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Rat cytomegalovirus: induction of and sensitivity to interferon

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Rat interferon (IFN) in a dose-dependent fashion inhibited the cytopathic effect of rat cytomegalovirus (RCMV) in rat cell cultures. Treatment of Lewis and Brown Norway (BN) rats with intraperitoneal injections of IFN reduced the amount of virus recovered from the spleens at 3 days post infection (p.i.) and from the salivary glands at 10 and 21 days p.i. In cell cultures, RCMV failed to induce detectable amounts of IFN. Small amounts of IFN were detectable in the serum of BN rats at 3 days p.i. In Lewis rats no IFN was found in serum at any time during the first month p.i.

cytomegalovirus; rat; interferon

Introduction

Human cytomegalovirus (CMV) causes a broad spectrum of clinical illness especially in patients with impaired immune responses [5]. We have described the isolation of a rat cytomegalovirus (RCMV) [1,2,8] from feral rats. Infection of laboratory rats with this virus results in a chronic inapparent infection with virus persistence in the salivary glands. The course of the infection is different in Lewis and Brown Norway (BN) rats in the number of virus particles recoverable from the salivary glands, in the duration of persistence of virus and in the extent of antibody production.

Interferon (IFN) is known to be an important constituent of host defense against most viruses. CMV have generally been regarded as being more resistant to the effect of IFN [7,9,10] than viruses regularly used for IFN assays. However, other authors have described IFN producton during mouse cytomegalovirus (MCMV) infection and have inferred that it has a protective role in this system [4,14,6].

In the present study we have examined the production and action of IFN by RCMV in rat cell cultures and in rats in vivo.

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Materials and methods

Viruses

Rat cytomegalovirus (RCMV) isolated in our laboratory [1,2,3] was passaged in rat embryo fibroblasts (REF) (cell culture virus) or in rats (salivary gland virus). The cell culture virus was obtained by harvesting the supernatant of the infected REF cultures showing 90–100% cytopathic effect (CPE). The virus suspension was assayed by a plaque test in REF and stored at −70°C. Vesicular stomatitis virus (VSV), Indiana strain, was obtained from the American Type Culture Collection. Stocks were prepared and assayed in L_{929} cells. The salivary gland virus was obtained from the salivary glands of 3- to 4-wk-old rats inoculated intraperitoneally (i.p.) with 10^6 plaque forming units (PFU) of virus. Virus was harvested 4 wk after the inoculation by homogenizing the salivary glands in 10% (w/v) Eagle's minimum essential medium (MEM) using a Ten Broek grinder. The homogenates were stored at −80°C.

Cell culture

Rat embryo fibroblasts prepared from 17-day-old Lewis or BN rat embryos were used at the third passage for plaque titrations of RCMV and for interferon (IFN) assays. Cells were grown in Eagle's MEM containing 10% newborn calfserum (NCS). All media contained penicillin (100 IU/ml), streptomycin (100 μg/ml) and amphotericin B (5 μg/ml). All experiments were conducted with cells grown in wells of 24 well-trays (Costar Europe Ltd., Badhoevedorp, The Netherlands) at 37°C in a humidified CO_2 incubator.

Animals

Four- to five-wk-old male rats, from two inbred (Lewis and BN) rat strains were used. The rats were bred under specific pathogen-free (SPF) circumstances at the Department of Experimental Animal Services of the State University of Limburg, Biomedical Centre (Maastricht, The Netherlands).

Virus assay

RCMV was assayed by employing a modification of a technique described by Wentworth and French [15]. Confluent REF monolayers grown in wells of a 24-well tray (Costar Europe Ltd., Badhoevedorp, The Netherlands) were infected in quadruplicate with 0.1 ml of 10-fold serial dilutions of the tissue homogenates. After an incubation period of 90 min at 37°C in a CO_2 incubator the agarose layer (0.25% agarose in BME-2 (Gibco Laboratories, Paisley, Scotland) with 5% NCS) was added. After 7 days incubation at 37°C in a CO_2 atmosphere, the cells were fixed with 10% formalin. Following fixation the solid base layer was removed and the cells were stained with 1% aqueous methylene blue and the plaques were counted.

Preparation of interferon

The rat IFN was prepared in Ratec cells induced with Sendai virus as described before [12]. Briefly, Ratec cells were grown to confluency in 850 m^2 roller bottles using 100 ml Dulbecco's modification of Eagle's minimum essential medium (DMEM) containing antibiotics and 10% foetal calf serum (FCS). The cells were washed with phosphate buffered saline (PBS) and incubated with 10 000 haemagglutinating units of Sendai virus in 10 ml DMEM for 1 h. The cells were again washed with PBS and incubated overnight in 50 ml DMEM containing 1% FCS. The supernatant was harvested and incubated for at least 5 days at pH 2.0 in the cold to inactivate residual Sendai virus. Then the crude IFN preparation was concentrated by precipitation with $(NH_4)_2SO_4$ or membrane filtration. The final preparation contained approximately 10^4 U/ml with a specific activity of around 10^5 U/mg protein.

Interferon assay

The IFN activity was assayed by a CPE inhibition assay with Ratec cells infected with VSV in microtiter plates using an internal laboratory standard as reference. Both the assay and the validation of the internal standard have been described extensively [12].

Effect of interferon on CPE

Rat IFN was applied to REF cultures in appropriate dilutions by adding 2 ml to each well which had been seeded with 10^5 cells 48–72 h previously. After given intervals, the medium with IFN was drained and the cultures were infected with dilutions of VSV or RCMV. Four replicate cultures were used for each IFN combination and virus dose. Before and after virus adsorption (2 h at 37°C) the cells were washed twice with medium and fed with medium containing IFN. The cells were examined daily for the development of CPE. To maintain the desired IFN concentration in the medium during the whole experiment, fresh medium was added to the cell cultures 5 days p.i.

Effect of interferon on virus production

Confluent REF monolayers grown in 6 well/2 cm^2 trays (Costar Europe Ltd., Badhoevedorp, The Netherlands) were infected in triplicate with RCMV at a multiplicity of infection (MOI) of 0.05. After adding 10 ml of medium (Eagle's MEM) containing 5, 50 or 500 U/ml of IFN to each well the cells were incubated in a CO_2 incubator at 37°C. At different intervals p.i. 1 ml of the supernatant was removed and assayed for the amount of virus using a plaque titration test.

Interferon induction in vitro

REF monolayers grown in 2 cm^2 wells (Costar Europe Ltd., Badhoevedorp, The

Netherlands) were inoculated with RCMV at MOI = 1. Following a 120-min adsorption period, the cultures were washed once, and 2 ml of fresh medium were added to each well. Control plates were treated in a similar way, but were not infected with RCMV. The plates were examined daily for the appearance of CPE and the medium from the plates was assayed for RCMV and IFN activity in the usual way.

Preparation of rat tissues for viral assays

Salivary glands and spleens from infected animals were prepared as described previously [2]. Briefly, salivary gland tissues from animals were excised at various times after infection. Homogenates (10% w/v) were prepared in MEM. Serial 10-fold dilutions of these homogenates were assayed for virus on REF cells using a plaque assay.

Interferon induction in animals

The IFN response to RCMV was determined in Lewis and BN rats that had been infected i.p. with 10^6 PFU of virus. Blood was obtained by retroorbital puncture from 3 rats at intervals up to 15 days p.i. Serum samples from individual animals were assayed for IFN. Spleens and salivary gland homogenates of the infected animals were also assayed for IFN at various times after infection.

Results

In vitro sensitivity

The sensitivity of RCMV to the inhibitory effect of IFN was determined by incubating the REF cultures with IFN for 24 h prior to infection. The relative sensitivity of RCMV to IFN was evaluated by comparison with VSV, the latter being among the most sensitive viruses known.

Figure 1 shows that in both cell types (Lewis and BN) IFN induced resistance to the CPE of RCMV; with decreasing doses of challenge virus the reduction of CPE became more pronounced. From a comparison of the data in Figs. 1 and 2 it appears that the reduction of CPE, following application of similar IFN doses, was much more pronounced for VSV than for RCMV. The dose of IFN needed to inhibit CPE by 50% was about 30 U/ml for RCMV and about 1 U/ml for VSV. The sensitivity of RCMV to IFN, as tested by CPE inhibition in REF cells was about 30-fold less than that of VSV. The effect of IFN on virus yield is shown in Table 1. In Lewis and BN (results not shown) cells IFN reduced the amount of extracellular virus. The IFN effect was dependent on the amount IFN used in the experiment; using 500 U/ml a significant reduction of the amount of extracellular virus was observed at 72 till 120 hp.i.

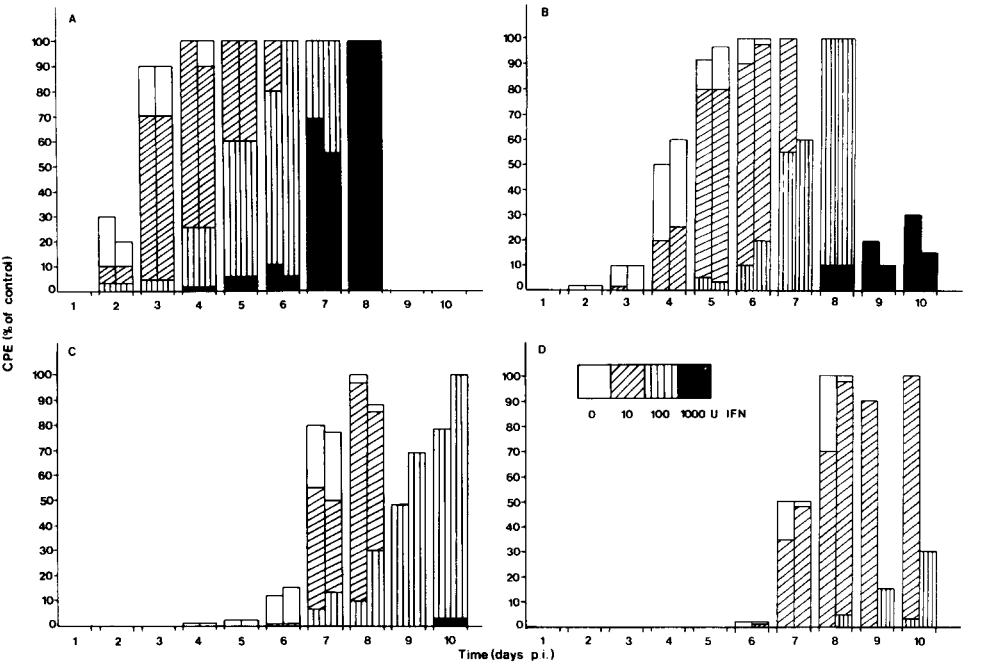

Fig. 1. Time course of CPE of RCMV in Lewis REF cells (left columns) and BN REF cells (right columns) at a MOI of 2×10^{-2} (A), 2×10^{-3} (B) 2×10^{-4} (C) and 2×10^{-5} (D) plotted as percent of the cell monolayer showing CPE in control cultures and in cultures containing 10, 100 and 1 000 U of rat IFN. Left columns represent averages of data of 3 experiments.

In vivo sensitivity

Lewis and BN rats were inoculated i.p. with 10^6 PFU of virus. IFN was administered in amounts of 200, 1 000 and 5 000 U/injection. The first dose was administered 36 h before virus inoculation, the second dose 4 h before virus inoculation and the third dose 24 h p.i. The effect of treatment was analysed by measuring the amount of virus in the spleens and the salivary glands in 3–5 animals at 3, 10 and 21 days p.i. Virus in the spleen homogenates was detected in the control BN and Lewis rats at 3 days p.i. In BN rats the spleen homogenates contained approximately 50 PFU/ml; in Lewis rats this was approximately 200 PFU/ml. The spleens of animals treated with IFN were negative 3 days p.i. The effect of IFN on the virus titers in the spleens at 10 and 21 days p.i. could not be measured because no virus was found in the spleens of the control animals. The effects of treatment on the virus titer in the salivary glands are summarized in Fig. 3a for the BN rats and Fig. 3b for the Lewis rats. Interferon treatment had no effect on the amount of virus recovered from salivary glands at day 3 p.i. On the other hand, reduced amounts of virus were found on days 10 and 20 p.i. This effect was more pronounced in BN than in Lewis rats. In BN rats the decrease of virus in the salivary glands was maximal at day 10 p.i. using 1 000 or 5 000 U of IFN. Thereafter the virus titre increased in the salivary glands reaching the same titre as in the control

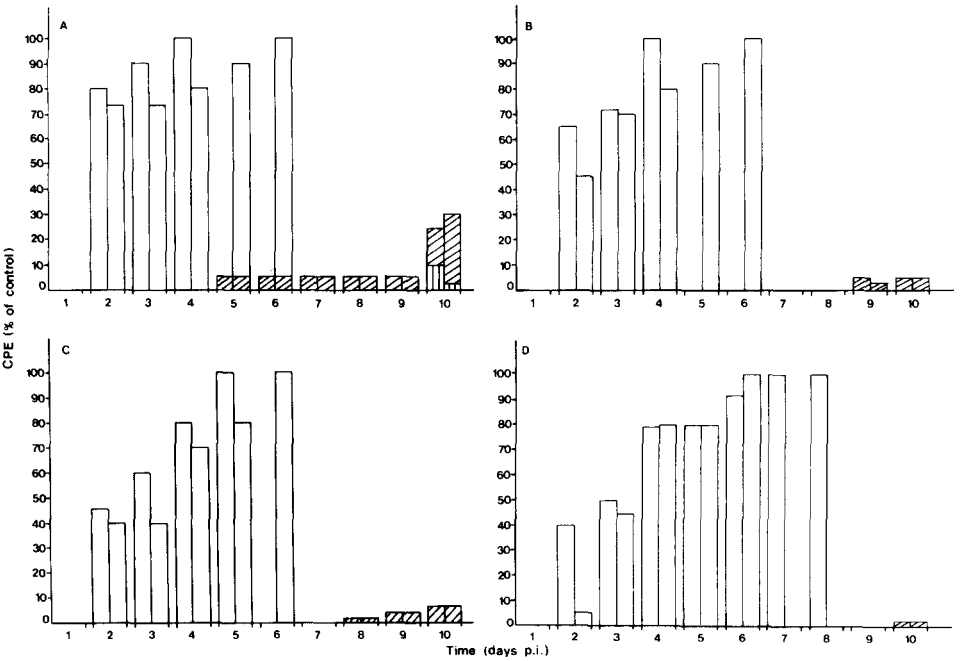

Fig. 2.Time course of CPE of VSV in Lewis (left columns) and BN REF cells (right columns) at a MOI of 2×10^{-2} (A), 2×10^{-3} (B), 2×10^{-4} (C) and 2×10^{-5} (D) plotted as percent of the cell monolayer showing CPE in control cultures and in cultures containing 10, 100 and 1 000 U of rat IFN. Left column curves represent averages of data of 3 experiments.

TABLE 1

Effect of rat interferon on the production of RCMV in REF[a]

Time (h) p.i.	Virus yields ($\log_{10}$ PFU/ml) Treatment with IFN at doses (U/ml) of:			
	0	5	50	500
24	0.78	0.78	0.90	0.00
48	2.28	2.26	2.11	2.11
72	3.80	3.67	3.63	3.58
96	4.43	4.45	4.18	4.11
120	5.38	5.32	5.11	4.45

[a] Lewis REF were infected with RCMV at MOI of 5×10^{-2}. The data are the mean titres of three experiments.

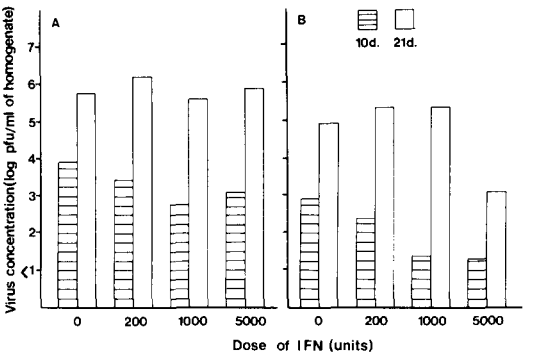

Fig. 3. Effect of treatment with interferon on RCMV concentration (expressed as log PFU/ml) in salivary glands of Lewis (a) and BN rats (b) at 10 and 21 days p.i. Indicated doses were given i.p. on each day. The first doses were administered 36 and 4 h before virus inoculation.

animals at day 20 p.i. for the doses of 200 and 1 000 U of IFN. The increase occurred more slowly in animals treated with 5 000 U of IFN. In Lewis rats the decrease of virus titre was smaller than in the BN rats.

Time courses of interferon production and of virus replication in vitro

To determine if RCMV induced production of IFN in vitro, REF cell cultures derived from either Lewis or BN rats infected with RCMV at a MOI of 1, were observed daily for CPE. The supernatant fluids harvested at varying time intervals were divided into two aliquots which were used for determination of virus and IFN production. Viral CPE became apparent 24 h p.i.; at 96 hp.i. 90–100% of the cells showed CPE. Virus production became detectable 2 days p.i. and increased until 3–5 days both in Lewis and BN rat cells. Thereafter the production did not further increase (Fig. 4). No IFN production was detected in the extracellular fluids at any time point of the entire observation period.

Production of interferon in infected rats

RCMV-infected rats were followed for interferon content in their serum for a period of 20 days p.i. In Lewis rats no IFN could be detected in the serum at any time. In BN rats an IFN response to RCMV occurred at 3 days p.i., but the levels of activity were low ($\approx$ 100 U/ml). In spleens and salivary glands of infected animals no IFN could be detected.

Discussion

Human and mouse CMV are reported to have a low sensitivity to the antiviral action of IFN [10,3,11] as shown by the reduction in CPE. The resistance is dependent

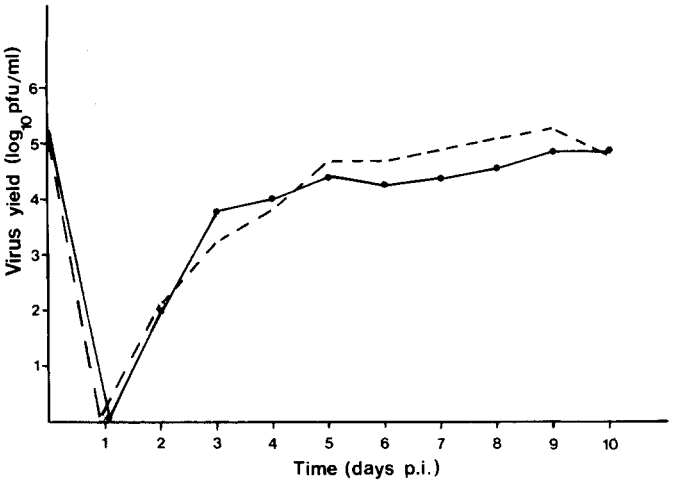

Fig. 4. Growth curves of RCMV in REF cells (●———● = Lewis; ○———○ = BN). (Data points represent averages over 3 experiments).

on the virus strain used [11] and on the challenge dose of virus used in the assay [9]. The last finding is also reported by Stitz and Schellekens [12] for other viruses. They found that the amount of IFN needed to protect cells of different species against the CPE of both DNA and RNA viruses is directly related to the amount of virus with which the cells are challenged. Our studies with RCMV showed that, at small challenge doses, RCMV was highly sensitive to IFN, but at higher viral doses proved to be markedly resistant.

Furthermore, no detectable IFN was present in the cell culture fluid from rat cells infected with RCMV. These results confirm the findings of certain authors with MCMV [10] and human CMV [3]. In contrast, other investigators found that moderate amounts of IFN were produced in mouse cell cultures infected with MCMV [9,6]. An explanation for these differences has not been found yet. Oie et al. [9] suggested the presence of an undetectable agent (e.g. polyomavirus) in the stock preparations which might interfere with the virus-induced IFN production. In our case no contaminating virus was detected in the preparations. Our results indicate that the rat virus probably differs from that of the mouse in the capacity to induce IFN in vitro, although the growth of RCMV in BN or Lewis fibroblast cell cultures was similar to that of MCMV in mouse embryo cells [9].

To evaluate the effect of the administration of IFN on the pathogenesis of the RCMV infection in BN and Lewis rats we measured the alteration in viral replication in the spleens and in the salivary glands at several times p.i. Our results suggest that IFN is effective in altering the course of the infection in rats by reducing the amount of virus in the spleens at 3 days p.i. and in the salivary glands at 10 days p.i. In both Lewis and BN rat spleens virus was undetectable at 3 days p.i., if the animals had been treated with IFN. The effect of IFN on the reduction of virus titers in the salivary glands was more significant in Lewis than in BN rats. These results indicate that the IFN effect was significant in the early period after the infection (3 days p.i. for the

spleens, 10 days p.i. for the salivary glands). The alteration of the virus production in other organs was not determined in these studies. Although direct comparison of these results obtained with RCMV with those obtained with MCMV are difficult due to differences in methods used (i.e. reduction of virus titre or the effect on mortality [4,7,10], the present results are in general agreement with those obtained in mice. With both viruses an effect of IFN on the infection in animals was observed.

An IFN response was seen in BN rats 3 days after infection with RCMV. In Lewis rats no IFN could be detected in the sera over a 20-day period p.i.. Similar experiments with MCMV in mice have revealed contradictory results. Thus Osborn and Medearis [10] did not find IFN in serum of mice infected with MCMV. An explanation for this could be the fact that they examined sera at 4, 5 and 20 h p.i. and not at later times. Although in the experiments of Grundy et al. [4] serum IFN levels were measured at 6 h p.i. Kelsey et al. [6] found serum IFN as early as 12 h p.i. with a peak at 36 h p.i. The amount of IFN in our experiments was low in BN rats at 3 days p.i. and undetectable in Lewis rats. In contrast, a vigorous IFN response in MCMV-infected mice was detected [14].

From these in vivo experiments together with the in vitro experiments we could conclude that RMCV is a poor IFN inducer.

The role of IFN in RCMV infection arising from this study suggests differences between strains of the rats used. Similar differences between BN and Lewis rats have also been reported in our previous studies [2]. The in vitro studies did not show this rat strain effect because the induction of IFN, the virus production and the sensitivity to IFN were nearly identical.

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