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Efficacy and Safety of Orally/Sublingually, Intranasally, and Intraperitoneally Administered Recombinant Murine Interferon in the Treatment of Murine Encephalomyocarditis Virus

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ABSTRACT

Interferons (IFN) have been shown to be effective in protecting animals against lethal viral infections when administered systemically in relatively high doses. Intraperitoneal (i.p.) injection of mice with encephalomyocarditis virus (EMCV) gives rise to a rapidly progressive fatal disease characterized by central nervous system involvement and encephalitis. IFN-α has been shown to be effective in protecting mice against lethal EMCV infection when given via parenteral and oral/sublingual routes. The current study was designed to explore the ability of orally/sublingually and intranasally (i.n.) administered IFN-α to treat mice infected with EMCV in support of a planned clinical trial to evaluate efficacy of oral IFN-α in human viral infections. The primary objective of the study was to determine the efficacy of recombinant murine IFN-α (rMuIFN-α) in the treatment of mice infected with 100 LD_{50} EMCV following oral, i.n., and i.p. administration at doses of 20,000 and 100,000 IU. The results of the current experiment did not indicate protection from infection with EMCV in mice that received IFN by the i.n. or oral/sublingual routes. The negative controls, infection of mice with 100 LD_{50} of EMCV followed by treatment with excipient via all three routes, resulted in death of nearly all mice, as expected. The positive control, treatment of EMCV-infected (100 LD_{50}) mice with rMuIFN-α via the i.p. route, was successful in protecting a significant number of mice from death compared with matched controls. This study points out the need to determine the optimum conditions for administration of oral/sublingual or i.n. IFN to insure maximum efficacy against viral infections.

INTRODUCTION

INTERFERONS (IFN) ARE AMONG THE CYTOKINES most widely used in clinical medicine today, with applications both in oncology and in the treatment of viral infections.[1] Although a number of routes of IFN administration, including intravenous (i.v.), subcutaneous (s.c.), intramuscular (i.m.), and intralesional (i.l.), are commonly used, the oral route rarely has been used clinically because IFN are proteins that are inactivated by digestive tract proteolytic enzymes, such as trypsin. In addition, IFN are not absorbed appreciably in their native form.[2–4] Nevertheless, multiple reports have demonstrated the effectiveness of administration of IFN via the oral/sublingual or intranasal (i.n.) routes in protecting animals from a wide variety of viral infections.[5] These results were compiled and summarized in a recent special issue of the *Journal of Interferon and Cytokine Research*.[5] A number of studies have failed to detect biologically active IFN in the blood following oral administration, suggesting the possibility of novel mechanisms of action.[2–4]

IFN have been shown to be effective in protecting animals against lethal viral infections when administered systemically in relatively high doses.[6] Intraperitoneal injection of mice with encephalomyocarditis virus (EMCV) gives rise to a rapidly progressive fatal disease characterized by central nervous system

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(CNS) involvement and encephalitis.[7] IFN-α has been shown to be effective in protecting mice against lethal EMCV infection.[6] Intranasal or oral/sublingual administration (or both) of IFN-α has been shown to successfully protect mice infected with EMCV.[8]

The current study was designed to confirm that oral/sublingual and i.n. IFN-α treatment protected mice infected with EMCV in support of a planned clinical trial to determine efficacy of oral/sublingual IFN-α in a human viral infection. The study consisted of two parts, the EMCV titration and the antiviral assessment. The objective of the EMCV titration was to determine the dose of EMCV required to kill 50% of the infected animals (LD_{50}). The primary objects of the antiviral activity assessment was to determine the efficacy of recombinant murine IFN-α (rMuIFN-α) in the treatment of mice infected with 100 LD_{50} of EMCV following oral/sublingual, i.n., and i.p. administration at doses of 20,000 and 100,000 IU rMuIFN-α. The primary measure of effect was defined as survival at 21 days, and the primary efficacy analysis was the relative survival at 21 days of each treatment group in comparison to its control group, which received only excipient.

In addition to the principal objectives, the study also was designed to (1) provide an additional evaluation of efficacy, where time to death following infection was measured, and (2) evaluate the safety of rMuIFN-α in the treatment of mice infected with 100 LD_{50} of EMCV following oral/sublingual, i.n., and i.p. administration at doses of 20,000 and 100,000 IU. Safety was evaluated both by clinical assessment and by gross pathologic assessment.

MATERIALS AND METHODS

Animals

Specific pathogen-free female Swiss mice were obtained from Charles River Laboratories (Wilmington, MA). All procedures were approved by the Institutional Animal Care and Use Committee of the Carolinas Medical Center. The mice were approximately 5 weeks old and weighed approximately 14–20 g when received for the EMCV titration. The mice were approximately 9 weeks old and weighed approximately 23–27 g when received for the antiviral assessment. The increase in weight was necessitated by the availability of mice from the supplier. On receipt, the animals were put into quarantine for 7 days. After 7 days of quarantine, the mice were retained in the study room.

Randomization

Groups of 44 mice were used to ensure sufficient power for appropriate statistical analysis. Mice were selected randomly and assigned sequentially to route: oral/sublingual, i.n., i.p., excipient only, safety only, or sentinel. IFN-treated groups were then assigned sequentially to dose (20,000 IU or 100,000 IU).

Reagents

rMuIFN-α was obtained from PBL Biomedical Laboratories (New Brunswick, NJ; lot number 1094). The rMuIFN-α was determined to be >95% pure by affinity chromatography. Unit activity was measured by the cytopathic effect inhibition assay on mouse L-929 cells challenged with Semliki Forest virus. The solution was filter sterilized. International units (IU) were calibrated against the NIH laboratory standard for MuIFN-α/β (Ga02-901-511). Individual aliquots of rMuIFN-α were stored at -70 to -80°C. Phosphate-buffered saline with bovine serum albumin (PBS/BSA, 0.01%) was prepared in the laboratory and was obtained from Sigma Chemical Co. (St. Louis, MO). The rMuIFN-α was diluted in PBS/BSA. EMCV (200 μl) obtained from the American Type Culture Collection (ATCC VR 129) (Rockville, MD) was injected i.p. into each mouse.

Administration of IFN

The first dose of IFN was given 1–2 h after inoculation with 100 $\times$ LD_{50} EMCV. Dosing always began in the morning and continued until the last animal scheduled to be dosed that day was dosed. The treatment groups were always dosed in approximately the same order so that dosing occurred at approximately the same time for each treatment group. Because of the unexpected loss of material during administration, only 80%–90% of the designated IFN dose was administered on day 1. Four mice in the low-dose (20,000 IU) i.n. IFN + EMCV group and 11 mice in the high-dose (100,000 IU) i.n. IFN + EMCV group mice did not receive any IFN on day 1 of the experiment. All mice received 100% of the designated IFN dose on days 2–4 of the experiment. Intranasal IFN was administered in a total volume of 10 μl—5 μl to each nostril. Oral/sublingual administration was carried out by placing 10 μl IFN under the tongue of the mouse. Additional IFN was administered in a volume of 200 μl via the i.p. route. PBS/BSA excipient was administered in equivalent volumes via each route to negative control animals.

Study design

EMCV titration. Groups of 12 female (approximately 48 days old) Swiss mice were uninfected and received i.p. 200 μl PBS only or were infected i.p. with 200 μl PBS containing 10-fold serial dilutions of EMCV ranging from 10^{-2} to 10^{-7}. After infection, the mice initially were observed four times daily, within an hour at 07:00, 12:00, 17:00, and 21:00, and any deaths were noted. The number of daily observations was reduced to two (08:00 and 16:00) when it appeared that no additional mice were expected to die. Day of death was defined as a 24-h day beginning at the time of EMCV inoculation for each animal (e.g., 09:00). A death that occurred between 09:00 on day 3 and up to 09:00 on day 4 was credited to day 3. Animals that were observed to be in distress and near death, as evidenced by ruffling of fur and moribund and inactive behavior, were immediately killed by cervical dislocation to minimize pain and suffering and were counted as dead animals. The animals were observed for 21 days, well beyond the time anticipated for EMCV infection to induce mortality. A Reed-Muench analysis[9] was carried out to determine the 50% end point of death, the LD_{50}.

Antiviral assessment. To maximize dose-ranging information by route and to minimize the number of animals required, oral/sublingual, i.n., and i.p. routes were evaluated in two doses (20,000 IU and 100,000 IU) to allow for comparison. The excipient only group served as a negative control. The i.p. route served as a positive control. The safety only group was ad-

ministered EMCV and served as a control for evaluation of safety in comparison with the EMCV treatment groups in which there was also drug treatment. The sentinel group served as a control for changes in environmental conditions.

The rMuIFN-α was administered once a day for 4 days. The first dose was given 1–2 h after inoculation with $100 \times LD_{50}$ EMCV, the dose used in the successful preliminary study.[8] Dosing always began in the morning and continued until the last animal scheduled to be dosed that day was dosed. The treatment groups were always dosed in approximately the same order so that dosing occurred at approximately the same time for each treatment group.

Clinical assessments for all animals were conducted after they were released from quarantine, and the findings were recorded. These observations included but were not limited to changes in skin and fur, eyes and mucous membranes, respiratory system, circulatory system, autonomic nervous system and CNS, somatomotor activity, and behavior pattern.

Mice were observed four times daily through day 21 postinoculation, and any deaths were noted. Observation conditions were similar to those described for the LD_{50}. Comparisons were made between the oral/sublingual and i.n. treatment groups and their respective excipient only group for survival.

The animals were monitored for survival for a period through 21 days after EMCV administration. Animals surviving 21 days of the study were killed on day 21 after the 08:00 observation using cervical dislocation.

Statistical analysis

Standard statistical methods were used. Descriptive statistics, including means and standard deviations (SD) or counts and percentages, were calculated. The principal analysis compared survival at day 21 in each group with its respective excipient only group (virus and no IFN). The groups differed by dose level of IFN-α and the route of administration. The one-tailed test was used, as the alternative hypothesis is that the survival rate will be higher than in the excipient only group (the mortality in the excipient only group was near 100%).

A secondary analysis compared the time to death in each group compared with its respective excipient only group. Kaplan-Meier survival curves were plotted, and the log-rank statistic was tested for differences among the groups. The gross pathology on each mouse was characterized as normal or abnormal. Safety was assessed through an evaluation of observation and gross pathology. Data were tabulated, and descriptive statistics, including means and SD or counts and percentages, were calculated.

The SAS software was used for all analyses. A p value of ≤ 0.05 was considered statistically significant. The expected percent survival at day 21 was $<5\%$ in the controls and 25% in the i.p. IFN-α treatment groups, based on previous work by Tovey and Maury.[8]

The sample size was based on the principal comparison of the percent surviving on day 21 of each group with the respective control group. A one-tailed chi-square test requires 39 animals per group to achieve a power of 80%, with an alpha level of 0.05. This calculation also assumes that the percent surviving in the control group is 5% and the clinical difference is 20% (i.e., at least a 25% survival in the other group). To allow for any deaths following EMCV inoculation, 44 animals were allocated to each group. Death counted toward the efficacy analysis if the animal had received at least one dose of IFN-α.

RESULTS

LD_{50} determination

Female Swiss mice, after quarantine and acclimation, were randomly assigned to groups of 12 mice each. One group received 200 μl PBS i.p. Six other groups of 12 mice each received 10-fold serial dilutions of EMCV in 200 μl PBS, yielding doses of 10^{-2}–10^{-7} dilutions of virus. The titer of the virus was determined to be 6.5×10^6 plaque-forming units (pfu)/ml by plaque assay on L-929 cells. Mice were observed for 21 days, four times daily for 14 days, and then twice daily for the last 7 days, when it became apparent that deaths from viral infection had ended. Animals that showed signs of paralysis were killed by cervical dislocation. Deaths ended by day 5 after infection (data not shown). The LD_{50} for the mice was calculated by means of a Reed-Muench estimation and was determined to be $10^{-5.6}$ dilution of viral stock (3.3 viral particles in 200 μl PBS). Based on the previous studies of Tovey and Maury,[8] a concentration of 100 LD_{50} of EMCV was used for future experiments. As the initial titer of virus was 6.5×10^6 pfu/ml, this was obtained by diluting the stock virus by 1:3981.

IFN titration

The IFN assay was based on a cytopathic effect using the Indiana strain of vesicular stomatitis virus (VSV). The assay was carried out in 96-well flat-bottomed microtiter plates. An international reference standard for rMuIFN-α (Ga02-901-511) was obtained from the NIH/WHO and used to calibrate the assay. An internal laboratory standard was prepared from a commercially obtained preparation of purified MuIFN-α/β (Sigma). This laboratory standard, calibrated in our assay against the international reference standard, was found to contain 33% of the expected IU. This standard was run with all further assays, and all values were multiplied by 3 to obtain IU values.

Commercial IFN was obtained from PBL Biomedical Laboratories and contained glycerol (MuIFN-α A, lot 1094, specified titer 1×10^7 IU/ml). In our assay, the titer was 64.8×10^7 IU/ml, substantially higher than specified. The IFN was assayed multiple times by different individuals, and similar results were obtained. The IFN was stored at -70°C.

Antiviral efficacy experiment

The animals were infected with virus and treated with IFN by the study director and research assistants. Treatments were staggered, and all animals were treated with IFN beginning 1 h after and completed within 2 h after viral infection. Because titration of the rMuIFN-α indicated a titer substantially greater than specified, a question arose as to which dose should be applied. Two alternatives were considered. One was to apply the IFN assuming the dose to be the same as specified by the supplier (PBL). The other was to apply the IFN assuming the dose indicated by the laboratory assay. If the PBL titer were used, the risk was that the actual dose would be so high that it could

TABLE 1. STATISTICAL ANALYSIS OF TREATMENT VS. CONTROLS (ALL ANIMALS)

Treatment group	*% Survival*[a]	*Control % survival*	p *value*
High-dose i.p. IFN	26/44, 59.1%[b]	5/44, 11.4%	<0.001
Low-dose i.p. IFN	32/44, 72.7%[b]	5/44, 11.4%	<0.001
High-dose i.n. IFN	1/44, 2.3%	1/44, 2.3%	NS[c]
Low-dose i.n. IFN	3/44, 6.8%	1/44, 2.3%	NS
High-dose sublingual/oral IFN	1/44, 2.3%	3/44, 6.8%	NS
Low-dose sublingual/oral IFN	4/44, 9.1%	3/44, 6.8%	NS

[a]Comparing different treatment groups with their appropriate control group.
[b]The survival rate for the group given high-dose IFN via the i.p. route was compared with the group given low-dose IFN via the i.p. route. The results were not statistically significant.
[c]NS, not significant.

be toxic to the mice. If the laboratory titer were used, the risk was that the actual dose would be so low that any potential benefit would be lost because of dilution of the IFN. A decision was made to treat two extra test mice with 100,000 U IFN based on the PBL specified titer. One mouse was treated via the i.p. route, and the other was treated via the oral/sublingual route. The mice were observed for 24 h. No obvious toxicity was observed, so we used doses specified by the manufacturer. The decision was made that the risk of diluting the IFN too much was greater than the risk of toxicity. Reports have indicated considerable variability in IFN assays,[10] and this remains a problem.

After dilutions were made with PBS/BSA, it became clear that IFN was being lost in the hubs of the syringes and on the sides of the tubes holding the IFN for the i.n. and oral/sublingual applications. It was noted that the IFN samples were very viscous, and it is possible that the glycerol could have accounted for this unexpected loss. As the dilution had already been made, it was impossible to provide the full dose of IFN on the first day. The IFN-treated mice received between 80% and 90% of the intended dose. This was likely to have minimal impact because, if anything, the actual IFN titer was higher than the PBL titer used to calculate the dose. Even the extreme measures taken did not allow us to provide a full dose to all mice that received IFN by the i.n. route. Four mice that received low-dose (20,000 IU) IFN via the i.n. route + EMCV and 11 mice that received high-dose (100,000 IU) IFN via the i.n. route + EMCV did not receive any IFN on day 1 of the experiment. Because we were aware of this difficulty, we were able to compensate for it on the following days. All mice received the full dose of IFN on days 2–4 of the experiment.

Several of the mice that received IFN via the i.n. route showed signs of lethargy for about 60 sec after administration. They recovered rapidly and showed normal behavior within 2–3 min after dosing. The i.n. dose may have momentarily interfered with breathing of the mice. The four times daily clinical

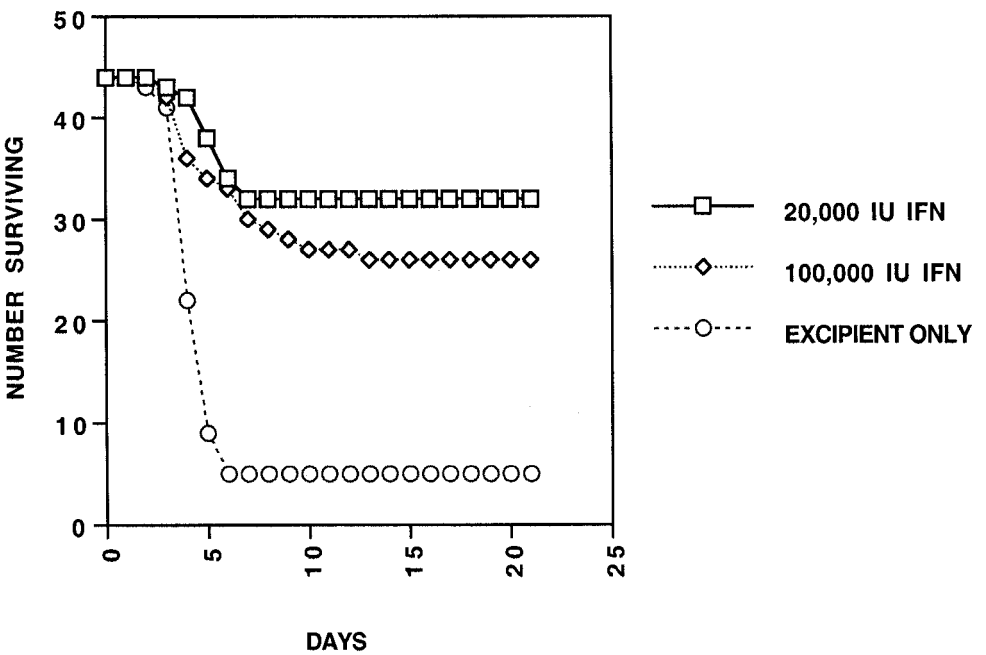

FIG. 1. Survival plot after IFN treatment via the i.p. route. All mice received EMCV.

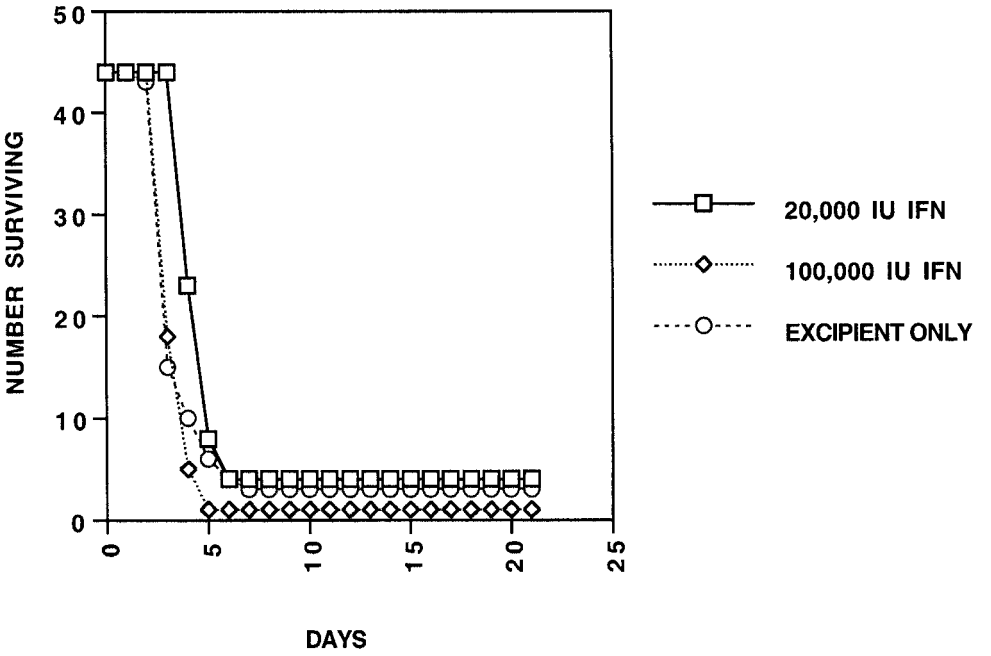

FIG. 2. Survival plot after IFN treatment via the oral/sublingual route. All mice received EMCV.

assessment showed no untoward signs other than occasional eye irritation and bleeding from the mouth. This did not appear to be linked to the treatment but rather to grooming and behavioral interactions of the mice. All mice remained grossly healthy until paralysis and death began as a result of the EMCV infection. All control mice remained grossly clinically healthy throughout the experiment until they were killed.

The results of the treatment are shown in Table 1. There was statistical significance for the differences in total survival for the mice that received IFN i.p. compared with appropriate controls but no statistically significant difference in survival between oral/sublingual or i.n. IFN-treated mice and matched controls (Table 1 and Figs. 1, 2, and 3). Mean survival time was enhanced for mice that received IFN via the i.p. route compared with matched controls ($p < 0.001$) but not between mice that received IFN via the oral/sublingual or i.n. route compared with

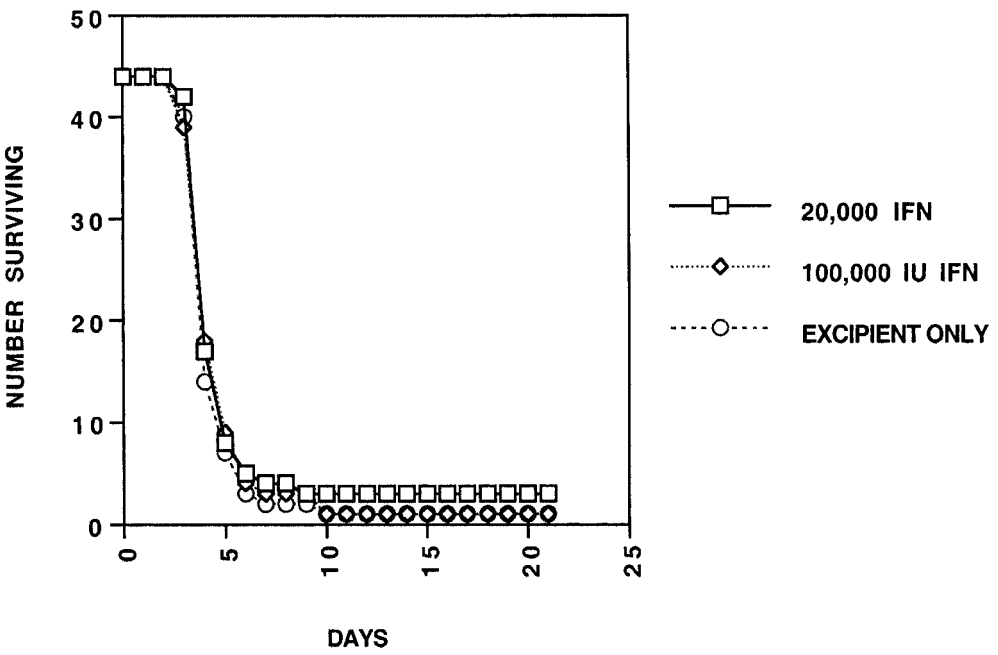

FIG. 3. Survival plot after IFN treatment via the i.n. route. All mice received EMCV.

TABLE 2. STATISTICAL ANALYSIS OF TREATMENT VS. CONTROLS (WITHOUT ANIMALS NOT GIVEN IFN ON DAY 1)

Treatment group	*% Survival*	*Control % survival*	p *value*
High-dose i.p. IFN	26/44, 59.1%	5/44, 11.4%	<0.001
Low-dose i.p. IFN	32/44, 72.7%	5/44, 11.4%	<0.001
High-dose i.n. IFN	1/33, 3.0%	1/44, 2.3%	NS[a]
Low-dose i.n. IFN	1/40, 2.5%	1/44, 2.3%	NS
High-dose sublingual/oral IFN	1/44, 2.3%	3/44, 6.8%	NS
Low-dose sublingual/oral IFN	4/44, 9.1%	3/44, 6.8%	NS

[a]NS, not significant.

matched controls (Table 1 and Figs. 1, 2, and 3). An analysis of the data not including mice that had not received IFN on day 1 showed that mice that received IFN via the i.p. route were protected against EMCV infection but mice that received IFN via the oral/sublingual or i.n. route were not protected (Table 2). Therefore, the study was so highly powered that statistical analysis proved that eliminating mice that had not received IFN on all days had no influence on the results (Table 2). Mean survival time of EMCV-infected mice was enhanced by i.p. IFN treatment, but IFN treatment by the oral/sublingual and i.n. routes had no effect (Table 3).

No gross pathologic problems beyond those induced by the virus were noted. After the 21-day 08:00 evaluation period, the mice were killed by cervical dislocation. No gross pathologic signs were observed in the euthanized mice.

The experiment was repeated comparing effects of IFN containing glycerol and IFN without glycerol. No significant differences were observed between the two types of IFN (data not shown).

DISCUSSION

These results do not indicate protection from infection with EMCV in mice that received IFN by the i.n. or oral/sublingual routes. This is in contrast to previous studies, including one with EMCV.[5,8]

The negative controls, mice infected with 100 LD_{50} of EMCV followed by treatment with PBS/BSA excipient via all three routes, nearly all died, as expected. Treatment of EMCV-infected (100 LD_{50}) mice with IFN via the i.p. route, the positive controls, was successful in protecting a significant number of mice from death compared with matched controls. This indicated that it was unlikely that the possible reduced dose of IFN received on the first day by most mice contributed to the lack of positive effect observed in the oral/sublingual and i.n. groups. Additionally, the study was sufficiently powered so that omission from the statistical analysis of mice that did not receive IFN via the i.n. route on the first treatment day did not affect the outcome of the analysis.

It appears, therefore, that the experiment was carried out successfully without major technical issues. The results of the present study do not confirm studies[5,8] that were not powered to the same extent.

The difference in results may be due to an inability to show statistically the same trends of the previous studies because of the greatly enhanced powering of the present study. However, an alternative explanation could be a difference in the procedures/reagents used. One possibility was that the glycerol content of the IFN used in the study interfered with the activity via the i.n. or oral/sublingual routes. This could have accounted for the observed high viscosity of the IFN and could have affected the distribution of small volumes of IFN via the oral/sublingual and i.n. routes. A repeat of this highly powered study using IFN without glycerol gave similar results, suggesting that glycerol was not the problem.

Several other factors could have been responsible for differences in the results between the present study and previous reports.[5,8] Such factors include differences in strains of mice, in the source and makeup of the IFN used, in viral strains, and in

TABLE 3. MEAN SURVIVAL TIME OF EMCV-INFECTED MICE TREATED WITH IFN

Treatment group	*Mean survival time (days)*[a]	p *value*[b]
High-dose i.p. IFN	15.25 ± 7.2	<0.005
Low-dose i.p. IFN	16.75 ± 7.05	<0.005
i.p. excipient only	6.32 ± 5.38	—
High-dose i.n. IFN	4.93 ± 2.77	NS[c]
Low-dose i.n. IFN	5.64 ± 4.33	NS
i.n. excipient only	4.84 ± 2.77	—
High-dose sublingual/oral IFN	4.86 ± 2.56	NS
Low-dose sublingual/oral IFN	6.07 ± 4.82	NS
Sublingual/oral excipient only	5.66 ± 4.32	—

[a]± SD.
[b]*p* value of treatment compared with matched excipient only control.
[c]NS, not significant.

experimental procedures. The results of the present study indicate that these differences must be identified to allow for the design of protocols that can assure standardized beneficial effects of orally/sublingually and i.n. administered IFN.

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