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Requirement for Macrophages for Interferon to be Effective Against Encephalomyocarditis Virus Infection of Mice

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Suppression of macrophages in mice by treatments with silica or auro-thio-malate (Myocrisin) reduced production of serum interferon by polyriboinosinic acid:polyribocytidylic acid by 85 to 90%, indicating that this double-stranded polynucleotide caused interferon production primarily in macrophages. Suppression of macrophages in mice by silica or Myocrisin treatment did not significantly affect the susceptibility of mice to encephalomyocarditis virus, although at virus doses around 20 times the 50% lethal dose they died about 48 h earlier. Macrophage interferon protected mice from encephalomyocarditis virus infection at much lower doses than fibroblast interferon, and treatment of mice with silica or Myocrisin abolished the protection conferred by macrophage interferon, whereas these treatments had a much smaller effect on the protection afforded by fibroblast interferon. The requirement for macrophages for interferon to be effective in mice can explain why macrophage suppression can cause normally nonlethal viruses to kill adult mice.

The cellular origin of circulating interferon produced in vivo has been established for several different inducers, but other factors necessary for such interferon to be effective have not been considered. The mechanism of action of interferon is now reasonably well understood at the intracellular level (11), but which cells in the whole animal accumulate interferon and which other factors provide additional barriers to infection necessary for the effect of interferon to be manifest is still unclear.

Lymphocytes appear to be the cells responsible for interferon production induced by Newcastle disease virus and Sindbis virus, and encephalomyocarditis (EMC) virus infection seems to cause interferon production in parenchymal cells of the brain and muscles (10). The double-stranded polynucleotide complex polyriboinosinic acid:polyribocytidylic acid [poly(I:C)] results in interferon production from radio-resistant cells derived from the hemopoietic system, and macrophages have been inferred to be the cells responsible (10). However, these studies have not indicated whether these or other cell types are important for protection of the whole animal by interferon, once produced. Moreover, the amounts of circulating interferon produced were not related to the degree of protection against infection conferred to the animals in these studies. In view of the heterogeneity of interferon produced in different cells (19), it is possible that the degree of protection conferred by different interferons in one cell type is different and that different cell types respond differently to any one species of interferon. Human fibroblast and leukocyte interferons of apparently identical titers confer different degrees of protection to fibroblast cells in culture against different viruses when the doses of the two types of interferon are varied (5). Moreover, different non-human cells respond differently to human interferon (7), and human interferons of different cellular origins confer different degrees of protection against infections in different human cell lines (A. A. Schwartz, in preparation).

The importance of macrophages in resistance to viral infection (12) prompted us to examine the role of macrophages in the antiviral effects of interferon against EMC virus infection of mice. To destroy macrophages in vivo, we used silica (Dorentrup 12, 25 mg/mouse intraperitoneally [i.p.] or 5 mg/mouse intravenously [i.v.]) or Myocrisin (auro-thio-malate, 8 mg/mouse or three treatments of 2 mg/mouse each, i.p.), both of which are known to be specifically cytotoxic for macrophages in mice under these conditions (2, 3). We report here further evidence in support of the notion that macrophages are the principal cells producing serum interferon after poly(I:C) treatment of mice. Interferon so induced is therefore referred to as macrophage interferon in this work. We demonstrate that macrophages are also necessary for macrophage interferon to be effective against EMC virus infections of

mice, but that fibroblast interferon is only partially dependent on macrophages for its antiviral activity against EMC virus in vivo.

MATERIALS AND METHODS

Virus. EMC virus was grown and stored as previously described (15).

Mice. Female white mice (BK:W), 6 to 10 weeks old and weighing 18 to 21 g, were obtained from Bantin & Kingman Ltd., Hull, U.K., and maintained at 22°C with unlimited access to water and standard rat and mouse breeding diet from Grain Harvesters Ltd., Wingham, Kent, U.K.

Cells. L-929 monolayer cultures were grown in RPMI 1640 medium containing 10% donor calf serum (Flow Laboratories, Irvine, Scotland).

Chemicals. The double-stranded complex poly(I:C) was obtained from P-L Biochemicals, Milwaukee, Wis. Sodium auro-thio-malate (Myocrisin) was obtained from May & Baker, Dagenham, Essex, U.K. Silica (Dorentrup 12) was a gift from R. Norpoth, Institut für Arbeitsmedizin, Münster, West Germany. This material was made up at 125 mg/ml, and after sonic treatment 0.2 ml was administered i.p. to mice. Suspensions of silica for i.v. administration were made up at 50 mg/ml, and after sonic treatment 0.1 ml was injected into a lateral tail vein. Dilutions of virus stocks and poly(I:C) were made up in 0.89% (wt/vol) NaCl, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.5), and volumes of 0.1 ml were injected i.p. or i.v.

Interferon preparations and assay. Crude, poly(I:C)-induced (macrophage) interferon was prepared by i.p. administration of 60 μg of poly(I:C) per mouse and bleeding out the mice by cardiac puncture 2 or 3 h later. After clotting the blood, serum was removed and stored at −20°C. Mouse fibroblast interferon was obtained from Bionetics Laboratory Products Inc., Rockville, Md. This interferon was derived from C-243-3 cells induced with Newcastle disease virus. Interferon determinations were carried out using a plaque reduction assay in L cells against EMC as the challenge virus. Confluent monolayers of L cells in 35-mm wells of Linbro FB-6-TC multidishes (Flow Laboratories) were treated for 17 h at 37°C with dilutions of the interferon samples. The cells were then infected with EMC and overlaid with RPMI 1640 medium containing 2% donor calf serum and 0.75% carboxymethyl cellulose (Sigma, high viscosity). Plaques were counted 2 days later after staining with neutral red. The interferon titer was taken as the dilution that inhibited plaque formation by 50%. Parallel assays of mouse reference interferon (catalog no. G002-904-511) obtained from the Research Resources Branch, National Institute of Allergy and Infectious Diseases, Bethesda, Md., were performed. In our system the international standard had a titer of 2,000 U/ml compared to the quoted titer of 6,485 U/ml. All the interferon titers in the text are the values determined in our assay multiplied by 3.24 to convert them to international units.

Statistical methods. The survival time of mice was obtained from records prepared twice daily. Records were made for 25 days from the day of infection, although no further deaths occurred after 18 days postinfection. Significant differences in the survival times of different groups of mice were tested for by calculating χ^2 values by the log-rank method, as described by Peto and Pike (13), on the survival data up to 18 days postinfection. This is a nonparametric analytical method and does not rely exclusively on either the number of surviving mice or delays in death. Paired comparisons are made between treated and control groups and between various treatments, and the method tends to underestimate probabilities. The significance levels of these χ^2 values, which have one degree of freedom, are indicated by asterisks as follows: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$. No asterisk indicates $P > 0.05$ and is generally taken as not significant. For convenience of presentation, where results are shown in the form of mortality curves, deaths occurring in any one day are shown at one time only.

We were concerned with studying the effects of our polynucleotide treatments against lethal doses of virus, but for the sake of accuracy virus titrations are given in terms of 50% lethal doses (LD_{50}). Titrations of our glycerol-stored virus stocks remained constant over many months. We have routinely found that a virus dose of 12 × LD_{50} kills virtually all mice in groups of 20.

RESULTS

Effect of silica and Myocrisin treatments of mice on infectivity of EMC virus and production of serum interferon of poly(I:C) in mice. The results in Fig. 1 show that Myocrisin treatment (8 mg/mouse i.p. 8 h before infection) does not alter the end-point titration of EMC virus in mice, although at a virus dose of 50 × LD_{50} mice die earlier. This difference is significant at $P = 0.05$ and is consistently observed around this virus dose, but not usually at higher or lower virus doses. The largest effect of Myocrisin on susceptibility to infection with virus doses below 1 × LD_{50} is also shown in Fig. 1. This difference is not generally apparent, but for the data shown in Fig. 1d it is significant (at $P = 0.05$) and we estimate that it represents an increase of 20-fold in lethality of the virus. Similar results have been obtained with mice treated with silica.

Serum obtained 2 h after treating mice with 60 μg of poly(I:C) per mouse was assayed for interferon by plaque reduction of EMC virus on L-cell monolayers. Myocrisin (8 mg/mouse i.p.) or silica (25 mg/mouse i.p.) given 4 or 22 h before poly(I:C) was found to reduce production of serum interferon by 85 to 90%, as indicated by the results in Table 1. The same serum samples from silica- or Myocrisin-treated mice also failed to confer protection to other mice when administered 6 h before infection with a lethal dose of virus (12 × LD_{50}). The results in Table 2 show that poly(I:C) (60 μg/mouse) given either i.p. or i.v. induces similar levels of serum inter-

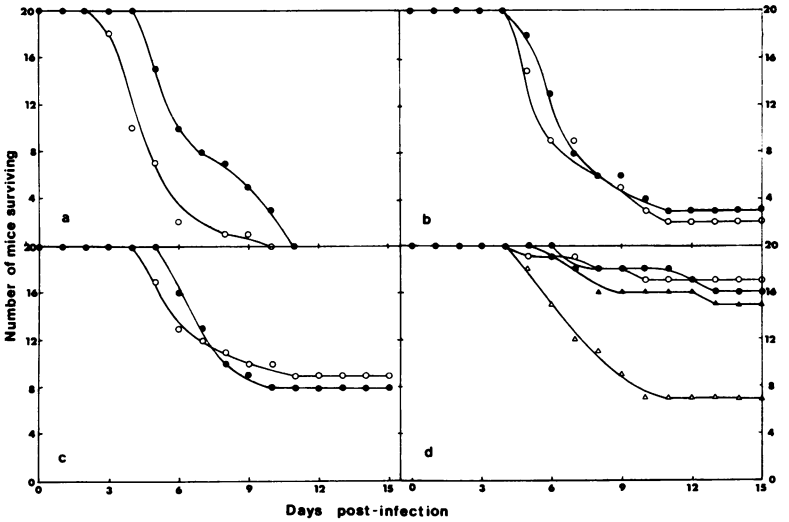

FIG. 1. *Effect of 8 mg of Myocrisin per mouse, given i.p. 8 h before infection of mice with EMC virus at (a) 50 × LD_{50}, (b) 10 × LD_{50}, (c) 1 × LD_{50}, and (d) 1/25 × LD_{50}. Symbols: (●) infected only; (○) Myocrisin treated and infected. Another experiment (d) shows the largest effect observed with Myocrisin treatment at 1/25 × LD_{50}. Symbols: (▲) infected only; (△) Myocrisin treated and infected.*

TABLE 1. *Effect of i.p. injection of silica (25 mg/mouse) or Myocrisin (8 mg/mouse) on serum interferon titers 2 h after administering 60 μg of poly(I:C) per mouse*

Treatment	Serum interferon (U/ml)
Poly(I:C) only	5,200
Poly(I:C) and silica at −6 h	640
Poly(I:C) and silica at −22 h	780
Poly(I:C) and Myocrisin at −6 h	710
Poly(I:C) and Myocrisin at −22 h	530

TABLE 2. *Effect of silica (25 mg/mouse i.p.) on serum interferon titers 2 h after administering 60 μg of poly(I:C) per mouse*

Treatment	Serum interferon (U/ml)
Poly(I:C) only, i.p.	5,200
Poly(I:C) only, i.v.	5,000
Poly(I:C) i.p., silica at −24 h	620
Poly(I:C) i.v., silica at −24 h	600

feron and that these interferon titers are greatly reduced by silica given by the i.p. route, regardless of the route by which the poly(I:C) is given. These results indicate that the treatments suppressing macrophages do not simply have their effect on interferon induction by poly(I:C) when the macrophage-suppressing agents are given by the same route as the interferon inducer.

Effect of suppressing macrophages on the antiviral activity of macrophage interferon. The results in Fig. 2 demonstrate that the pronounced protection achieved with 500 U of poly(I:C)-induced (macrophage) interferon given 4 h before infection of mice with EMC virus is virtually abolished on treating the mice with silica 8 h before the time of infection. It is difficult to assess whether the silica treatment completely abolished the protective effect of the interferon, since silica treatment at the virus dose used (20 × LD_{50}) caused the infected mice to die about 48 h earlier, and the mice treated with silica and interferon did not die at this earlier time but rather around the time of death of the infected mice given no treatments. The interferon treatment was highly protective compared with the infected control group (χ^2 = 25.87***), and the group treated with silica and interferon was not significantly different from the infected-only group but was significantly different, at the 5% level, from the infected group that had also been treated with silica. Essentially complete abolition of protection by macrophage interferon has also been observed at lower doses of interferon and by silica treatments at 24 h before infection, and also when the interferon was administered i.v. and the silica was administered i.p.

The effect of Myocrisin treatment on protection of mice against EMC virus infection by macrophage interferon was also examined after a single Myocrisin treatment of 8 mg/mouse 16 h before infection or three treatments of 2 mg/mouse each on alternate days, the last treatment being at 8 h before infection. In all cases these treatments abolished or very greatly re-

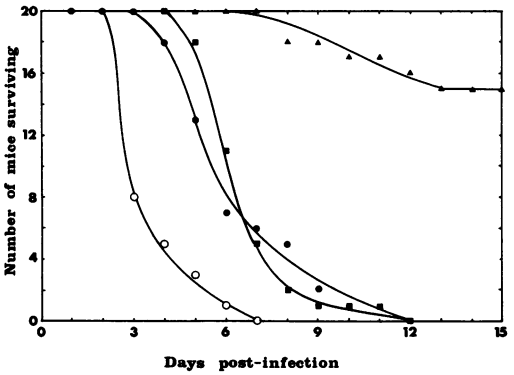

FIG. 2. *Effect of 25 mg of silica per mouse, given i.p. 8 h before infection, on the protective effect of 500 U of macrophage interferon given i.p. 4 h before infection of mice with 20 × LD_{50} of EMC virus. Symbols:* (●) *infected only;* (○) *infected and silica treated;* (▲) *infected and interferon treated;* (■) *infected and interferon and silica treated.*

duced the protective effect of the macrophage interferon.

To examine the possibility that silica or Myocrisin treatments simply increase the susceptibility of mice to EMC virus, thereby rendering macrophage interferon treatment ineffective, we checked the protective effect of the interferon against different virus doses. The greatest increase in susceptibility of mice to EMC virus observed by silica or Myocrisin treatment was about 20-fold (see Fig. 1d), and the protective effect of 500 U of macrophage interferon was virtually abolished by silica treatment (Fig. 2). We therefore determined the protective effect of a lower dose of macrophage interferon (250 U/mouse) against virus doses of 1 and 20 × LD_{50}. The results (Fig. 3) clearly show that a 20-fold increase in virus dose does not abolish the protective effect of the interferon treatment. The log-rank χ^2 comparisons between the infected controls and interferon-treated groups in Fig. 3a and b were 5.24* and 15.64***, respectively.

Effect of suppressing macrophages on the antiviral activity of fibroblast interferon. Mouse fibroblast interferon (4,000 U/mouse) confers obvious protection against EMC virus infection of mice when administered 6 h before infection. The results in Fig. 4a show the effect of this dose of interferon against 17 × LD_{50} of EMC virus (χ^2 comparison between the infected group and the group also treated with interferon = 16.3***). Figure 4a also shows that 8 mg of Myocrisin per mouse at 24 h before infection has no effect on the protective effect of this dose of interferon. A higher dose of fibroblast interferon (8,000 U/mouse) confers slightly greater protection (χ^2 comparison with infected-only group = 22.0***), and at this dose the Myocrisin treatments partially suppress the antiviral effect of the interferon treatment (see Fig. 4b, χ^2 comparison between interferon-treated group and group treated with interferon and Myocrisin = 4.1*). Higher doses of fibroblast interferon (10,000 U/mouse) did not confer greater protection than 8,000 U/mouse. At 2,000 U/mouse, the protective effect of fibroblast interferon was just significant ($P = 0.05$), but 1,000 U/mouse did not confer significant protection. In this experiment (Fig. 4) Myocrisin treatment did not affect the course of infection: the mortality curve for the Myocrisin-treated, infected group was the same as that for the infected-only group.

The effect of Myocrisin treatment on the antiviral activity of fibroblast interferon contrasts with the virtual elimination of the antiviral effect of macrophage interferon by Myocrisin or silica treatment. As part of the experiment just described to ascertain the effect of Myocrisin on fibroblast interferon, we also examined the effect of Myocrisin treatment on the antiviral activity of macrophage interferon. The protective effect of a dose of macrophage interferon protecting all but one of 20 mice was virtually abolished

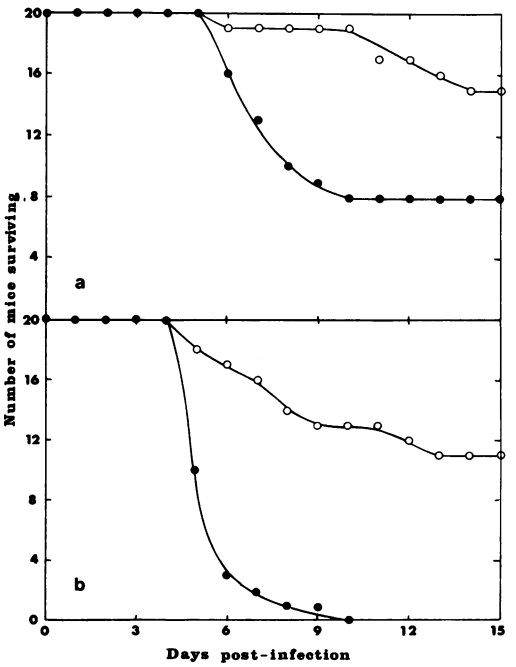

FIG. 3. *Protective effect of 250 U of macrophage interferon administered i.p. 4 h before infection of mice with (a) ca. 1 × LD_{50} and (b) ca. 20 × LD_{50} of EMC virus. Symbols:* (●) *infected only;* (○) *interferon treated and infected.*

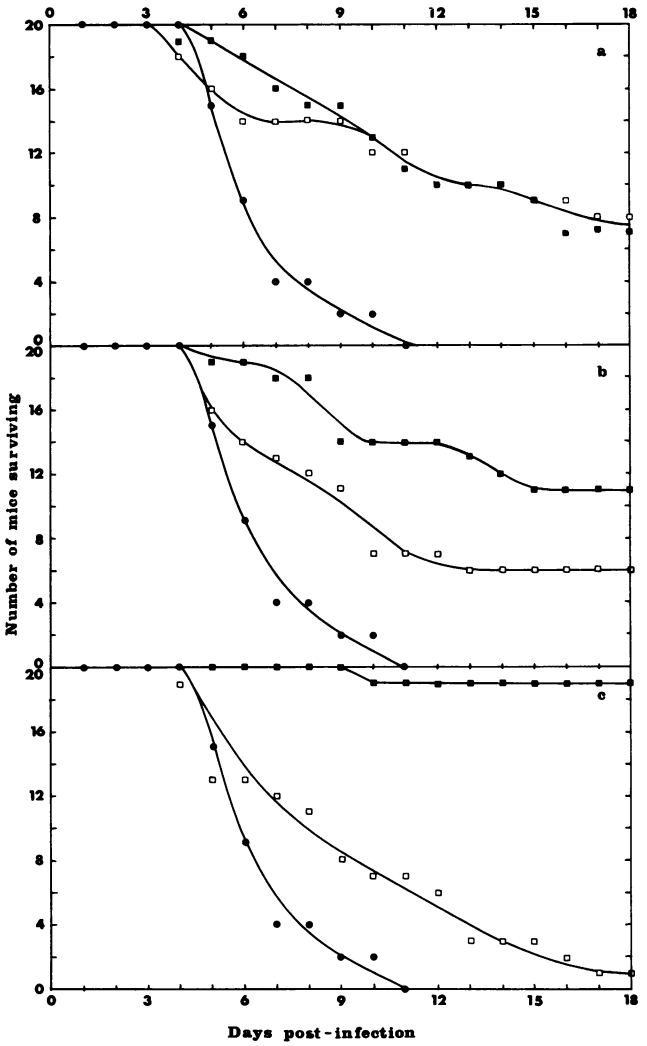

FIG. 4. *Protective effect of (a) 4,000 U of fibroblast interferon, (b) 8,000 U of fibroblast interferon, and (c) 250 U of macrophage interferon against 17 × LD_{50} of EMC virus infection of mice. Symbols (●) infected-only controls; (■) infected and treated with interferon 6 h before infection; (□) infected and treated with 8 mg of Myocrisin per mouse 24 h before infection and treated with interferon at 6 h before infection.*

by the Myocrisin treatment (log-rank χ^2 comparison between the infected control group and the group treated with macrophage interferon and Myocrisin was not significant) (Fig. 4c). It should be noted that macrophage interferon is considerably more protective than fibroblast interferon against EMC virus infection of mice (compare the top lines in Fig. 4a and c). This difference is significant (χ^2 = 15.2***) and has been consistently observed in several experiments.

DISCUSSION

The results reported here demonstrate the necessity for macrophages for poly(I:C)-induced interferon to show significant antiviral activity in mice and that production of this interferon is dependent on macrophages. It is possible that macrophages are the cells in which interferon is induced by poly(I:C) in mice, as suggested by other work (10), or that macrophages are required in some way for induction in other cells, such as lymphocytes. Since purified interferon was not used in the studies reported here, it is possible that macrophages are required for antiviral activity of factors other than interferon that may be present in our serum and medium preparations.

The results in Fig. 4 show that macrophage

interferon is considerably more effective against EMC virus infection of mice than fibroblast interferon: in this particular case the difference is 16-fold, since 250 U of macrophage interferon proved to be more effective than 4,000 U of fibroblast interferon. This unexpected difference indicates that host factors can determine the efficacy of different interferons or that assaying macrophage interferon in fibroblasts underestimates its efficacy in the whole animal. We conclude that although the mode of action of interferon and its induction are now reasonably well understood at the molecular level, the antiviral effects of interferon on cells in culture may be no guide to their efficacy in the whole animal where overriding factors exist. Moreover, although various agents may induce interferon in cells in culture, these agents may not cause interferon induction in the same cell types in the whole animal. If these observations prove to be generally true, then the choice of interferon type or inducer for treatment of a particular virus disease must take into account the particular cell types in which the virus replicates and cell populations important for the antiviral activity of interferon to be expressed in vivo.

The titration of EMC virus (LD_{50}) in mice is not greatly affected by silica or Myocrisin treatment: generally there is no effect on the titration of the virus, and the greatest increase in susceptibility was 20-fold. However, a 20-fold increase in the virus dose does not eliminate the protective effect of macrophage interferon (Fig. 3), so the elimination of the antiviral activity of this interferon by silica or Myocrisin cannot be due simply to increased susceptibility to infection. Moreover, the protective effect of fibroblast interferon is largely unaffected by macrophage suppression (Fig. 4). These observations show that antiviral treatments are not all eliminated by suppressing macrophages and that macrophages normally play only a minor role in determining the outcome of EMC virus infections of mice.

Although the outcome in terms of deaths is not greatly affected by suppressing macrophages in EMC virus-infected mice, the course of the disease is clearly different. EMC virus-infected mice generally die with obvious central nervous system involvement, but this is seldom the case in mice with suppressed macrophages: these mice die earlier, probably from more extensive systemic virus replication. A similar hastening of death of EMC virus-infected mice occurs in mice treated with anti-interferon serum, implying that interferon produced during virus replication normally delays death and alters the course of the disease (8).

The requirement for macrophages for the antiviral effect particularly of poly(I:C)-induced interferon to be manifest and the greater efficacy of macrophage interferon could be explained if EMC virus initially replicates in macrophages, as reported by others (1). However, at this time we cannot find any evidence for significant replication of EMC virus in macrophages of the mice used in the present studies, and we therefore consider such a mechanism unlikely. Macrophages are clearly important for resistance of mice to infection by several viruses, and suppressing macrophages of adult mice may render them susceptible to otherwise nonlethal viruses (9, 14, 22). A correlation has been observed between susceptibility of macrophages from different strains of mice and susceptibility of the whole animals (4, 6, 21), indicating that macrophages are the real primary target cells for these viruses, and their susceptibility determines the outcome of infection in the whole animal. However, no such correlation was found for arbovirus infections of mice (20). Our results also show no such correlation for EMC virus infection and suggest an alternative explanation of the cited correlation: the viruses that are only lethal in adult mice when their macrophages are suppressed normally cause sufficient interferon production during replication to suppress the infection, but macrophages are essential for this interferon to be effective. The correlation between susceptibility of macrophages and the whole animals from which they are derived may therefore be largely fortuitous or merely augment a relationship between the amount of interferon produced during virus replication and its ability to suppress replication of the virus in conjunction with macrophage-mediated effects.

The mechanism whereby macrophages are involved in the antiviral effects of interferon in vivo is obscure. A requirement for macrophages has also been observed for polynucleotides that are antiviral without inducing interferon (16, 17). It is likely that the requirement for macrophages does not involve mechanisms specific for interferon, and possibly no antiviral agent is effective against systemic virus infections of mice unless macrophages are present. We have previously suggested (18) that antiviral single-stranded polynucleotides that do not induce interferon require macrophages in vivo because (i) some directly antiviral macrophage activity is stimulated or an adverse activity abrogated, or (ii) protection of mice occurs only because of an additional independent macrophage-mediated mechanism such that neither mechanism alone is protective. Interferon may require macrophages in vivo for similar reasons.

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