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Interferon-α protects mice against lethal infection with St Louis encephalitis virus delivered by the aerosol and subcutaneous routes

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Abstract

In common with other flaviviruses, there is no specific therapy for St Louis encephalitis (SLE) virus infections. A number of cases have occurred where infection may have been acquired by the aerosol route in laboratory accidents. The recombinant human interferon hybrids IFN-α A/D (Roche Laboratories) and IFN-α B/D (Ciba-Geigy) have activity in murine models. Given for several days around the time of exposure to the virus or shortly after, these compounds reduce the mortality from SLE virus administered to mice subcutaneously by up to 70%. In an aerosol model of SLE disease, the mortality was reduced to 30–50% compared to 100% in controls, depending on the challenge level of virus. These results suggest that interferon-α could be used to reduce the mortality from SLE infection after known exposure to the virus.

Keywords: Interferon; Therapy; Flavivirus; Aerosol; St. Louis encephalitis

1. Introduction

St Louis encephalitis virus (SLE) is endemic in much of the Americas, and causes significant outbreaks of disease in southern USA. The virus belongs to the flavivirus family, and is transmitted by mosquitoes. Man is an accidental host, the normal reservoir being in passeriform and columbiform birds which harbour the organism without developing overt disease (Monath and Heinz, 1996). Serological studies suggest that between 1 in 100 to 1000 individuals exposed to the virus develop clinical disease, with a case mortality rate of 5–15%. Significant neurological sequelae are common amongst survivors (Tsai and Mitchell, 1988). Several flaviviruses have been shown to be infectious by the aerosol route

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(Daneš et al., 1961; Nir et al., 1965) and we have recently demonstrated this for SLE virus using a small animal model of disease (Phillpotts et al., 1997). Laboratory personnel are at risk from accidental inoculation with SLE or from accidental inhalation during aerosol generating procedures, and the virus is assigned to hazard group 3 in the US (Subcommittee on Arbovirus Laboratory Safety of the American Committee on Arthropod-borne Viruses, 1980) and by the Advisory Committee on Dangerous Pathogens in the UK for this reason (Advisory Committee on Dangerous Pathogens, 1995).

Some flaviviruses are moderately sensitive to interferon-α in vitro (Vithanomsat et al., 1984), but large quantities of purified native human interferon are very expensive. Recombinant human interferon-α is now available commercially, but the actions of interferon-α are usually species specific, so the preparation cannot be used in most laboratory models of infection. The hybrid interferons, interferon-α A/D (Roche) and interferon-α B/D (Ciba-Geigy) contain sequences from various human and murine interferon-α subtypes, and are active in tissue culture on cells of both species, and in vivo in mice (Weck et al., 1982; Kramer et al., 1983; Gangemi et al., 1989). It has been demonstrated that they have some prophylactic effect against Banzi virus (Kende, 1992) which is a member of the flavivirus group. Here we show that given prophylactically, interferon-α can prevent disease in animals exposed to both subcutaneous and inhalational challenge with SLE virus.

2. Materials and methods

2.1. Animals

Locally produced Porton outbred TO strain mice (at age 3–4 weeks, weight < 18 g) were used for the virus challenge experiments and for the preparation of suckling mouse brain suspensions containing infectious SLE virus. Balb-c mice at 3–4 weeks age (Charles Rivers, UK) were used in a comparative trial with Porton mice in one experiment.

2.2. Virus preparations and assay

We used the MS1-7 strain of SLE (kindly supplied by Dr E.A. Gould of the NERC Institute of Virology, Oxford, UK) throughout. All virus samples were stored frozen at −70°C. A stock virus was prepared for inoculation experiments in Vero cells infected at a multiplicity of infection of 0.1 and incubated at 37°C for 4 days before harvesting and clarification using low speed centrifugation. The virus stock had a titre $10^{6.2}$ plaque forming units/ml (pfu/ml). For the preparation of infected mouse brain suspension, suckling mice were inoculated intracerebrally with approximately 1000 pfu of SLE virus in 25 μl of Leibovitz L15 medium containing 2% foetal calf serum. Brains were harvested when the animals developed signs of paralysis (3–4 days) and a 10% w/v suspension prepared in L15 medium as above. Trehalose (Sigma, UK) was added as a stabiliser to virus suspensions to a final concentration of 2% before aerosolisation.

SLE virus was titrated by plaque assay in BHK21 cells grown on Eagles MEM supplemented with 10% foetal calf serum and 10% tryptose phosphate broth. The plaques were allowed to develop for 4 days under an overlay of L15 medium containing 1.5% carboxymethyl cellulose and 2% foetal calf serum before formalin fixation and staining of the residual cell sheet with crystal violet.

2.3. Interferon (IFN) preparations

Recombinant human IFN-α A/D was a gift from Hoffman La Roche (Welwyn Garden City, UK) and was diluted to 10^6 units/ml in 10% normal mouse serum in Saline for Injection BP (Antigen Ltd.). Diluted IFN was stored at −70°C and thawed before use. Recombinant human IFN-α B/D was a gift from Ciba-Geigy (Basel, Switzerland) and was prepared in the same way. IFN was administered as a single daily dose of 100 μl of IFN containing 10^5 units given intraperitoneally. This dose was chosen as trial experiments using 10^4 units of purified mouse IFN α/β gave equivocal results (data not shown). Groups of 20 mice were given a full course (six

doses) of these IFN-α regimens without apparent ill effects as judged by activity level, piloerection or visible weight loss.

2.4. Experiments using subcutaneous challenge with virus

Groups of mice were challenged with SLE virus given as a 100 μl injection subcutaneously at the scruff of the neck. At the 10^{-4} dilution of stock virus used each mouse received about 15 plaque forming units (pfu) of virus, corresponding to about five median lethal doses (MLD). Various groups received different regimens of IFN-α A/D on the day before virus exposure, and on a number of days after exposure starting on the first day after exposure. A smaller number of combinations was used to test IFN-α B/D based on the experience gained with first experiment. Control groups for virus alone were given diluent for the maximum number of doses. In a separate experiment, IFN was started at day 5 after exposure, just before clinical signs of infection normally appear in mice (day 6–7).

Balb-c mice have inactive genes to at least one IFN effector protein (the Mx protein system) (Doolittle et al., 1996). In a separate experiment to check for differences between mouse strains, 20 Balb-c mice and 20 Porton mice were treated with IFN-α B/D on the day preceding virus inoculation, 6 h after inoculation, and daily for 5 days thereafter. Twenty controls of each strain were given normal mouse serum in the same regimen.

2.5. Inhalational challenge with virus

The apparatus for whole body aerosol exposure of small rodents has been described previously (Phillpotts et al., 1997). Briefly, it consists of a metal box supplied with virus spray from a Collison atomiser, exhausting through a HEPA filter. The box is fitted with an AGI30 impinger to sample the virus level, and can be shown to reach a steady state in 15 min. The Collison spray was operated with an air flow of 8 l/min. The impinger was charged with 60 ml of glass beads and 40 ml of maintenance medium, and the flow rate was set at 1 l/min. Animals were exposed in groups in wire cages for 20 min each, and the dose varied for each group by changing the dilution of virus in the spray. Samples were taken from the virus stock, the Collison apparatus after spraying and the impinger for each dosage regimen, and stored at −70°C for later analysis by plaque assay.

A range of log virus dilutions from −1 to −6 was used to establish an approximate median lethal dose. To test the efficacy of IFN α B/D, groups of animals were given IFN at a dose of 10^5 units i.p. daily, starting on the day before exposure or the day of exposure and continuing for 10 days. Controls were given diluent only. The protection afforded by IFN was determined at three virus dilutions (10^{-1} to 10^{-3}). Samples were taken for analysis as before. Animals were observed for 16 days, when the survivors were culled.

To confirm that animals were dying from SLE infection, the brains of several dying animals were removed and stored at −70°C for later analysis. The brain was homogenised in L15 medium using a Griffiths tube and assayed by plaque assay. An aliquot of the suspension was seeded into a 25-ml flask of Vero cells and incubated for 4 days at 37°C. The cells were scraped off, washed in PBS and used to prepare slides for immunofluorescence. The slides were fixed in acetone, and the presence of a flavivirus confirmed by immunofluorescent staining using the monoclonal antibody F7/3 (which is specific for flavivirus envelope protein) and polyclonal rabbit anti-SLE serum. Dr Ernie Gould from the Institute of Virology, Oxford, kindly supplied both of these reagents.

2.6. Histological studies

A limited histological study was undertaken on animals apparently dying from SLE. The brain, liver, spleen, heart, lungs and kidney were removed and fixed for at least 7 days in 10% neutral buffered formalin (Pioneer Research Chemicals, Colchester, UK) before embedding in paraffin wax. Sections were cut at 4 μm and stained with haematoxylin and eosin and examined by light microscopy.

Table 1
Subcutaneous virus challenge, IFN-α A/D treatment

Treatment[a]	Survivors/total	% survivors	Mean days to death ± S.D.	Significance/virus control[†]
IFN pre only	11/20	55	9.1 ± 1.05	<0.01
IFN pre/post	18/20	90	10.5 ± 0.71	<0.001
IFN post, 2 doses	12/20	60	10.13 ± 2.16	<0.001
IFN post 5 doses	13/19	68	11.17 ± 2.31	<0.001
Virus control	1/20	5	8.79 ± 0.79	–
IFN control, uninfected	20/20	100		–

[a] Pre, single dose of 10^5 units on day preceding infection; pre/post, doses of 10^5 units IFN on day before infection, and days 1–5 after; post 2 and post 5 are daily doses on days 1–2 or 1–5 after infection.
[†] Significance of mortality by χ^2 with Yates correction.

3. Results

3.1. Subcutaneous challenge

Animals infected subcutaneously with 5 × MLD of SLE virus became ill after an incubation period of 6–8 days. The illness progressed through a lethargic state to a hunched posture with increasing paucity of movement, then hind-limb paralysis, followed by front limb paralysis, coma and death. Paralysed animals were culled to prevent additional suffering as previous studies showed that they invariably died.

Tables 1 and 2 show the results of prophylactic therapy with IFN-α A/D and B/D respectively. The pattern of response was similar, in that the best protection from disease was seen with IFN given both before and after exposure to the virus. For both IFNs, significant protection was still given by post-exposure prophylaxis. For IFN-α A/D there is no significant difference between the protection afforded by two or five daily doses (Table 1). Table 3 shows the effects of giving IFN late on in the incubation period: there was no significant protection.

Balb-c mice are also protected against SLE by IFN-α B/D given both pre- and post-exposure, 100% (20/20) surviving compared to none of the controls.

3.2. Inhalational challenge

The course of the disease by inhalational exposure to SLE paralleled that of subcutaneous injection of virus, although the incubation period was a day shorter for the highest doses of virus. Animals became sick on the sixth or seventh day post-exposure, and developed progressive neurological disease as described above. Paralysed animals were culled.

The median lethal dose determined by probit slope analysis was $10^{3.6}$ pfu/ml of virus in the spray fluid (95% confidence limits 3.2–3.9). The three virus dilutions used for infecting the treated and control animals corresponded to 100, 1000 and 10 000 median lethal doses. Table 4 shows the protection offered by IFN B/D for each infectious dose. Significant protection was afforded against the lower dose of virus when the IFN B/D was given before or immediately after exposure to the virus. As the virus dose increased the protection decreased, as might be expected. No protection was afforded against the two higher levels of virus by IFN B/D given after exposure, but significant protection was still achieved by pre-dosing with IFN B/D.

3.3. Demonstration of virus in the brain

Virus was isolated from the brains of control animals dying from SLE by both routes of exposure. In both cases the titre of virus isolated was between 10^8 and 10^9 plaque forming units (pfu) per brain. Immunofluorescent stains confirmed that the virus was SLE. The brains of several of the survivors (no signs of illness at 14 days post-challenge) from the aerosol experiment were also examined. Virus was isolated from one animal

Table 2
Subcutaneous virus challenge, IFN-α B/D treatment

Treatment[a]	Survivors/total	% survivors	Mean days to death ± S.D.	Significance/virus control[†]
IFN pre/post	16/19	90	10.7 ± 1.15	<0.001
IFN post 5 doses	13/20	68	9.3 ± 2.36	<0.01
Virus control	3/20	5	8.7 ± 1.83	–
IFN control, uninfected	20/20	100		–

[a] Pre/post, doses of 10^5 units IFN on day before infection, and days 1–5 after; post 5, received daily doses on days 1–2 or 1–5 after infection.
[†] Significance of mortality by χ^2 with Yates correction.

which had been sick but not paralysed for 3 days before the experiment was terminated. This brain contained a low level of virus (1.4 $\log_{10}$ pfu in the whole brain). The other eight brains tested were negative by viral culture.

3.4. Histology

The histology of animals dying from SLE is similar for aerosol and subcutaneous infection, and between the different doses of virus administered by aerosol is very similar. In animals that died with acute disease (before the eighth day post-exposure), the brain had changes of a florid, non-suppurative encephalomyelitis. This was characterised by a diffuse lymphocytic infiltration of the pia mater and cerebral cortex, marked perivascular cuffing by small lymphocytes and focal areas of neuronal necrosis surrounded by significant cerebral oedema and haemorrhage. The liver, lungs, spleen and kidneys all showed a marked lymphocytic infiltration. The brains from animals culled later in the disease had developed a florid glial cell proliferation and necrosis of the endothelial cells lining small blood vessels in the substance of the brain. These changes are consistent with those described in other species (Monath and Heinz, 1996).

Table 3
Effect of late administration of IFN with subcutaneous challenge

Treatment[a]	Survivors/total	% survivors	Significance
rHuIFN-α A/D	3/20	15	Not significant
rHuIFN-α B/D	2/20	10	Not significant
Virus control	1/20	5	–

[a] Animals received daily doses of 10^5 units of IFN-α staring on day 5 after infection, for up to 5 days or until death.

4. Discussion

The results indicate that both IFN-α A/D and IFN-α B/D are effective as prophylactic treatment for SLE virus in mice. In our initial experiments we administered IFN intraperitoneally both before and after subcutaneous challenge with a low dose of virus, as we considered that these conditions would give us the maximum chance of observing a beneficial effect of IFN treatment. Protection of between 90 and 100% can be achieved if the IFN is given pre- and post-exposure. Significant benefit is still conferred even if the treatment is started 24 h after exposure to the virus. Late treatment begun at 5 days is not effective, and at this point significant titres of virus (up to 10^5 pfu/g of tissue) can be isolated from the brain, spleen and lungs of mice infected with SLE (T. Brooks, unpublished observations). IFN does not appear to be effective enough to overcome this level of viral load.

The level of virus we administered by the aerosol apparatus was very high compared to the subcutaneous challenge, and the duration of IFN treatment was increased in the hope of obtaining good protection on the basis of the results of the subcutaneous inoculations. Significant protection was obtained if IFN is given before and after

Table 4
Results of IFN B/D treatment of SLE infection by aerosol

Treatment[a]	Virus dose (MLD)	Survivors/total (%)	Mean days to death ± S.D.	Significance/virus control[†]
Low virus, pre/post IFN	100	7/10 (70)	10.3 ± 1.58	<0.001
Low virus, IFN post only	100	3/10 (30)	10.1 ± 1.34	Not significant
Low virus control	100	0/15 (0)	8.6 ± 0.63	–
Mid virus, pre/post IFN	1000	5/10 (50)	8.8 ± 0.84	<0.05
Mid virus, IFN post only	1000	0/10 (0)	9.2 ± 1.23	–
Mid virus control	1000	0/14 (0)	8.1 ± 0.62	–
High virus, pre/post IFN	10 000	4/10 (40)	9.0 ± 1.26	<0.05
High virus, IFN post only	10 000	0/10(0)	7.2 ± 1.23	–
High virus control	10 000	0/15(0)	6.9 ± 0.7	–

[a] Pre-post IFN: IFN given on day before exposure, 6 h after exposure, and daily for 10 days; post only, IFN starting at 6 h after exposure to virus, and daily for 10 days.
[†] Significance of mortality by χ^2 with Yates correction, relative to control at same virus dose.

exposure to the virus at both 100 and 1000 median lethal doses. Some protection was obtained by therapy at 100 median lethal doses, but this was not statistically significant.

The high virus dose used in airborne challenges may have contributed to the reduced protection observed. The time course and pattern of disease after aerosol infection or intranasal infection is very similar to that for subcutaneous infection, and SLE replicates readily in mouse lung. However, it has long been assumed that SLE could enter the brain through the olfactory nerve (Monath and Heinz, 1996), and aerosol exposure would favour this route. In an experimental aerosol infection of mice with West Nile virus, Nir et al. (1965) showed that virus appeared in the olfactory bulb before any other part of the brain. Virus entering the brain in this way would be shielded from the immune system, and may also be exposed to lower levels of the administered IFN in the CSF.

One treated animal in the aerosol experiment (low virus dose, post treatment only) appeared to develop a more chronic infection lasting several days with a low level of virus in the brain. This is most unusual, and we have not seen this phenomenon in animals with SLE infection before, the vast majority dying within 24 h of visible signs. It is possible that IFN induced a chronic infection in this animal, perhaps by controlling virus replication until a degree of immunity developed. Our experiments did not examine whether virus was present at an earlier stage in the brains of apparently well, IFN-treated animals. Persistent infection has been described in children infected with the serologically related virus of Japanese encephalitis (Monath and Heinz, 1996). The other treated animals examined all cleared the infection and no virus was recovered from their brains. The later stage of histological changes seen in surviving animals could represent the cellular components of the immune response clearing the virus from the brain. Residual neurological lesions have been described in animals many years after infection with flaviviruses (Monath and Heinz, 1996).

Flaviviruses have been shown to be sensitive to IFN in vitro (Vithanomsat et al., 1984), and in vivo (Kende, 1992) and our experiments support this, with SLE being about 20 times more sensitive in vitro than the antigenically similar virus of Japanese encephalitis (JE) (T. Brooks, unpublished data). Harinasuta et al. (1984) showed in cell culture that the effect of IFN added to cells infected with JE virus diminished as the interval between inoculation and the addition of IFN was increased from 0 to 6 h, although the dose could be increased to overcome this effect. This phenomenon is mirrored in our results. As the incubation period progresses, the viral load will

increase until it overcomes the ability of IFN to suppress it. There are a number of possible reasons why this occurs, and these will be considered briefly.

The IFN mechanisms responsible for controlling flavivirus infection are not known, but as the virus replicates in the cytoplasm, the Mx system is unlikely to be involved. This is borne out by the efficacy of IFN-α in Balb-c mice, which are deficient for this gene. Because the effector proteins are synthesised in response to IFN-α, pre-treatment or early treatment with IFN-α will give the host a head start in its race with the virus, as the anti-viral proteins will be in place when the virus enters the cells. Prevention of the early stages of viral replication could limit the spread of the disease and either arrest the virus completely or allow the immune system time to mount an effective defence.

IFN-α also acts in synergy with other cytokines on the immune system, generally enhancing the expression of MHC class 1 antigens (de Maeyer and de Maeyer-Guignard, 1991), as well as enhancing NK and T cell toxicity, modulating antibody production and possibly promoting a T-helper cell type 1 (T_H1) response (Beladelli and Gresser, 1996). Part of the action of IFN may be through non-specific activation of NK cells, and through stimulation of a cytotoxic T-cell response. Early selection of an appropriate immune response would favour survival, and treatment with interleukin-12 (IL-12) gives similar results to IFN-α (T. Brooks, in preparation). IL-12 also promotes a T_H1 response and may act synergistically with IFN-α (Romagnani, 1992).

The dose of IFN used (10^5 units) approximates to the highest levels of recombinant IFN given to humans (30 million units daily) for acute disease (Freireich et al., 1966). It was chosen to offer the best protection in the light of our preliminary findings and other published work. This dose was used by Weck et al. (1982), who also showed that better protection was afforded against encephalomyocarditis virus in mice by 10^5 units prophylactically compared to 10^4 units. A similar dose-response effect was found by Kramer et al. (1983) in mice infected with herpes simplex type I virus, Semliki forest virus and encephalomyocarditis virus, and by Morrill et al. (1989) in monkeys exposed to Rift Valley Fever.

The results presented here suggest that SLE infection could be treated with IFN-α if the drug was given early enough. Further examination of dose level and routes of administration e.g. directly into the respiratory tract, may enhance the level of protection we observed. Prophylaxis is likely to be even more effective, but is of limited use in a normal setting, where exposure to SLE is most likely to occur either as a result of a laboratory accident or during an outbreak. Although the subcutaneous route corresponds to the natural route of infection (mosquito bite) our challenge dose was much lower than might be expected. In addition the equivalent route of IFN administration in man would be intravenous. Clearly our experiments do not indicate a possible means of prophylaxis against infected mosquito bite, although by extrapolation from the results of animals exposed to high levels of virus by the aerosol route, such protection might be inferred.

Only after an accident is the time of the exposure likely to be known, and our data support the idea that early use of IFN could prevent individuals developing the disease after inoculation or aerosol exposure. The IFN treatment of human flavivirus disease has not been documented. However a 'therapeutic window' has been observed for a number of other virus diseases, especially respiratory viruses, where although it has not proved possible to affect the course of acute disease, early therapy may prevent the appearance of symptoms (Finter et al., 1991). A similar period may exist after SLE virus infection. IFN-α has proven useful in the treatment of chronic diseases, including hepatitis C which like SLE is caused by a flavivirus.

IFN prophylaxis after known exposure to SLE virus by injection or by aerosol would be beneficial in preventing disease, and should be considered as a serious option in such cases.

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