ROLE OF INTERFERON IN THE PATHOGENESIS OF VIRUS DISEASES IN MICE AS DEMONSTRATED BY THE USE OF ANTI-INTERFERON SERUM

I. Rapid Evolution of Encephalomyocarditis Virus Infection*

By ION GRESSER, MICHAEL G. TOVEY, MARIE-THÉRÈSE BANDU, CHANTAL MAURY, AND DANIÈLE BROUTY-BOYÉ

(From the Institut de Recherches Scientifiques sur le Cancer, Villejuif, France)

The role of interferon in host resistance to viral infection has not been established. Although administration of interferon can confer a marked protection on virus-infected animals (1), the evidence that it is important in limiting virus infection has been indirect, based for the most part on temporal associations between the presence of virus and interferon in different tissues (2). Two direct means of determining the role of interferon in experimental or natural virus infections would be: (*a*) use of an animal incapable of producing interferon or insensitive to its action (such an animal has not been described); and (b) use of a selective inhibitor of interferon production or action. Metabolic inhibitors which inhibit both interferon production and action also affect many host functions and are therefore not selective. An anti-interferon antibody should however combine selectively with extracellular interferon produced by viral-infected tissues and prevent interferon from protecting uninfected cells. Fauconnier has in fact shown that inoculation of sheep anti-mouse interferon serum of low potency was associated with a more rapid onset of disease and an increased mortality in mice infected with very small amounts of Semliki Forest virus (3, 4). The development of techniques for the production of potent and partially purified mouse interferon (5) enabled us to obtain sheep anti-mouse interferon serum 1,000-fold more potent than that previously used. Administration of this serum to mice markedly altered the evolution of several virus diseases, clearly demonstrating the importance of interferon in the early response to some viral infections. We present in some detail in this article the results of our experiments using encephalomyocarditis virus and in the accompanying article the results of experiments using several other viruses.

Materials and Methods

Virus. The origin, methods of preparation, and assay of the encephalomyocarditis (EMC)[1] virus used in these experiments have been previously described (6). Mice were injected intraperitoneally (i.p.) or subcutaneously (s.c.) with 0.2 ml of the given viral dilution.

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[1] *Abbreviations used in this paper:* EMC, encephalomyocarditis; HA, hemagglutination; NDV, Newcastle disease virus; PBS, phosphate-buffered saline; poly-I·C, polyriboinosinic-polycytidylic; $TCID_{50}$, mean tissue culture infective dose; VSV, vesicular stomatitis virus.

Mice. Swiss and C3H mice from pathogen-free colonies at the Institut du Cancer were used in these experiments.

Preparation of Mouse Interferon. Mouse interferon was prepared from suspension cultures of Swiss mouse C-243 cells (5) induced with Newcastle disease virus (NDV) and was concentrated, semipurified, and assayed as previously described (5). The sp act of the mouse C-243 cell interferon preparations ranged between 2.8×10^6 and 1.2×10^7 reference units/mg protein. One of our mouse interferon units as quoted in the text equals four mouse interferon reference units.

Immunization Procedures. Sheep no. 1 was immunized by subcutaneous injection with 1 ml of mouse interferon preparations titering 1.6×10^{-5} to 8×10^{-5}. It received a total of 24 injections in the course of 13 mo, at which time the serum anti-interferon-neutralizing titer was 6.4×10^{-3}. Following techniques described by Mogensen et al. (7), the sheep was then injected twice s.c. with 8×10^6 units of mouse interferon admixed with Freund's complete adjuvant. This procedure increased the serum-neutralizing titer to 2.4×10^{-6}.

Sheep no. 4 received eight injections of mouse interferon of comparable titer over a period of 3 mo, at which time the serum anti-interferon-neutralizing titer was 2.5×10^{-1}. The serum of this sheep served as one of the control sera (see Fig. 1 B). As a further control, the serum globulin fraction of sheep (Iivar) immunized with human leukocyte interferon (7) was used, kindly provided by Dr. E. Mogensen.

Partial Purification of Antisera. For the majority of experiments anti-interferon and control sera were processed as follows: (*a*) 50 ml of each heat-inactivated (56°C for 30 min) serum were absorbed three times with a total of 9×10^9 C-243 cells. Serum-cell mixtures were incubated with agitation for 30 min at 37°C and 30 min at 4°C. (*b*) Sera were then adsorbed on a suspension of Swiss mouse spleen and thymic cells (6×10^9 cells for 50 ml of serum), for 30 min at 37°C and 30 min at 4°C. (*c*) Sera were centrifuged to eliminate cells and then ultracentrifuged at 100,000 *g* for 1 h. (*d*) The globulin fractions were precipitated with ammonium sulfate (39% saturation) and the protein content was determined by the Lowry method (8) using bovine serum albumin as a standard. The protein content of the globulin fractions prepared from the sera of sheep no. 1 was 44 mg/ml; of sheep no. 4, 62.5 mg/ml; and of a normal sheep, 32 mg/ml.

The degree of purification of the globulin fraction of the serum of sheep no. 1 was determined by passive hemagglutination (HA) on tanned sheep erythrocytes according to the method of Boyden (9). The HA titers of crude antiserum (before absorption) and of the corresponding serum globulin (after absorption) were, respectively, 1:163,840 and 1:10,240 against an extract of C-243 cells; 1:160 and <1:5 against NDV-infected allantoic fluid; and 1:81,920 and <1:5 against calf serum. The crude sheep anti-interferon serum (before absorption) had a cytotoxic titer of 1:80 for C-243 cells (in the presence of rabbit complement), whereas the globulin fraction (after absorption) exerted no toxicity at a dilution of 1:10.

No appreciable loss of anti-interferon antibody was observed after absorption and precipitation of the globulin fraction. Neither the crude serum nor the serum globulin from a normal sheep contained antibodies to the above substances.

Assay of Sera for Anti-Interferon-Neutralizing Antibody. Serial twofold dilutions of serum were incubated for 1 h at 37°C and 2 h at 4°C with 8 units of mouse C-243 cell interferon. Mixtures were then incubated with monolayer cultures of L cells for 18 h before challenge with approximately 100 mean tissue culture infective doses ($TCID_{50}$) of vesicular stomatitis virus (VSV). In each instance a simultaneous titration of the interferon used was included in the test. The neutralizing titer was taken as the highest dilution of antibody that inhibited by 50% the protective activity of at least 8 units of interferon. The anti-mouse interferon titer of serum globulin from sheep no. 1 was 1.2×10^{-6} and from sheep no. 4, 2.5×10^{-1}. The anti-human interferon titer of serum globulin from sheep Iivar was 3×10^{-5} [assayed as previously described (7)] and its anti-mouse interferon titer was 10^{-1}.

Experimental Plan. Unless otherwise indicated 1-mo-old male or female Swiss mice were inoculated in the tail vein with 0.1 ml (44 mg protein/ml) of serum globulin from sheep no. 1 or 0.14 ml (34 mg protein/ml) of serum globulin from a normal sheep, and then immediately inoculated i.p. or s.c. with the virus suspension. Mice were observed for at least 2 wk.

Determination of EMC Virus Content in Tissues of Mice. Mice were first exsanguinated and the heart, liver, spleen, kidney, and brain removed individually. A 10% wt/vol homogenate in phosphate-buffered saline (PBS) was made for each organ [except for the liver (33% wt/vol)] and then centrifuged at 500 *g* for 15 min. The virus content of the supernate was titered in monolayer cultures of L cells.

Determination of Interferon Content in Tissues of Mice. The same tissue homogenates assayed for EMC virus content were assayed for interferon after ultracentrifugation at 100,000 *g* for 1 h. The supernate was then processed as follows: One part was dialyzed at pH 2 for 2 days to inactivate residual EMC virus. Another part was not subjected to pH 2 but was incubated with a 1:50 or 1:250 dilution of mouse anti-EMC virus serum (titer 1:40,000 against 10^3 $TCID_{50}$ of EMC virus). Interferon was assayed on monolayer cultures of mouse L cells challenged with VSV (5).

Results

Inoculation of 0.1 ml of a crude sheep anti-mouse interferon serum titering 1.2×10^{-6} or the globulin fraction of this serum (see Materials and Methods) markedly altered the response of 1-mo-old Swiss mice to i.p. infection with approximately 100 LD_{50} of EMC virus. Control virus-infected mice appeared healthy until the 4th day, when they developed neurologic signs of disease. 25–50% of these mice died with signs of encephalitis on the 4th day and 50–75% were dead by the 5th day. In contrast, mice treated with anti-interferon serum appeared severely ill 24–36 h after viral infection. They remained almost immobile although neurologic signs of disease were not apparent. In most experiments virtually all of these mice were dead by the 2nd day.

Effect of Varying Amounts of Anti-Mouse Interferon Globulin on Infection of Mice with EMC Virus. Mice were infected i.p. with approximately 100 LD_{50} of EMC virus and then injected with 10-fold dilutions of anti-interferon globulin. Undiluted anti-interferon globulin (titer 1.2×10^{-6}) exerted a marked effect on EMC infection in mice (Fig. 1 A). A significant but less marked effect was observed in mice injected with anti-interferon globulin titering 1.2×10^{-5} and 1.2×10^{-4} but virtually no effect was observed in mice injected with globulin titering 1.2×10^{-3} (Fig. 1 A). In all the following experiments mice were injected intravenously (i.v.) with 0.1 ml of the anti-interferon globulin (or normal sheep globulin) diluted 1:3 in PBS to give an anti-interferon titer of 4×10^{-5}.

Effect of Different Sheep Globulin Preparations on Infection of Mice with EMC Virus. To determine the specificity of the effect of the sheep anti-mouse interferon globulin on EMC virus infection, mice were infected i.p. with approximately 100 LD_{50} of virus and then injected i.v. with 0.1 ml of either sheep anti-mouse interferon globulin (sheep no. 1) (titer of 4×10^{-5}); normal sheep serum globulin; the serum globulin of the incompletely immunized sheep (no. 4); or the serum globulin from sheep (Iivar) immunized with human leukocyte interferon. Only mice injected with potent anti-mouse interferon globulin showed the accelerated response to EMC virus infection (Fig. 1 B).

Clearance and Absence of Toxicity of Sheep Anti-Mouse Interferon Globulin in Swiss Mice. 1-mo-old Swiss mice were injected i.v. with anti-mouse interferon globulin (titer, 4×10^{-5}) and sacrificed in the ensuing days. As can be seen in Fig. 2, significant anti-mouse interferon-neutralizing titers were still detected in the serum at 25 days.

Crude anti-interferon serum or anti-interferon globulin exerted no discernible effects on uninfected mice kept for several months. Furthermore, splenic lymphocytes harvested 48 h after inoculation of sheep anti-mouse interferon globulin were as sensitive to phytohemagglutinin stimulation of tritiated thymidine incorporation as splenic lymphocytes from control mice.

Effect of Sheep Anti-Mouse Interferon Globulin on Varying Amounts of EMC

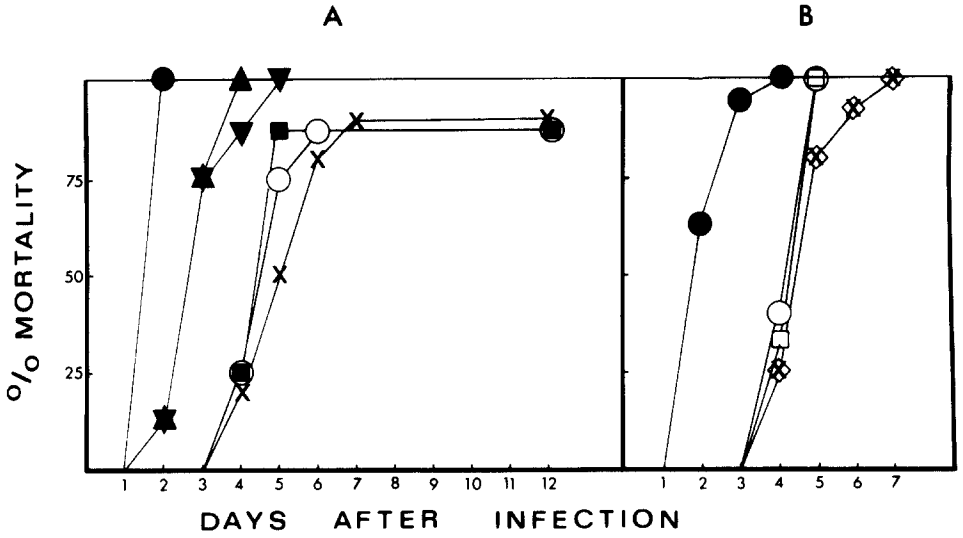

FIG. 1. (A). Effect of varying dilutions of sheep anti-mouse interferon globulin on EMC virus infection (100 LD_{50} i.p.) in 1-mo-old Swiss mice. Mice were injected i.v. with 0.1 ml of anti-mouse interferon globulin titering 1.2×10^{-6}, (●); 1.2×10^{-5}, (▲); 1.2×10^{-4}, (▼); 1.2×10^{-3}, (■); normal sheep serum globulin, (○); or left untreated, (X). There were eight mice per group. (B) Effect of different sheep globulin fractions on EMC virus infection (100 LD_{50} i.p.) in 1-mo-old Swiss mice. Mice were injected i.v. with 0.1 ml of serum globulin from sheep no. 1 (anti-mouse interferon titer, 4×10^{-5}), (●); from sheep no. 4, (□); from a normal sheep, (○); and from sheep Iivar (anti-human leukocyte interferon), (◇); or left untreated, (X). There were 15 mice per group.

Virus Inoculated i.p. or s.c. Eight experiments were undertaken to determine the effect of sheep anti-mouse interferon globulin on the evolution of EMC virus infection. Injection of anti-interferon globulin resulted in an earlier onset of disease, and at lower dilutions of virus an increased mortality (Fig. 3). This was especially marked when EMC virus was injected subcutaneously (Exp. II, Fig. 3), (the EMC virus titer was $10^{-6.5}$ LD_{50}/0.2 ml for control mice and $10^{-8.4}$ LD_{50}/0.2 ml for mice injected with anti-interferon globulin). Although most experiments were undertaken with Swiss mice, comparable results were also obtained in one experiment with C3H mice.

EMC Virus Content in Tissues of Mice Injected with Sheep Anti-Mouse Interferon Globulin. In view of the overwhelming course of EMC virus infection in mice injected with anti-interferon globulin, it was of interest to determine the amount of EMC virus in the different organs. Accordingly, 1-mo-old Swiss mice were infected with EMC virus and then inoculated i.v. with either anti-interferon globulin or normal sheep globulin. Four mice from each group were sacrificed every 12 h.

EMC virus was found earlier and in considerably greater titer in the serum, spleen, liver, kidney, and heart of mice treated with anti-mouse interferon globulin than in mice treated with normal sheep globulin (Fig. 4). Of particular interest was the finding that as early as 24 h after viral inoculation the titers of EMC virus in the livers of two anti-interferon-treated mice were $10^{-5.7}$ and $10^{-6.5}$

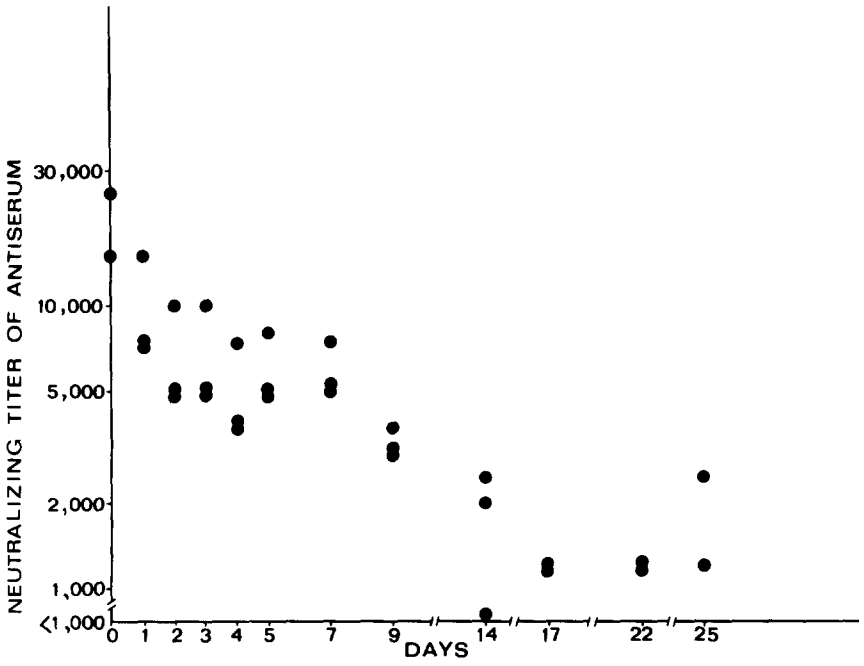

FIG. 2. Mice were injected i.v. with 0.1 ml of sheep anti-interferon globulin (anti-interferon titer, 4×10^{-5}) and at various times thereafter the serum anti-interferon-neutralizing titer was determined on individual mice (●). (No anti-mouse interferon-neutralizing activity was present in a 1:10 dilution of uninoculated mice.)

$TCID_{50}$/0.2 ml of homogenate and at 36 h the virus titers in the livers of all four mice were greater than $10^{-5.3}$ $TCID_{50}$/0.2 ml. In contrast, EMC virus multiplication was minimal or not detected at this time in the livers of mice treated with normal sheep serum globulin. In view of the clinical course of disease in antiserum-treated mice referred to above, it was of interest that at the time of death (36–48 h) the EMC virus titers in the brain were considerably below those found in control mice dying of EMC virus encephalitis (see values for the brains of control mice at 96 h) (Fig. 4).

Presence of Interferon in Tissues of Virus-Infected Mice Injected with Anti-Mouse Interferon Globulin. The same tissue homogenates assayed for EMC virus content were also assayed for the presence of interferon (with the exception of serum).

MICE INJECTED WITH NORMAL SHEEP GLOBULIN. Interferon was detected only in the brains of mice injected with normal sheep globulin. Titers of 1:60 to 1:160 were present in homogenates of brain in 2/4 mice at 72 h and in 2/3 mice at 96 h. Significant levels of interferon (>1:20) were not detected in any of the homogenates of liver, spleen, kidney, or heart from EMC virus-infected mice harvested between 12 and 96 h. (All specimens were assayed before and after treatment at pH 2, see Materials and Methods.)

MICE INJECTED WITH ANTI-INTERFERON GLOBULIN. When liver homogenates were assayed for interferon before treatment at pH 2 (but after ultracentrifuga-

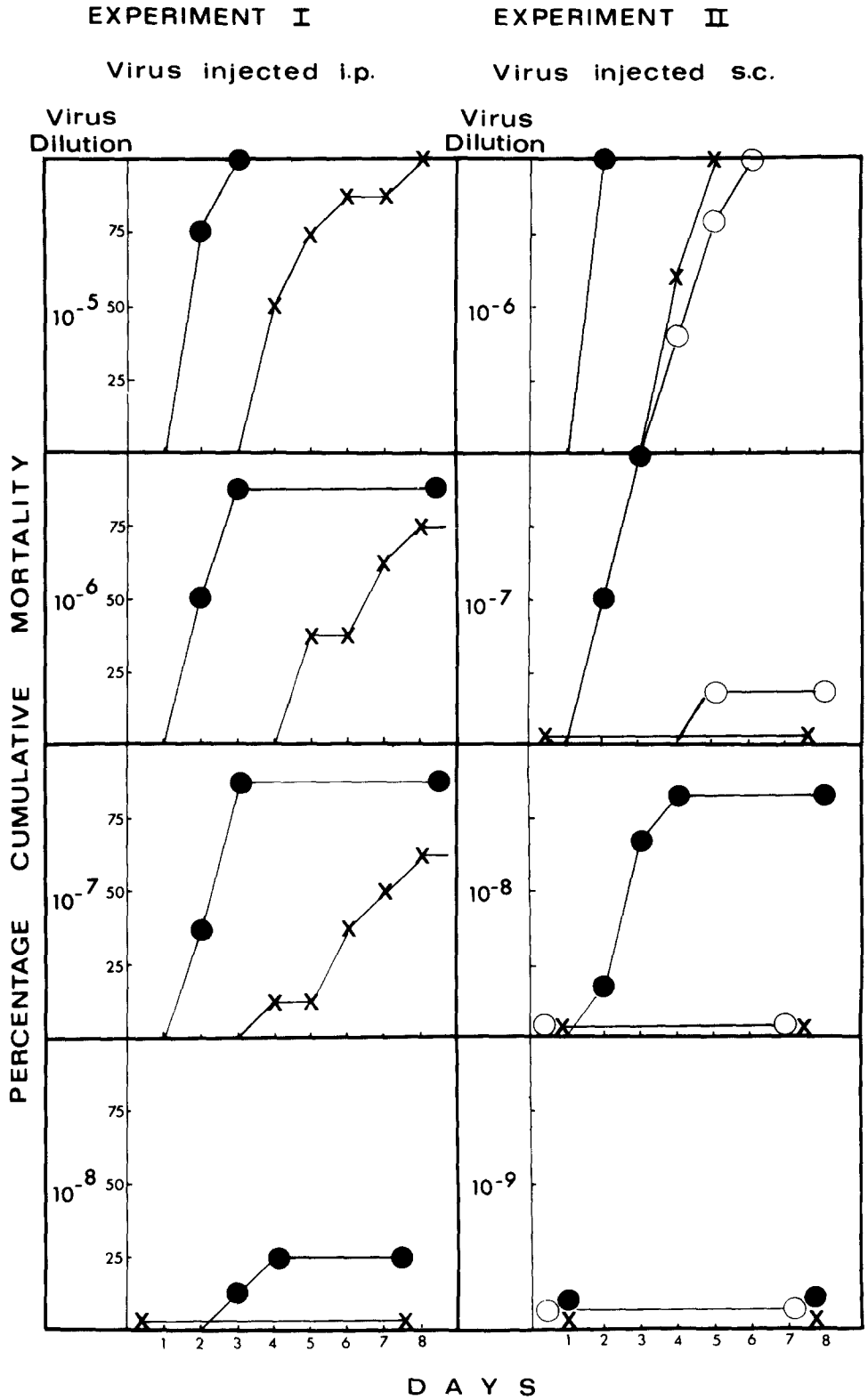

FIG. 3. 1-mo-old Swiss mice were injected i.v. with 0.1 ml of anti-interferon globulin (titer, 4×10^{-5}), (●); or with normal serum globulin, (○); or left untreated, (X). All mice were inoculated i.p. or s.c. with 0.2 ml of viral dilutions as indicated. There were eight and six mice per treatment group for each virus dilution in experiments I and II, respectively.

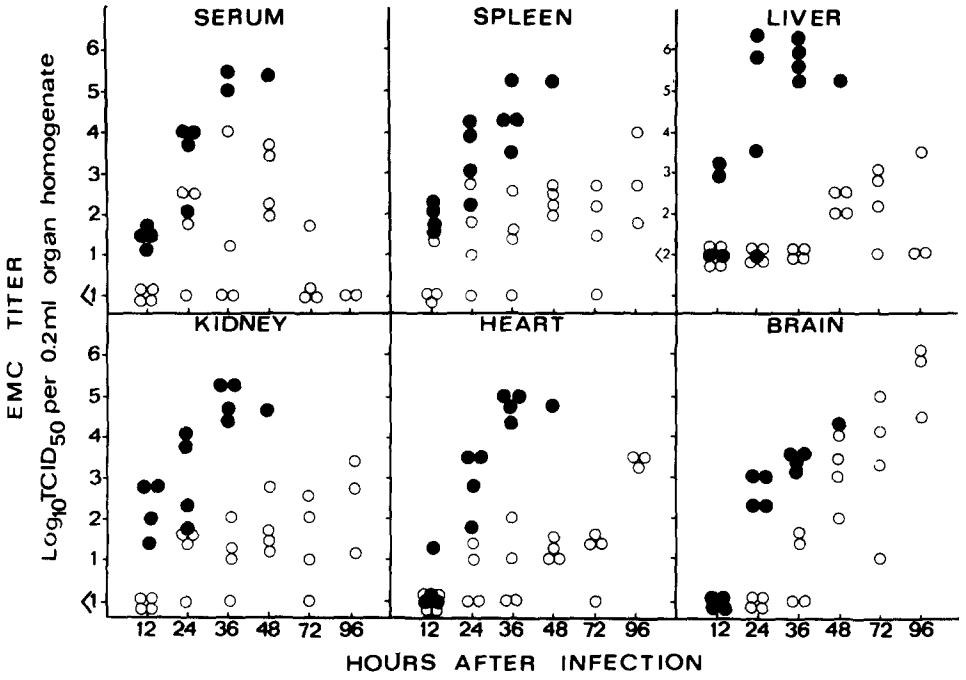

FIG. 4. EMC virus content in tissues of mice injected i.v. with 0.1 ml of anti-interferon globulin, (●) (titer, 4×10^{-5}); or normal sheep globulin, (○). All mice were inoculated i.p. with approximately 100 LD_{50} of EMC virus.

tion of the homogenate and addition of mouse anti-EMC virus serum) interferon was either not detected or the titers were low (open squares, Fig. 5). However when these same liver homogenates were assayed after treatment at pH 2, high levels of interferon were detected (closed squares, Fig. 5). Interferon was also present in the homogenates of kidney, heart, and spleen but there appeared to be little difference in titer between pre- and post-pH 2-treated samples.

The amount of interferon in a given organ (after passage of the sample at pH 2) was related to the virus titer. For example, at 24 h the EMC virus titers of the liver homogenates of two mice were $10^{-6.5}$ and $10^{-5.7}$ and the interferon titers were 1:650 and 1:80, respectively, whereas the virus titers in the livers of two other mice at this time were $10^{-3.5}$ and $<10^{-2}$ and no interferon was detected in these homogenates.[2]

The relationship between the virus titer in the liver, kidney, heart, or spleen of virus-infected mice treated with anti-mouse interferon globulin and the corresponding interferon titer (post-pH 2 treatment) in the homogenate is shown in Fig. 6. Interferon was only detected when the virus titer was 10^4 $TCID_{50}$/0.2 ml of homogenate or greater.

Lack of Toxicity for Mice of Mixtures of Mouse Interferon and Anti-Interferon

[2] Furthermore it was of interest that anti-interferon-neutralizing activity was present in pre-pH 2 homogenates of liver of these latter two mice, but could not be detected in the liver homogenates containing significant levels of interferon (two mice at 24 h, four mice at 36 h, and 1 mouse at 48 h).

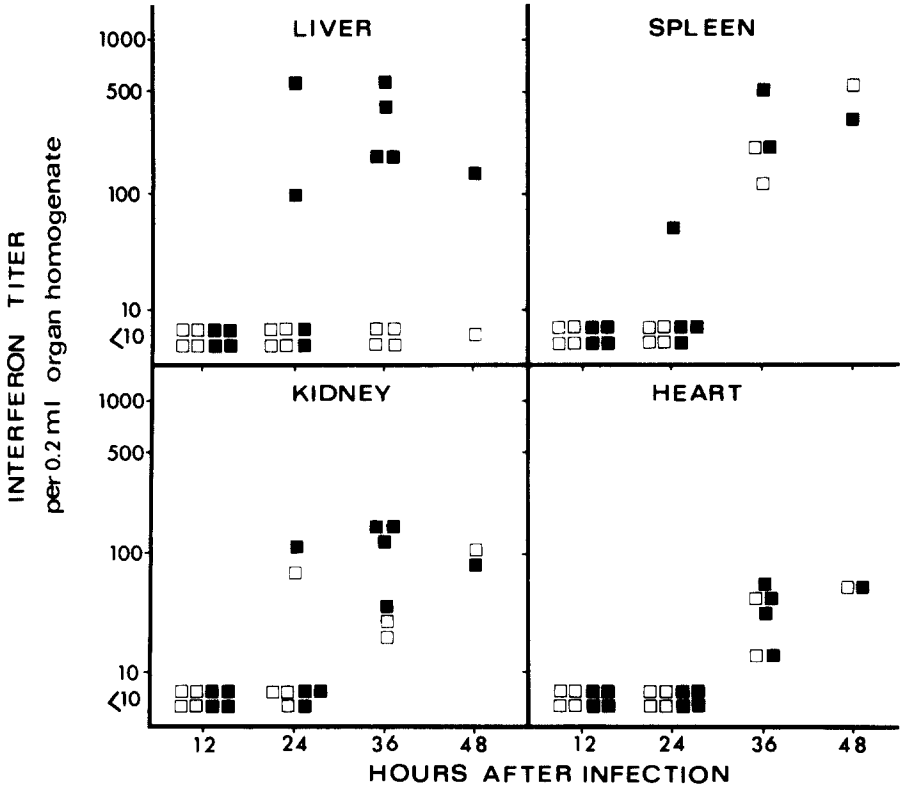

FIG. 5. Interferon content in tissues of mice inoculated with EMC virus and treated with anti-mouse interferon globulin. □, specimen tested before treatment at pH 2; ■, specimen tested after treatment at pH 2.

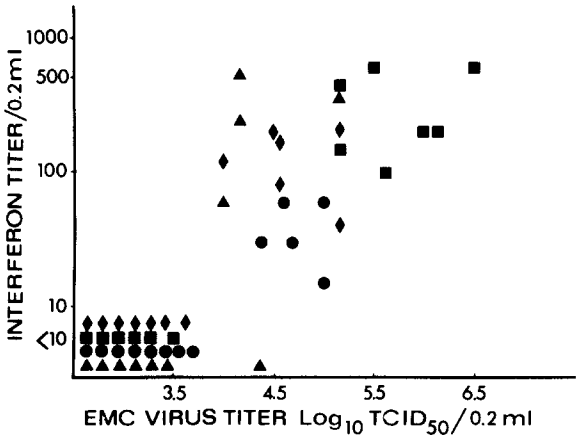

FIG. 6. Relationship between interferon content (post-pH 2) in kidney, (◆); liver, (■); heart, (●) and spleen, (▲); and EMC virus content in the corresponding tissue homogenates from mice treated with anti-mouse interferon globulin.

Globulin. Although the rapid onset of extensive viral multiplication in the organs of anti-interferon globulin-treated mice appeared responsible for the overwhelming evolution of disease and early death, it was considered of interest to determine whether interferon-anti-interferon serum complexes might exert any ill effects. Two types of experiments were undertaken:

(*a*) To induce the production of endogenous interferon, mice were injected with NDV in four experiments or with polyriboinosinic-polycytidylic acid (poly-I·C) in six experiments. Some mice were immediately inoculated with normal sheep globulin or PBS, and others were inoculated with anti-interferon globulin. Interferon was only detected in the serum of mice injected with interferon inducer and normal globulin or PBS (1:4,000 to 1:24,000 units for NDV and 1:640 to 1:1,280 units for poly I·C inoculated mice), and not in the serum (<1:50) of mice injected with inducer and anti-interferon globulin. No ill effects were noted in any of the mice over a prolonged observation period.

(*b*) 10-fold dilutions of mouse interferon were incubated with a large amount of anti-interferon globulin and 10-fold dilutions of anti-interferon globulin were incubated with a large amount of interferon. Each mixture was injected into three mice. No ill effects were noted in any of the mice during a 1 mo observation period.

Discussion

Mice infected either s.c. or i.p. with 100 LD_{50} of EMC virus die with signs of central nervous system (CNS) disease towards the 4th to 5th day. In contrast, the course of the disease was entirely different in EMC virus-infected mice inoculated with a potent sheep anti-mouse interferon globulin. Signs of disease were apparent in these mice as early as 24 h. At 36 h all mice were moribund without any obvious signs of CNS disease and at 48 h almost all mice had died. This rapid evolution of disease was never seen in untreated mice, nor in mice treated with the serum globulin from a normal sheep, nor from a sheep exhibiting a low anti-mouse interferon-neutralizing titer, nor from a sheep having a high titer of antibody to human leukocyte interferon (Fig. 1 B).

In control infected mice, EMC virus was either not detected or was present only in low titers in the serum and visceral organs, but attained high titers in the brain towards the 4th to 5th day at which time mice died with an encephalitis. In contrast, in mice treated with anti-mouse interferon globulin, EMC virus multiplied to high titer in several visceral organs (i.e., heart, liver, spleen, and kidney) 24–36 h after viral inoculation (Fig. 4). These mice appeared to die of an overwhelming systemic infection before virus had multiplied to high titer in the brain.

We infer therefore from the use of anti-interferon serum that the production of interferon in visceral organs is an important early event that inhibits viral multiplication in these organs.[3] Thus, in the anti-interferon globulin-treated mice, virus multiplies in the visceral organs and interferon is produced but can not protect neighboring cells because of the ubiquitous presence of high levels of

[3] Although we were unable to demonstrate interferon in liver, spleen, kidney, and heart homogenates of control mice in the experiment reported herein, we have been able in previous experiments with EMC virus to demonstrate its presence in very small amounts in these organs.

anti-interferon globulin (Fig. 2). Virus multiplication ensues unchecked, attaining very high titers especially in the liver (in control mice only minimal amounts of virus can be recovered from the liver).

It was of interest to note that interferon was either not detected or detected in only small amounts in crude homogenates of livers from anti-interferon globulin-treated mice. However, when these same homogenates were assayed after treatment at pH 2 it was apparent that relatively high levels of interferon were in fact present. Presumably prolonged treatment at pH 2 dissociated or destroyed the antibody-interferon complex, thus permitting the detection of the interferon present. Thus, anti-interferon globulin did not block the synthesis of interferon by viral-infected cells but rather neutralized its activity extracellularly.[4] (We have at present no explanation for the finding that no significant difference was observed in the interferon titers of pre- and post-pH 2 treatment of homogenates of other organs.)

Although it may seem a paradox that interferon was found in the organ homogenates of mice treated with anti-interferon globulin, (and not in control virus-infected mice except in the brains), this may be explained by the extent of viral multiplication in these organs. It has been reported that the amount of interferon in a given tissue is often directly related to the extent of viral multiplication (10), and our experimental results clearly support these observations (Fig. 6). Fauconnier also observed that the amount of serum interferon was greater in Semliki Forest virus-infected mice treated with anti-interferon serum than in control mice (4).

It is important to emphasize that neither the crude sheep anti-mouse interferon serum nor the globulin fraction prepared after absorption of this serum on known contaminants (of the interferon preparations used to immunize the sheep) exerted any ill effects in mice kept for extended periods of time. Furthermore, all our results indicate that the anti-mouse interferon serum acts in vivo by neutralizing interferon. We have no evidence that complexes of interferon-anti-interferon antibody contribute in any way to the fulminating disease observed. Thus mixture in different proportions of interferon and antibody exerted no discernible effect on mice. Likewise, large amounts of endogenous interferon were induced in NDV- or poly-I·C-inoculated mice and no ill effects were observed when this interferon was neutralized in vivo by injection of anti-interferon globulin. We conclude therefore that the sheep anti-interferon globulin used acts selectively to neutralize extracellular interferon, and that the overwhelming disease in these EMC virus-infected mice attests to the importance of interferon in limiting viral multiplication and delaying the evolution of this experimental viral disease.

Summary

The role of interferon in the pathogenesis of encephalomyocarditis (EMC) virus infection was determined by treating mice with potent, partially purified

[4] The efficacy of our anti-interferon serum in neutralizing large amounts of endogenous interferon was further emphasized by the apparent absence of significant levels of interferon in the serum of anti-interferon globulin-treated mice injected with NDV or poly-I·C in contrast to the high levels of serum interferon found in control mice inoculated with these inducers.

sheep anti-mouse interferon globulin. In control mice, EMC virus was present in low titers in various visceral organs but attained high titers in the brain towards the 4th to 5th day, at which time mice died with signs of central nervous system disease. In mice treated with anti-mouse interferon globulin, virus was present in high titer in visceral organs 24–36 h after viral inoculation and virtually all mice were dead by 48 h. This rapid evolution of EMC virus infection was not observed in mice treated with the globulin fraction prepared from a normal sheep, from a sheep exhibiting a low anti-mouse interferon-neutralizing titer, nor from a sheep having a high titer of antibody to human leukocyte interferon. The experimental results indicated that anti-interferon globulin neutralized the interferon liberated by virus-infected cells, thus permitting extensive virus multiplication in several visceral organs. We conclude that interferon is an important early component of host resistance to this virus infection.

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