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Antiviral Activity of Recombinant Rat Interferon Gamma in Immunologically Impaired and Immunosuppressed Rats

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SUMMARY

Treatment of Wag/Rij rats with recombinant rat interferon gamma (rRIF-γ) resulted in complete protection against a lethal pseudorabies virus (PRV) infection. To investigate whether the protection resulted from direct inhibition of virus replication or from a stimulation of immune mechanisms, we tested rRIF-γ activity in naturally immunocompromised and artificially immunosuppressed rats. The antiviral effect of rRIF-γ was not abolished in silica- and carrageenan-treated, phagocyte-depleted rats. Immunologically immature newborn and T cell-deficient nude rats were also protected under a regime of rRIF-γ treatment as well as whole body gamma-irradiated rats. Sera of the protected rats were devoid of PRV-neutralizing antibodies. Our results indicate that the protective activity of rRIF-γ is based on direct inhibition of virus replication; stimulation of the immune system is not required but may be responsible for protection upon challenge several weeks after infection.

INTRODUCTION

The number of animal studies aimed at investigating the antiviral activity of interferon has, until recently, been limited due to its scarcity. Since large amounts of recombinant human and mouse interferons have become available most studies *in vivo* have been performed in mouse and monkey model systems. After successful cloning and expression of the rat interferon gamma gene in Chinese hamster ovary cells (Dijkema *et al.*, 1985; Van der Meide *et al.*, 1986) we are now able to study the properties of recombinant rat interferon gamma (rRIF-γ) in the rat.

We have examined rRIF-γ activity in rats experimentally infected with pseudorabies virus (PRV). From earlier studies we know that infection with PRV is an excellent model for testing the antiviral activity of purified rat interferon (Schellekens *et al.*, 1984). PRV or Aujeszky's disease virus is a neurotropic alphaherpesvirus which infects swine and cattle (Nara, 1982), often leading to economic losses in farms. In laboratory rodents and lagomorphs, the virus causes mostly lethal infections of the nervous system (Fraser & Ramachandran, 1969).

Interferon-γ is a lymphokine produced only by T lymphocytes in response to antigens and mitogens (O'Malley *et al.*, 1982). Apart from its different cellular origin, IFN-γ differs in its physicochemical and biological properties from IFN-α and IFN-β (Trinchieri & Perussia, 1985). IFN-γ binds to cell surface receptors distinct from those binding IFN-α and IFN-β (Branca & Baglioni, 1981; Czarniecki *et al.*, 1984); in addition, it is capable of enhancing the effects of the other interferon types (Czarniecki *et al.*, 1984). Interferon-γ characteristically exerts strong immunomodulating activity. It has been shown to alter natural killer cell function (Shalaby *et al.*, 1985), modulate cell-mediated cytotoxicity (Morris *et al.*, 1982), affect antibody production (Sidman *et al.*, 1984), activate macrophages (Steeg *et al.*, 1982) and induce or enhance expression of major histocompatibility complex class I and/or class II antigens on a variety of cell types (Rosa & Fellous, 1984). In order to test whether the antiviral activity of rRIF-γ is based on involvement of the host immune system, we examined rRIF-γ activity in immunologically impaired and immunosuppressed rats during experimental infection with PRV.

METHODS

Animals. Except when stated otherwise, female 4- to 5-week-old specific pathogen-free Wag/Rij rats from the colony of the REPGO Institutes (Rijswijk, The Netherlands) were used. Animals, housed 10 per cage, had free access to food and water. Experiments were performed under quarantine conditions. The animals were observed daily for signs of illness until a steady condition was apparent. Nude rats (Wag/Rij rnu/rnu) were obtained from the same source whereas Rowett hooded homozygous (rnu/rnu) and heterozygous (rnu/+) nude rats were purchased from the Harlan-Centraal Proefdier Bedrijf (Austerlitz, The Netherlands).

Virus. A field isolate of PRV was obtained from the Centraal Diergeneeskundig Instituut, Lelystad, The Netherlands; the third and fourth passages in Ratec cells (rat embryo fibroblasts) were used. All inoculations described in this study were performed intraperitoneally (i.p.). With the exception of the experiment with newborn rats, all infections were with 200 p.f.u. per animal. Infected rats showed signs of paralysis, accelerated respiration rate and neurological symptoms (spasms and difficult walking). Death ensued within 48 to 72 h after infection. Differences in the survival times between groups of rats were tested for significance by calculating χ^2 values according to the method of Kaplan & Meier (1958; Peto *et al.*, 1976, 1977).

Interferon. rRIF-γ produced as previously described (Dijkema *et al.*, 1985; Van der Meide *et al.*, 1986) was assayed in a vesicular stomatitis virus c.p.e. inhibition assay on Ratec cells. Activity is expressed in units standardized against a laboratory reference. The preparations had activities of $0{\cdot}5 \times 10^6$ to $2{\cdot}0 \times 10^6$ units/ml and a specific activity of $4{\cdot}0 \times 10^6$ units/mg protein. Freeze-dried interferon preparations were diluted in phosphate-buffered saline (PBS) pH 8·0 to a final concentration of $12{\cdot}5 \times 10^4$ units/ml prior to i.p. injection into rats. In all the experiments 500 units/g body weight were applied per animal per day.

Drugs. Silica particles (0·012 μm; Sigma) were suspended in PBS pH 8·0 to a concentration of 2 mg/ml and ultrasonicated (1·5 A for 20 min) before i.p. injection of 0·5 ml on days -1, 0 and $+1$. Rats were infected with 200 p.f.u. PRV on day 0.

Carrageenan type V (Sigma) was dissolved in PBS pH 7·2 to a concentration of 6 mg/ml and injected i.p. in a volume of 0·5 ml on 4 consecutive days starting 2 days before infection. The macrophage-depleting effect of this treatment was confirmed by testing for a reduction in peritoneal macrophage numbers.

Cell transfer. Rat peritoneal cells were obtained by washing the peritoneal cavity with a total volume of 10 ml PBS pH 7·2; they were centrifuged at 1000 r.p.m. (225 ***g***) for 10 min, resuspended in Iskov's medium and counted. An average of $3{\cdot}0 \times 10^6$ peritoneal cells per rat were obtained. Interferon activity remaining in the supernatant was less than 10 units/ml. The suspension consisted of about 60% macrophage-type cells and 40% lymphoid cells, as determined by morphology (May Grünwald–Giemsa staining) and non-specific α-naphthylacetic esterase staining. Vitality was more than 95%. For the transfer experiments, cell suspensions of three donor rats were pooled and injected i.p. into syngeneic recipients in a final volume of 1·0 ml, 24 h before infection.

Virus neutralization test. Blood samples of surviving rats were taken from the periorbital plexus on days 5, 11 and 18 after infection and on consecutive days beginning on day 32 until day 39 after infection. Blood samples of rats surviving a second infection were collected on days 7, 8, 9 and 14 after challenge. All sera were inactivated at 56 °C for 30 min and assayed for neutralizing antibodies in a microtitre system (Costar) 50% plaque reduction test using Ratec cells; serum dilutions were mixed with an equal volume of 50 p.f.u. PRV and incubated for 24 h at 37 °C. All serum dilutions were also tested with guinea-pig complement added in an equal volume to a final test concentration of 1:50.

Whole body gamma irradiation. Groups of 10 rats were placed in an animal container which was flushed with air during irradiation. The radiation unit (Gammacell 20 Small Animal Irradiator; Atomic Energy of Canada Ltd.) at the Institute of Radiobiology TNO, Rijswijk, The Netherlands, houses twin ^{137}Cs gamma ray sources located one above and one below the irradiation compartment, thus providing an approximately homogeneous dose distribution over the animals (maximum variation 2%). Absorbed dose measurements were made using a calibrated ionization chamber. The dose rate was about 0·9 Gy/min and 4·5 Gy was applied per rat. These radiation doses have been shown to produce haematopoietic death and lymphocytotoxic effects (Broerse & Zoetelief, 1984; Vriesendorp & van Bekkum, 1980).

RESULTS

Protection by rRIF-γ against PRV infection

Wag/Rij rats were found to be highly sensitive to i.p. PRV infection, with approximately 1 p.f.u. leading to death within 9 days. In rats infected with higher doses (125 to 8000 p.f.u.), first symptoms occurred after 48 to 72 h; these rats died within 4 to 5 days after infection.

The antiviral effect of rRIF-γ was studied in rats infected with 200 p.f.u. of PRV, which resulted reproducibly in 100% mortality within 5 days or earlier. Rats receiving rRIF-γ for 3 consecutive days starting 1 day before infection were completely protected (Fig. 1). Day -1 was

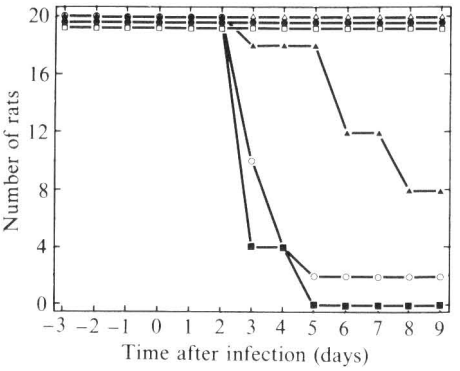

Fig. 1

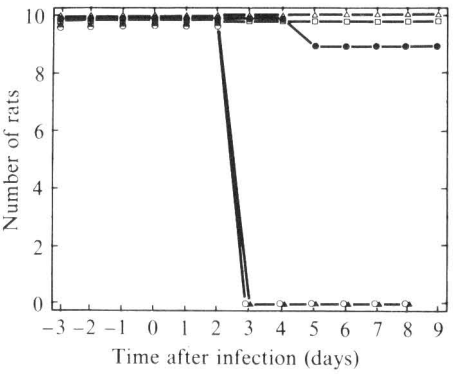

Fig. 2

Fig. 1. Antiviral activity of rRIF-γ in rats infected with PRV. Effect of daily administration of rRIF-γ starting at different times for each group. Regimes were days $-3/+1$ (△), $-2/+1$ (●), $-1/+1$ (□), $0/+1$ (▲), $+1$ (○). Untreated rats served as controls (■).

Fig. 2. Effect of silica treatment on the antiviral activity of rRIF-γ in silica-treated infected rats (○) and silica-treated rRIF-γ-treated infected rats (●). Untreated rats (▲) served as controls; the statistical significance of difference between these and the untreated virus control group was $P < 0{\cdot}01$. Other groups of rats were treated only with silica (□) and rRIF-γ-treated infected (△).

the critical time when administration had to be started to confer complete protection. Treatment on the day of infection resulted in partial (40%) protection, whereas treatment 1 day after infection had no effect.

Rats protected by rRIF-γ were challenged 28 days after the first infection with the same lethal dose of 200 p.f.u. PRV. The survival rate was 60% in the group of re-infected rats as compared to zero in the age-matched control group of animals.

Role of peritoneal cells during rRIF-γ-mediated protection

Silica-treated rats received rRIF-γ for 5 consecutive days beginning 3 days before infection. As shown in Fig. 2, silica pretreatment did not influence the antiviral effect of rRIF-γ. Comparable results were observed in carrageenan-treated, phagocyte-depleted rats (data not shown). These results suggest that peritoneal phagocytes are not required for rRIF-γ-mediated protection of rats against PRV infection.

Since both PRV and interferon were administered i.p., we examined the role of peritoneal cells in protection. In these experiments, $6{\cdot}5 \times 10^6$ to 15×10^6 peritoneal cells obtained from rRIF-γ-treated donor rats were transferred to untreated syngeneic recipients of the same age; these were infected 24 h later. Donor rats had received daily i.p. injections of 500 units rRIF-γ/g body weight for 4 days. There were no significant differences in the survival rates between rats that had been given the cell suspension and those of the virus control group receiving peritoneal cells from PBS-treated donor rats.

Protection of newborn rats by rRIF-γ

rRIF-γ was administered to newborn rats for 5 consecutive days starting 3 days before infection with 50 p.f.u. PRV on day 0. At the time of infection the animals were 5 days old. Interferon treatment resulted in complete protection in a group of 13 rats, whereas 11 of 13 rats in the untreated virus control group succumbed to the infection. Our results show that immunocompetent adult rats as well as neonatal immunologically immature rats can be protected from a lethal PRV infection by rRIF-γ treatment. The rRIF-γ-treated surviving newborn rats showed no resistance to a challenge infection (200 p.f.u. PRV/rat) given 28 days after the first infection.

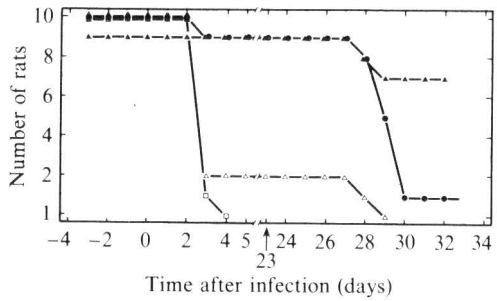

Fig. 3

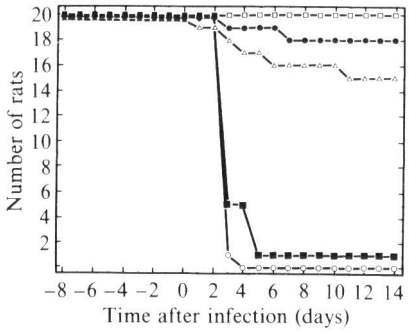

Fig. 4

Fig. 3. Survival rates of nude rats (Rowett hooded rnu/rnu) infected with PRV and the effect of rRIF-γ treatment. Groups of heterozygous (▲) and homozygous (●) nude rats were treated daily with rRIF-γ for 6 consecutive days starting 4 days before infection. Untreated heterozygous (□) and homozygous (△) nude rats served as the virus control group. The statistical significance of difference between treated groups and untreated groups was $P < 0{\cdot}01$. After challenge with PRV on day 25, surviving heterozygous nude rats were significantly protected ($P < 0{\cdot}05$) as compared to homozygous nude rats.

Fig. 4. Effect of whole body gamma-irradiation on the antiviral activity of rRIF-γ. Whole body irradiation was applied 6 days before infection. rRIF-γ was administered daily for 6 consecutive days starting at day −3. Untreated virus control group (○); irradiated infected rats (■); irradiated rRIF-γ-treated infected rats (△) (statistical significance of difference between treated and untreated virus control group: $P < 0{\cdot}01$); irradiated untreated uninfected rats (●); rRIF-γ-treated infected rats (□).

Protection of immunologically impaired nude rats by rRIF-γ

rRIF-γ was given to Rowett hooded nude rats on 6 consecutive days starting 4 days before infection. The treatment resulted in protection against infection of nine out of ten animals. From the ten untreated control rats only two survived the infection (Fig. 3). These observations suggest that an intact T cell system is not necessary for IFN-γ-mediated protection. From the nine rRIF-γ-treated surviving nude rats only one survived a challenge 25 days after the first infection (Fig. 3); administration of the same challenge dose to rRIF-γ-protected heterozygous nude rats resulted in significant protection (seven of nine; Fig. 3). Similar results were obtained from experiments with Wag/Rij nude rats.

Antiviral activity of rRIF-γ in whole body gamma-irradiated rats

In order to test the antiviral activity in heavily immunosuppressed rats we made use of total body irradiation. Immunosuppression was confirmed by peripheral blood cell counts which revealed a strong depletion of leukocytes. At the time of infection the numbers of peripheral blood lymphocytes, granulocytes and eosinophils were reduced by 83, 82 and 100% respectively. Three days after irradiation, rRIF-γ treatment was started; it was administered on 5 consecutive days starting on day −3. Significant protection of the irradiated rats was seen (Fig. 4). A radiation dose of 5·5 Gy/rat proved to be lethal for 5-week-old Wag/Rij rats within 10 days after irradiation. Whole body irradiation of 4·5 Gy, however, resulted in death only 16 to 20 days after irradiation (results not shown), a period long enough to study the antiviral effect of rRIF-γ. These results suggest that the protective effect of the lymphokine is not a result of an induced immune function.

Neutralizing antibodies in surviving rats

In view of the rapid effect of rRIF-γ within 3 to 4 days after infection, a humoral immune response is very unlikely to be responsible for the protection. Nevertheless, we examined the sera of surviving rats for the presence of neutralizing antibodies. All the sera tested had neutralizing titres below 5, as determined with or without addition of complement. From these observations we conclude that neutralizing antibodies are not responsible for protection of rRIF-γ-treated rats.

DISCUSSION

The precise mechanism of the antiviral activity in an organism as a result of interferon administration is not known. In addition to direct inhibition of virus replication in the target cell, interferon may exert indirect effects on the host's defence system. Several observations in support of this assumption have been reported (Gresser *et al.*, 1975; Schellekens *et al.*, 1979, 1981, 1984; Bolhuis *et al.*, 1981). In view of the pleiotropic effects of IFN-γ on cells of the immune system we investigated its effects in immunologically impaired and immunosuppressed rats.

Our results demonstrate that rRIF-γ protects both newborn and adult rats against an otherwise lethal PRV infection. The observation that rRIF-γ is also able to protect neonatal, immunologically immature rats suggests that an antiviral state may be induced directly in the target cell of PRV. It should be noted, however, that interferon may stimulate or accelerate maturation of immune functions (Cruz *et al.*, 1981).

Fig. 3 shows that rRIF-γ administration can also protect nude rats against a lethal PRV infection. However, some T cell functions can be taken over by other cell populations; in addition it has been reported that spleen cells from nude mice have extraordinarily high natural killer cell activity (Kiessling *et al.*, 1975). Our results show that T lymphocytes are not essential for IFN-γ-mediated protection. This conclusion is supported by the results of experiments with whole body gamma-irradiated rats (Fig. 4). These animals are protected by rRIF-γ treatment against a lethal PRV infection with approximately the same efficiency as immunologically unimpaired rats. The suppressive effect of whole body irradiation on the immune functions has been well documented (Anderson & Warner, 1976; Durum & Gengozian, 1978). Whole body gamma irradiation resulted in a pronounced decrease of peripheral blood leukocytes, especially lymphocytes, in our experiments; in addition, natural killer cells, polymorphonuclear leukocytes and peripheral blood monocytes are highly sensitive to radiation (Anderson & Warner, 1976; Taliaferro *et al.*, 1964). However, tissue macrophages and mononuclear phagocytes are relatively resistant to the doses used in our experiments (Benacerraf *et al.*, 1959; Anderson & Warner, 1976). To investigate the possible involvement of phagocytes during interferon-mediated protection, we examined rRIF-γ efficacy in silica- and carrageenan-treated rats; both substances are selectively cytotoxic to phagocytes (Catanzaro *et al.*, 1971; Wirth *et al.*, 1980), and silica is also cytotoxic to natural killer cells *in vitro* (Sibbitt *et al.*, 1986). In our model system the antiviral effect was not influenced by either substance. In addition, passive transfer of peritoneal cells from rRIF-γ-treated donors did not protect syngeneic recipients against a lethal dose of PRV given 24 h later.

Since protection is effected within 3 days after infection, it was anticipated that IFN-γ-stimulated neutralizing antibody would not have developed by that time. Indeed, we found no neutralizing antibodies in the sera of the surviving rats, in agreement with the observations of Maes *et al.* (1979).

Re-infection of surviving rats with the same dose of PRV resulted in significant survival as compared to the untreated virus control group. It is unlikely that the observed protection against challenge resulted from maintenance of an antiviral state in the target cell of PRV, since the rRIF-γ administration occurred 28 days before. Neutralizing antibodies were not detected in the sera of these surviving rats; interferon-induced cell-mediated processes are possibly of major importance. This suggestion is supported by the results of experiments with nude rats surviving a lethal PRV infection. These rats did not survive the challenge infection, whereas for euthymic protected rats, survival was about 75%. Also rRIF-γ-protected newborn rats did not survive the challenge; this can be explained by the absence of a mature immune system. It is possible that in addition to inhibition of viral replication that would diminish the antigenic stimulus, rRIF-γ enhances immunogenicity by providing a trigger signal to antigen-presenting cells or T lymphocytes (Schellekens *et al.*, 1981).

Our conclusion is that rRIF-γ does not protect rats via an activation of the immune system which, however, may be responsible for survival after challenge. It is, of course, impossible to prove the absence of a phenomenon. Our evidence is cumulative and based on the lack of any gradual response to our immunosuppressive manipulations. The observed antiviral effect is

most probably based on direct inhibition of viral replication in the target cell. Preliminary results are in support of this hypothesis.

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