harmonic mean survival times of different groups of mice were determined by calculating log rank X^2 values and accounting for surviving mice by the method of Peto and Pike (1973). *P* values less than 0.05 were considered significant.

Results

Inhibition of MCMV replication in mouse embryo fibroblasts treated with murine IFN-γ

The sensitivity of MCMV to IFN-γ treatment in vitro was investigated using an infectious virus yield reduction assay; murine IFN-γ pretreatment of mouse embryo fibroblasts for 24 h prior to infection resulted in no significant reduction in infectious MCMV yield from cells infected at a high multiplicity of infection (10 PFU/cell) even at IFN-γ concentrations as high as 100 ng/ml. However, when the amount of input virus used to infect cells was reduced to 0.1 or 1.0 PFU/cell, greater than 90% inhibition of infectious MCMV yield was observed at relatively low IFN-γ concentrations (< 1 ng/ml: Fig. 1). Murine IFN-γ pretreatment at 100 ng/ml reduced infectious virus yield to approximately 2% of that observed in untreated mouse embryo cells. Treatment of cells with doses of IFN-γ greater than 100 ng/ml did not inhibit replication of MCMV further at any multiplicity of infection (data not shown).

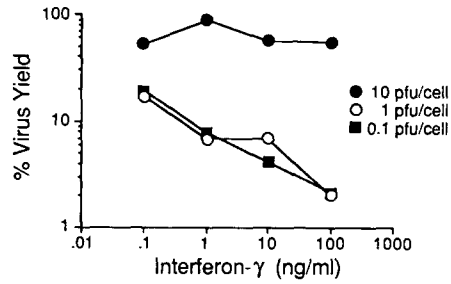

Fig. 1. IFN-γ inhibition of MCMV replication in primary mouse embryo cells. Primary mouse embryo cells were treated for 24 h prior to MCMV infection with the indicated concentrations of IFN-γ. Infectious MCMV yields from infected cells were determined by plaque assay and are expressed as the percent of yield from untreated cells. Each curve represents results from parallel experiments performed using the indicated multiplicities of infection for MCMV.

Enhanced survival of IFN-γ treated mice following MCMV infection

The effect of murine IFN-γ treatment on the course of MCMV infection in vivo was investigated in an acute systemic infection of weanling Swiss Webster mice using mortality as an endpoint. When IFN-γ was administered by intraperitoneal in-

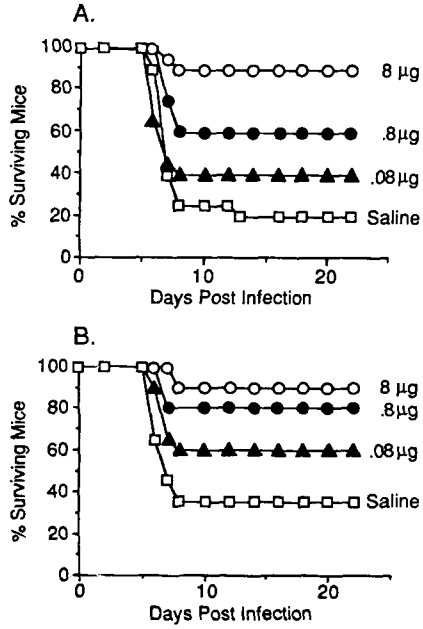

Fig. 2. Enhanced survival in MCMV infected mice as a result of murine IFN-γ treatment. Mice were infected for 12 consecutive days beginning 1 day prior to MCMV infection with the indicated concentrations of murine IFN-γ. Control mice were treated with saline according to the same schedule. Groups of 20 animals were monitored daily for survival but some observation points are omitted for clarity when there was no change from the previous day. (A) Murine IFN-γ was administered by intraperitoneal injection. (B) Murine IFN-γ was administered by intramuscular injection.

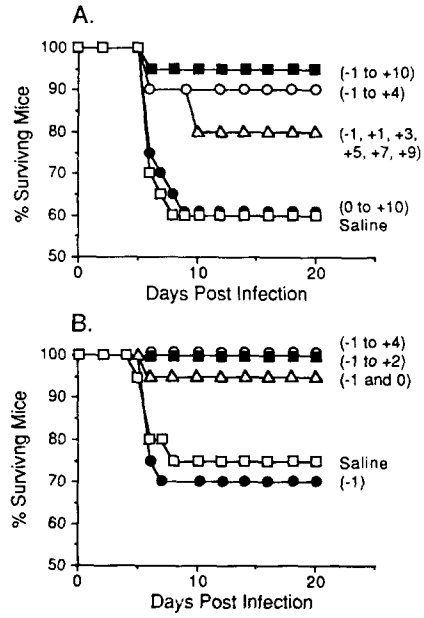

Fig. 3. Optimum dosing regimen for efficacy of murine IFN-γ in MCMV infected mice. (A) MCMV infected mice ($N = 20$) were treated with 8 μg of murine IFN-γ administered by intramuscular injection on the indicated days relative to MCMV infection. Control mice received intramuscular injections of saline on days −1 to +10 relative to infection. (B) MCMV infected mice ($N = 20$) were treated with 8 μg of murine IFN-γ administered by intraperitoneal injection on the indicated days relative to MCMV infection. Control mice received intraperitoneal injections of saline on days −1 to +4 relative to infection.

jection for 12 consecutive days beginning the day prior to infection, a dose-dependent enhancement of survival in MCMV infected mice was observed (Fig. 2A). Ninety percent of mice treated with the highest dose of IFN-γ tested (8 μg) survived the viral infection, while only 20% of saline-treated animals survived. Survival in mice receiving daily doses of 0.8 or 0.08 μg of IFN-γ was 60 and 40%, respectively. Survival of mice treated with daily doses of 8 or 0.8 μg of IFN-γ was significantly enhanced compared to control treated mice ($N = 20$; $P < 0.01$).

When IFN-γ was administered by intramuscular injection rather than by intraperitoneal injection, a similar dose-dependent enhancement of survival was observed (Fig. 2B). In this experiment 35% of untreated mice survived the MCMV infection, but 60, 80 and 90%, respectively, of mice treated with daily doses of 0.08, 0.8, or 8 μg of murine IFN-γ, respectively, survived. Survival of mice treated with the 2 highest doses of IFN-γ by the intramuscular route was significantly enhanced compared to the control treated animals ($N = 20$; $P < 0.01$).

Optimum IFN-γ dosing strategy for prevention of mortality in MCMV infected mice

Several experiments were performed to determine optimum dosing regimens for efficacy of IFN-γ in MCMV infections. In an initial experiment using intramus-

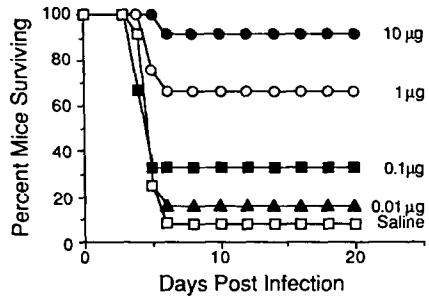

Fig. 4. Dose-dependent prevention of mortality in MCMV infected mice as a result of murine IFN-γ pretreatment. Groups of 12 mice were treated with murine IFN-γ administered by intraperitoneal injection at 24 h and 4 h prior to MCMV infection. Each injection contained the indicated doses of murine IFN-γ in saline. Mice in the control group received injections of saline only.

cular administration of murine IFN-γ, 6 doses of 8 μg per mouse administered daily beginning the day prior to MCMV infection prevented mortality due to MCMV infection just as efficiently as 12 doses (Fig. 3A). However, when mice were treated for 11 consecutive days beginning on the day of infection, mortality was indistinguishable from that of a saline-treated control group. When mice were treated every other day beginning the day prior to infection, some reduction in mortality was observed, but the difference was not statistically significant compared to the control group.

In a second experiment in which murine IFN-γ was administered by intraperitoneal administration, 4 to 10 doses of murine IFN-γ beginning the day prior to MCMV infection provided equivalent significant protection from the challenge (Fig. 3B). Only a slight and statistically insignificant decrease in efficacy was observed for those animals treated with only 2 doses of murine IFN-γ, one on the day prior to infection and one on the day of infection.

A dose-response experiment performed using only 2 doses of murine IFN-γ (days −1 and 0; Fig. 4) resulted in a 50% efficacious dose (ED_{50}) (1.0 μg) that was nearly identical to that observed using 12 daily doses of IFN-γ (0.8 μg; Fig. 2A). Survival was significantly enhanced in those mice treated with 1 or 10 μg of IFN-γ on days −1 and 0 compared to control mice ($N = 12$; $P < 0.05$).

Reduction of MCMV titers in selected organs of mice treated with murine IFN-γ

Titers of infectious MCMV in selected organs of infected mice were monitored to determine the effect of IFN-γ treatment on in vivo virus replication. Mice receiving a lethal inoculum of MCMV were treated by intraperitoneal injection for 10 consecutive days beginning the day prior to infection with either 8 μg of murine IFN-γ or saline per animal. Five mice in each group were sacrificed at intervals for titration of virus in specific organs. In this experiment, only 12% of animals in the control group survived to day 8 while 60% of IFN-γ treated animals survived. Infectious virus titers of serum, salivary glands, livers, spleens and kidneys from IFN-γ treated mice were lower than corresponding titers in control mice (Fig. 5). For

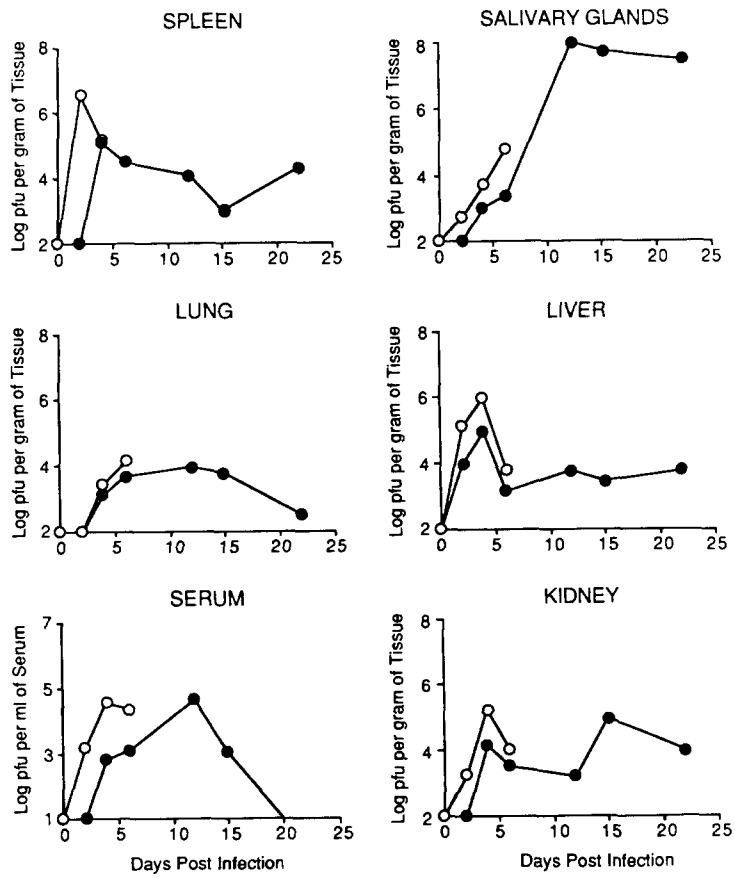

Fig. 5. Infectious MCMV titers in selected organs of control and murine IFN-γ treated MCMV infected mice. Mice received intraperitoneal injections of either sterile saline (○) or 8 μg of murine IFN-γ (●) on days −1 to +9 relative to MCMV infection. Only 12% of animals in the saline treated control group survived to day 8, while 60% of murine IFN-γ treated animals survived. Five mice in each group were sacrificed on indicated days and 10% (w/v) homogenates of pooled organs from each group were assayed for infectious virus by plaquing on mouse embryo cells. The minimum detectable $\log_{10}$ titer/gr of tissue for spleen, salivary glands, lung, liver and kidney was 2.0. The minimum detectable $\log_{10}$ titer/ml of serum was 1.0.

most of these organs, peak infectious virus titers during the first 6 days after infection were approximately 1 log less than in the saline-treated control mice. There was also a delay in the time of onset of detectable infectious virus in serum, salivary glands, spleens and kidneys of IFN-γ treated mice. No reduction of virus titer or delay in the onset of detectable infectious virus was observed in the lungs of IFN-γ treated animals. Infectious MCMV was not eliminated from organs of surviving IFN-γ treated mice, but persisted in most organs until at least 22 days after infection. MCMV titers in the salivary glands of IFN-γ treated mice continued to increase until 12 days after infection and remained at that elevated level until the last observation (day 22).

Discussion

The experiments presented here demonstrate that prophylactic parenteral administration of murine IFN-γ can prevent mortality due to MCMV infection in weanling mice. Measurable efficacy requires dosing on the day prior to infection as well as on the day of infection. Treatments subsequent to infection do not significantly enhance efficacy resulting from pretreatment of infected mice and have no observable effect on mortality of MCMV infected mice alone. In 3 separate experiments (Figs. 2A and 4, and additional data not presented here) reductions in mortality of 50% where achieved with daily doses of 0.8 to 1.0 μg of murine IFN-γ administered by intraperitoneal injection. The ED_{50} was comparable regardless of the number of daily doses administered as long as critical doses on the day prior to infection and on the same day as infection were included (compare Fig. 2A and Fig. 4). In a single dose-response experiment using intramuscular administration of murine IFN-γ, the ED_{50} was estimated to be less than that for intraperitoneal injection (0.2 μg, Fig. 2B) demonstrating that this route is at least as effective as the intraperitoneal route.

The mechanism by which IFN-γ prevents mortality due to MCMV infection is not known. One possible explanation for the reduced mortality in IFN-γ treated mice may be that MCMV replication is inhibited as a consequence of the direct antiviral activity of IFN-γ. Infectious MCMV titers in many organs of IFN-γ treated mice were reduced relative to titers in control mice by approximately 1 log: a factor comparable to that observed for inhibition of MCMV replication in mouse embryo cells. A reduction of this magnitude in the amount of MCMV used to infect mice initially reduces mortality due to MCMV infection in a manner comparable to that observed following IFN-γ treatment (mean of 82% reduction in mortality in 3 separate experiments; data not shown). A 1 log reduction of infectious virus in critical organs may therefore account for the observed reduction in mortality. Efficacy in vivo also requires prophylactic treatment prior to infection, just as manifestation of the antiviral properties of interferons in vitro requires pre-incubation of host cells prior to infection (Stewart, 1979).

However, alternative explanations exist. For example, reduced mortality due to MCMV infection in IFN-γ treated mice may be due to immunoenhancement as a result of IFN-γ treatment. IFN-γ can enhance B-cell differentiation and proliferation (Leibson et al., 1984; Sidman et al., 1984; Nakagawa et al., 1985), expression of class I and class II major histocompatability antigens (King and Jones, 1983; Wong et al., 1983; Skoskiewicz, 1985; Chang and Lee, 1986), and antibody production (Nakamura et al., 1984; Playfair and De Souza, 1987; Anderson et al., 1988). In addition, IFN-γ can activate phagocytic cells important for defense against viral infections (Nathan et al., 1983; Shalaby et al., 1985b). Any of these effects could lead to an enhanced or more rapid immune response to MCMV infection resulting in reduced virus titers in critical organs and enhanced survival.

IFN-γ treatment has also been reported to enhance natural killer (NK) cell activity in mice (Svedersky et al., 1984; Shalaby et al., 1985c). Differences in NK cell function of inbred strains of mice have been linked to differences in the sus-

ceptibility of these strains to MCMV infection (Bancroft et al., 1981; Quinnan and Manischewitz, 1987). Thus, enhancement of NK cell activity by IFN-γ treatment in normally susceptible mice could also account for the enhanced survival and reduced MCMV titers observed in IFN-γ treated animals. Enhancement of NK cell activity by type 1 IFN induced by MCMV infection has been implicated as an important host defense mechanism in MCMV infections (Grundy et al., 1982).

Although human CMV and MCMV are clearly distinct viruses, enough similarities in genomic structure, morphology and pathology of the two viruses exist to suggest that results obtained in the MCMV model may be predictive of results with human CMV (Hudson, 1979; Osborn, 1982). Based on information presented here, therapeutic use of human IFN-γ in infected human patients would probably not be effective. However, exposure to human CMV can often be predicted and prophylactic treatment might therefore be useful in some circumstances. Serious CMV infections of humans often occur as a result of organ transplants from donors harboring latent CMV infections to previously unexposed individuals. CMV infections of this nature are more likely to lead to serious complications since organ transplant recipients are often artificially immunosuppressed to prevent rejection of the transplanted organ. Prophylactic human IFN-α treatment has been used successfully to reduce the incidence and severity of human CMV infections in kidney transplant recipients (Cheeseman et al., 1979; Hirsch et al., 1983). IFN-γ treatment is likely to be at least as effective as IFN-α treatment in this situation since pretreatment of human cells with human IFN-γ is more effective in preventing replication of human CMV in vitro than pretreatment with human IFN-α (Yamamoto et al., 1987). However, potential adverse effects on transplant survival resulting from the immunomodulatory activities of IFNγ should be thoroughly investigated prior to treatment of this patient population.

Life-threatening CMV infections can also occur in infants receiving contaminated blood transfusions, probably due to the general immaturity of the immune systems in newborns. However, lymphocytes of human neonates are deficient in their ability to produce IFN-γ in response to mitogenic stimulation (Lewis et al., 1986; Wilson et al., 1986), suggesting an alternative explanation for their susceptibility. If enhanced susceptibility of human neonates to CMV infection is related to their inability to produce IFN-γ, then prophylactic IFN-γ treatment in neonates receiving transfusions or transplants might also be clinically useful.

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