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Interferon Aerosol Suppression of Vesicular Stomatitis Virus Replication in the Lungs of Infected Mice

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Mice were inoculated intranasally with vesicular stomatitis virus 16 to 22 h after being exposed to small-particle aerosols of saline, natural mouse alpha interferon, recombinant human alpha interferon A, or hybrid recombinant human alpha interferon A/D bgl for 2, 4, or 8 h. Compared with comparably inoculated, untreated mice, significantly reduced levels of vesicular stomatitis virus were observed in the lungs of animals treated with any interferon preparation for 8 h and in groups treated with mouse alpha interferon or hybrid recombinant human alpha interferon A/D bgl for 4 h. No significant reductions in lung virus titers were observed in any group treated with interferon for 2 h or in any of the groups treated with saline.

Many efforts have been made to use preparations of interferon or interferon inducers to treat experimentally induced respiratory virus infections (1–4, 8, 13, 14, 18, 19, 22). The results of these tests have been inconsistent, but generally suggest that with improved methods of delivery and larger doses, interferons might be useful in the prophylaxis or treatment of respiratory viral infections. Recent advances in DNA recombinant technology have allowed the production of high-titered interferons in relatively large quantities (15). We have been testing delivery of such interferons directly to the respiratory tract in small-particle aerosols. This method of delivery has been used with the antiviral agents amantadine and ribavirin to successfully treat experimental (9, 23, 24) and naturally acquired (6, 7, 10, 12, 20) influenza and respiratory syncytial virus infections. In a previous report, we described the deposition, respiratory clearance, and antiviral activity of small particles of interferons in the lungs of mice exposed to interferon aerosols continuously over a 4-day period (25). We have also reported that small-particle aerosols of recombinant interferons delivered 24 h a day for several days could protect cotton rats (*Sigmodon hispidus*) from vesicular stomatitis virus (VSV)-induced lung disease (C.-S. Sun, P. R. Wyde, S. Z. Wilson, and V. Knight, J. Interferon Res., in press). In this report, we compare the protective effects of single 2- to 8-h exposures of small-particle aerosols of a natural mouse alpha interferon (MoIFN) and two recombinant human alpha interferons on VSV replication in lungs of mice inoculated intranasally with this virus. Similar efforts to use small-particle aerosols of interferons to ameliorate influenza virus pulmonary infections have thus far been unsuccessful.

MATERIALS AND METHODS

Mice. Male Swiss outbred mice 6 to 9 weeks old were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, Mass.). These mice were housed in cages covered with barrier filters and fed mouse chow and water ad libitum.

Virus. Stocks of VSV were prepared by infecting flasks of mouse fibroblast (L929) cells with seed virus and harvesting the cells and media when more than 90% cytopathic effect (CPE) was observed. After one freeze-thaw cycle, the resulting suspensions were centrifuged (100 × *g*) to remove cellular debris, portioned, and stored at −70°C. The titer of this pool was 2×10^7 PFU/ml.

Infection of the animals. Mice were lightly anesthetized with ether and inoculated intranasally with 10^2 PFU of VSV in 0.05 ml. This dose was usually not lethal, but produced an asymptomatic disease that was not evident except for the growth of virus in infected lungs or by observing histopathological changes in hemotoxylin- and eosin-stained sections of lung tissue from infected animals.

Tissue cultures. Starting cultures of L929 cells were obtained from Flow Laboratories (McLean, Va.; no 03-439); Madin Darby bovine kidney (MDBK) cells were provided by the Department of Experimental and Applied Biology, Hoffman-La Roche, Inc., Nutley, N.J. Cell cultures of both lines were routinely passaged when confluent by using Eagle minimal essential medium supplemented with 2 mM L-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 0.2% sodium bicarbonate, and 10% fetal calf serum.

Interferons. Both recombinant human alpha interferon A (rIFNαA) and hybrid recombinant human alpha interferon A/D bgl (rIFNαA/D) were obtained from the Department of Experimental and Applied Biology, Hoffmann-La Roche, Inc. The construction and specific molecular activities of these interferons have been described in detail previously (16). Briefly, when VSV was used as the challenge virus both rIFNαA and rIFNαA/D had equivalent activity in MDBK and WISH (a human amnion-derived cell line) cells. However, in mouse L929 tissue cells the antiviral activity of rIFNαA/D was nearly 100-fold that of rIFNαA. In later studies with influenza A/HK/68 virus and primary mouse embryo cells, a similar higher antiviral activity for rIFNαA/D as compared with rIFNαA was noted (25).

MoIFN was obtained from Lee Biomolecular Research Laboratories, Inc. (San Diego, Calif.; no. 22051). Human type alpha (no. G-023-901-527) and mouse type 1 (no. G-002-904-511) interferon reference reagents were obtained from June K. Dunnick and John R. LaMontagne of the Antiviral Substance Program of the National Institute of Allergy and Infectious Diseases, Bethesda, Md.

Measurement of antiviral activity. Assays were performed in 96-well round-bottom plates (no. 76-013-05; Flow Laboratories, McLean, Va.). Each assay was performed in duplicate and in parallel in L929 and MDBK cells. Briefly, 0.05 ml of medium, test sample, or interferon reference standard was

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added to the first well of a row and serially diluted (twofold) with microtiter loops (Rotatiter, no. 002-961-0100; Dynatech Laboratories, Inc., Alexandria, Va.). After the appropriate tissue culture cells (approximately 2×10^4 cells per well in 0.05 ml) were added, the plates were incubated (5% CO_2, 37°C) overnight. The medium was then removed with a suction device, and VSV (approximately 100 50% tissue culture infective doses in 0.1 ml) was added to all wells but those reserved as tissue controls. Virus control wells (wells containing medium plus virus) were observed daily. When the CPE was 90 to 100% in these wells (usually at 48 h), the degree of CPE in all test wells was recorded. Interferon titers were expressed as units ($\log_2$) with 1 U being defined as the reciprocal of the last dilution of each sample that inhibited CPE 50% as compared with virus control wells. The titers of the interferon reference standards used in these assays rarely varied more than twofold from assay to assay. All titers obtained in L929 cells were adjusted according to the stated titer of the mouse National Institutes of Health interferon reference standard; similarly titers obtained in MDBK cells were calculated relative to the National Institutes of Health human interferon reference standard.

Collection of lungs. Lungs were collected from four mice in each group immediately after ceasing aerosolizations, just before virus inoculation, and at 24-h intervals on days 1 through 4 after virus inoculation. The mice were first anesthetized with sodium pentobarbitol given intraperitoneally and then exsanguinated by cutting the aorta. Lungs were then removed intact with thoracic trachea, trimmed of detectable lymph nodes, and rinsed in sterile saline. Finally, they were ground in glass homogenizers and centrifuged (100 $\times$ *g*) to remove cellular debris. The resulting supernatants were frozen at −70°C until assayed for interferon and virus.

Aerosol therapy. Aerosol generators containing Collison nebulizers modeled after a design described by May (11) were used to generate continuous small-particle aerosols of interferon. The use of these generators to deliver other antiviral agents has been described previously (23, 24). In these experiments, rIFNαA/D and rIFNαA were diluted in sterile saline (0.9% sodium chloride; Travenol Laboratories, Inc., Deerfield, Ill.; no. 0338-0049-02) to either a concentration of 5×10^3 or 1.7×10^5 U/ml and placed in delivery reservoirs (500-ml polypropylene beakers, no. 146706; Spectrum Medical Industries). The MoIFN was delivered similarly, but only at a concentration of 5×10^3 U/ml.

Each interferon aerosol was administered for 2, 4, or 8 h to mice held in sealed plastic cages. At the end of each interval, the interferon remaining in the reservoirs was checked for titer and microbial sterility. In each experiment there were groups of mice exposed to aerosols of sterile saline not containing interferon or not exposed to any aerosol. At 24 h after initiation of the interferon administration, all mice were inoculated intranasally with VSV.

Pulmonary interferon and virus assessments. To determine interferon levels in fluids from infected lungs, portions of the test samples were irradiated with UV light (germicidal bulb at a distance of 30 cm for 7 min) to kill residual virus, serially diluted (twofold dilutions), and tested in parallel in MDBK and L929 tissue cells as described above. In preliminary experiments it was determined that such treatment killed all virus and that even the addition of as much as 2×10^6 PFU of VSV inactivated with UV irradiation did not induce detectable levels of interferon in wells containing either L929 or MDBK cells. By testing in parallel it was often possible to identify the nature of the interferon present in lung homogenates, since both MoIFN and endogenously produced interferons were not active in MDBK cells. rIFNαA was not detectable in L929 cells at the concentrations of this material found in lungs of test mice, and rIFNαA/D was uniquely active in both MDBK and L929 cells (25).

Virus titers in lungs of test animals were determined by testing samples in parallel in both MDBK and L929 cells. Briefly, clarified lung homogenates were serially diluted with serial half-$\log_{10}$ dilutions in 96-well tissue culture plates (Flow Laboratories; no. 76-013-05). L929 or MDBK cells were then added, and the plates were placed in a 37°C 5% CO_2 incubator. Each well was observed daily for the next 5 days for CPE. Interference by interferons present in lung homogenates was not a problem, since no exogenous interferons could be detected in any lung fluids 28 h after ceasing delivery of these materials, and all of the interferons except rIFNαA/D were active at the levels found in lungs only on one of the two tissue cell lines used to assess virus titers. Virus titers were expressed as the $\log_{10}$ of the reciprocal of the last dilution of lung fluid that exhibited CPE.

Statistics. Each geometric mean titer (GMT) and standard deviation for pulmonary virus and interferon levels presented in this manuscript was derived by testing lung samples from four different animals. Student's *t* test (17) was used to compare GMTs from different groups. *P* values of ≤ 0.05 were considered statistically significant.

RESULTS

Effects of concentration. Table 1 compares titers of VSV in lungs of mice exposed for 4 h to small-particle aerosols of saline, MoIFN, rIFNαA, or rIFNαA/D and then challenged intranasally 20 h later with 100 PFU of VSV. The reservoir concentration of MoIFN used (5×10^3 U/ml) was the maximum concentration of this material that could be delivered practically. Groups of mice exposed to MoIFN or either concentration of rIFNαA/D had significantly lower pulmonary virus titers than comparably inoculated mice that were exposed to just saline (Table 1). In contrast, no significant reduction of VSV was observed in lungs of mice exposed to rIFNαA (1.5×10^5 U/ml). The only group in which virus was not detected in lungs was the group exposed to the higher dose of rIFNαA/D.

Effects of exposure time. Figure 1 compares levels of VSV in lungs of mice exposed to small-particle aerosols of

TABLE 1. Pulmonary titers of VSV in lungs of mice exposed for 4 h to small-particle aerosols of saline, MoIFN, rIFNαA, rIFNαA/D 20 h before virus challenge[a]

Interferon	Interferon concn in reservoir (U/ml)	Estimated interferon dosage[b] (U/mouse)	Pulmonary virus titer[c]	*P*[d]
None (saline)			5.8 ± 0.6	
MoIFN	5.0×10^3	150	2.3 ± 0.2	<0.001
rIFNαA/D	5.0×10^3	150	1.4 ± 1.2	<0.001
rIFNαA/D	1.7×10^5	5,100	0	<0.001
rIFNαA	1.7×10^5	5,100	6.2 ± 0.6	>0.05

[a] All mice were inoculated intranasally with approximately 100 PFU of VSV 20 h after ceasing aerosolization. One day after virus inoculation these mice were killed, and their lungs were assessed for virus titers.

[b] Estimated dosage is the respiratory volume of mice per min [5] × (60 min) × (4 h) × (estimated retention volume [22]) × (reservoir concentration of interferon) × (output coefficient of the aerosol generator); e.g., for MoIFN above: 25 cm^3/min × 60 min × 4 h × 0.5 × 5,000 U/cm^3 × (1×10^{-5}) = 150 U.

[c] GMT ($\log_{10}$/lung) ± standard deviation for 4 mice per group.

[d] Determined by comparing each GMT ± standard deviation with 5.8 ± 0.6 by using Student's *t* test.

rIFNαA, rIFNαA/D, or MoIFN for 2, 4, or 8 h. Starting reservoir concentrations of rIFNαA, rIFNαA/D, and MoIFN were 1.7×10^5, 1.7×10^5, and 5×10^3 U/ml, respectively. Significant decreases in virus levels were observed in mice exposed to rIFNαA for 8 h, but there were no significant decreases in lung virus titers in mice exposed to rIFNαA for 2 or 4 h compared with pulmonary titers in the saline control group (Fig. 1). In the saline control mice, VSV levels in the lungs peaked on day 1 after inoculation (GMT, 6.5 $\log_{10}$/lung) and then decreased until day 4, when only very low levels of virus were detected. This pattern of replication in saline control mice was typical of the pattern observed in numerous experiments and was virtually identical to the growth kinetics observed in infected, untreated controls (data not shown).

Virus was not detected in lungs of mice exposed to rIFNαA/D for 8 h and was found only on day 1 at significantly reduced levels in the group exposed to aerosols of this recombinant interferon for 4 h (Fig. 1). No differences in virus titers were observed in the lungs of mice exposed for 2 h to rIFNαA/D aerosols and saline-treated controls (all P values were >0.05).

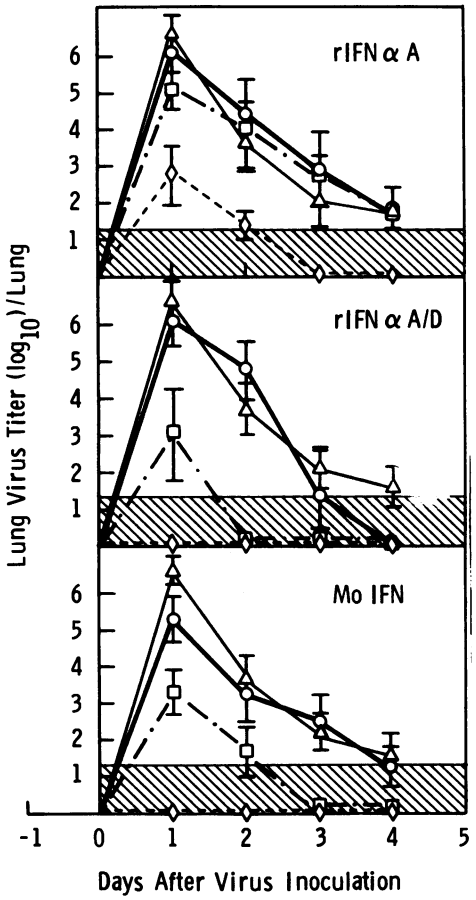

FIG. 1. Virus titers in lungs of mice inoculated intranasally with 10^2 PFU of VSV 16 to 22 h after exposing mice to small-particle aerosols containing rIFNαA, rIFNαA/D, or MoIFN. The intervals for each interferon exposure were 2 h (○), 4 h (□), or 8 h (◇). Also depicted in each panel are lung virus titers in mice exposed only to physiological saline (△). Each point represents the geometric mean titer ($\log_{10}$) with four mice per point. Shaded areas represent the detection limits of the assay. The GMT of all groups were compared with the GMT of untreated, inoculated mice with Student's t test. Significantly lower GMTs were observed only in the groups given rIFNαA for 8 h (P values for days 1 through 3 were ≤0.01) and in the groups given rIFNαA/D or MoIFN for 4 or 8 h (P values on days 1 through 3 were ≤0.01).

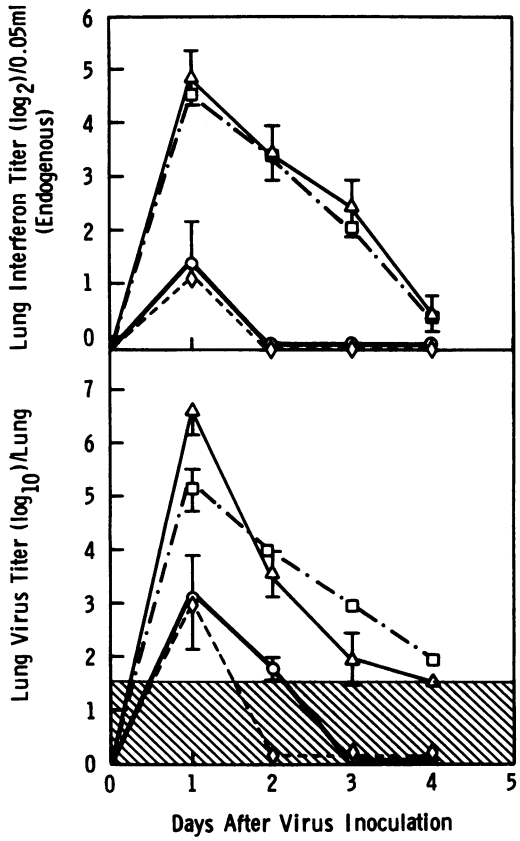

FIG. 2. Lung virus and endogenous interferon titers in mice exposed for 4 h on day −1 to small-particle aerosols containing saline (△), rIFNαA (□), rIFNαA/D (◇), or MoIFN (○) and inoculated intranasally on day 0 with 10^2 PFU of VSV. The GMT ± standard deviations are shown; the number of animals per point is 4. The virus GMTs for the groups exposed to rIFNαA/D or MoIFN were significantly lower than the GMTs of the virus-infected, saline-exposed group on days 1 through 3 ($P \leq 0.01$) when GMTs were compared with Student's t test. No significant differences were observed between the GMTs for the saline control and the group exposed to rIFNαA on any day of observation. Similarly the interferon GMTs were significantly lower ($P \leq 0.01$) on days 1 through 3 compared with values for control mice only in the groups exposed to rIFNαA/D and MoIFN.

Similarly (Fig. 1), no reductions in virus titers were observed in lungs of mice exposed to MoIFN for 2 h. Mice exposed to MoIFN for 4 h, however, had virus isolated on days 1 and 2. Those exposed for 8 h had no virus isolated.

Endogenous lung interferon levels. Figure 2 compares virus and endogenous interferon levels in lungs of the three groups of mice exposed to the different interferons for 4 h. The levels of endogenous interferon in these lungs correlated well with the degree of VSV replication. Thus, on day 1 after virus inoculation, relatively high concentrations of endogenous interferons (GMT, 4.5 to 4.8 $\log_2$/0.05 ml of lung homogenate) were observed in lungs of mice exposed to

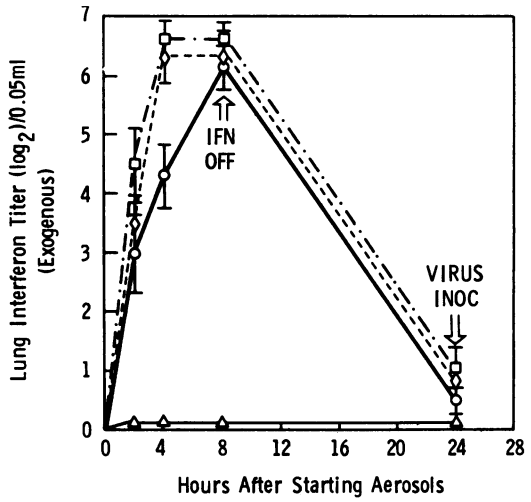

FIG. 3. Levels of exogenous interferon in lungs of mice exposed to small-particle aerosols of saline (△), rIFNαA (□), rIFNαA/D (◇), or MoIFN (○). The GMTs are depicted; the number of animals per point is 4. With Student's *t* test, no significant differences between the GMTs of the different groups were observed at any time interval. The maximum differences were observed at 4 h (GMT for MoIFN of 4.3 ± 0.5 versus GMT for rIFNαA of 6.5 ± 1.0; $P = 0.1$).

either saline or the less active rIFNαA, whereas only minimal concentrations of endogenous interferons (GMT, 1 to 1.2 log_2/0.05 ml of lung homogenate) were detected in lungs of mice exposed for 4 h to aerosols of rIFNαA/D or MoIFN. No endogenous interferons were detected in lungs of mice exposed for 8 h to rIFNαA/D or MoIFN, and they were only found on day 1 in lungs of mice exposed to rIFNαA for 8 h (data not shown).

Exogenous lung interferon levels. Figure 3 compares levels of exogenous interferon in lungs of mice exposed to aerosols of these materials for 8 h. The levels of exogenous interferon in groups of mice exposed for 2 or 4 h (data not shown) had kinetics up until 2 or 4 h, respectively, that were similar to those shown in Fig. 3. However, no interferon was detectable in lungs from mice in either the 2- or 4-h exposure groups at the 24-h test interval (just before virus inoculation). There was no detectable antiviral activity in lung homogenates from mice exposed only to saline aerosol (Fig. 3). In contrast, significant levels of interferon were detected in lungs of mice in all interferon groups by 1 h after starting the aerosolization. The levels of exogenous interferons rose in each group and were maximum by 4 h in the groups given either rIFNαA or rIFNαA/D and by 8 h in the group exposed to MoIFN. Levels of exogenous interferons 24 h after the start of the aerosolizations (just before virus challenge) were low and not statistically significant from saline control values for all three interferon groups (all GMTs were ≤1.2 log_2/ml). The values shown for MoIFN were determined in L929 cells (in MDBK cells all values were equal to 0), whereas the values shown for rIFNαA/D and αA were determined in MDBK cells; values for rIFNαA/D in L929 cells were quite similar to the values shown, whereas values for rIFNαA in L929 cells were all equal to 0.

DISCUSSION

In these studies, mice exposed to small-particle aerosols containing MoIFN or either rIFNαA or IFNαA/D for one 8-h interval, 16 h before virus challenge, had significantly reduced replication of VSV in their lungs than did comparably challenged control mice. The interferon doses and 8-h exposures used appeared to provide near-threshold values for efficacy, since under these conditions the MoIFN and rIFNαA/D were totally protective and the rIFNαA was partially so (Fig. 1), but when given at the same concentrations for 4 h, MoIFN and rIFNαA/D were only partially protective and rIFNαA was not significantly protective (Table 1, Fig. 1). Moreover, in experiments not presented, we have observed that lower interferon concentrations are significantly protective only when administered for longer intervals.

In an earlier study we showed that interferon concentrations in the lungs of mice exposed continually to aerosols of interferon rose rapidly after the start of aerosolization and plateaued at levels comparable to those seen for endogenous interferons at the peak of natural influenza virus infection. In these studies the maximum levels of exogenous interferon reached (Fig. 3) were approximately twofold that observed for maximum levels exogenous interferon induced after VSV inoculation (Fig. 2). This may have occurred because of the relatively low inoculum of VSV used in these experiments. Regardless, the data from both studies suggest that the use of higher interferon doses or longer exposure intervals may not be of further benefit. Conversely, the use of dosages of interferon near threshold levels could avoid the potential toxic effects due to the presence of excessive interferon.

rIFNαA appeared to be the least potent of the three interferons tested, as indicated by pulmonary virus titers in mice given equivalent dosages of the three interferons (Table 1), by dose-response studies (Fig. 1), and by examining levels of endogenous interferon levels in lungs of test mice after challenge with VSV (Fig. 2); i.e., after 4-h aerosolizations and virus challenge, endogenous interferon production was greatest in the lungs of mice exposed to aerosols of saline or the less active rIFNαA and least in animals exposed to rIFNαA/D and MoIFN for this period. These results correlated with results of in vitro tests where rIFNαA was shown to be 100-fold less effective in protecting mouse (L929) cells from VSV infection than was rIFNαA/D (16, 25). The partial protection that was observed after the aerosolization of relatively high doses of rIFNαA for 8 h may have been due to the forcing of heterologous interferon molecules into tissue cell receptors by mass action (16).

Both MoIFN and rIFNαA/D showed similar activity in suppressing VSV pulmonary infection (Table 1) when used at equivalent concentrations and exposure periods. However, only when higher concentrations of rIFNαA/D were used was VSV replication in lungs reduced to undectable levels. MoIFN was not tested at higher concentration because of the limited availability and excessive costs of higher-titered MoIFN. These findings point out the primary advantages of recombinant interferons: their high titer and availability.

Although the greater efficacy of 8-h aerosols over 4-h intervals could be due to the shorter interval between ceasing aerosolization and challenging with virus that occurred during the longer aerosolizations, this was not likely. In studies not presented here, we have found that we can wait 3 days before challenging mice and still get significant protection (data not shown).

At least two other groups of investigators have reported some success in using aerosols of interferon to reduce pulmonary virus infection (3, 21). In the first study (3), variable but overall significant reduction of rhinovirus replication in human volunteers was observed after treatment

with rIFNαA by aerosol for 1 h. In the second study (21), some protection (≤50%) of mice from lethal influenza virus infection was achieved, but only when very specified administration regimens were utilized. With the majority of regimens used in the latter studies, the test interferon was either ineffective or appeared to cause increased mortality. In our first efficacy studies (Sun et al., in press), rIFNαA and rIFNαA/D were both effective in reducing VSV lung infection in cotton rats (rIFNαA/D was significantly more protective than rIFNαA); however, prolonged interferon treatments (23.5 h/day starting before and continuing after virus challenge) were necessary to obtain a protective effect, since neither rIFNαA or rIFNαA/D is very active in cotton rat tissues. Thus, in the present study the successful use of single treatment periods of 4 or 8 h before virus challenge represents an advance over our previous effort. The increased efficacy can be explained by the increased activities of rIFNαA and rIFNαA/D in mouse tissue cells. We did not attempt to give interferons after virus challenge since VSV replication in the lungs occurs so rapidly and is at maximum levels within 24 h after inoculation (Fig. 1).

We have tried in numerous experiments to use MoIFN and rIFNαA/D administered as small-particle aerosols to treat avirulent and virulent influenza virus lung infections in mice. To date, despite many varied administration regimens and doses, we have not obtained any positive results. We are currently seeking the reasons for the differences seen in the VSV and influenza models, and we hope that the contrasts themselves will be informative.

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