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Interferon Independence of Genetically Controlled Resistance to Flaviviruses

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Flavivirus-resistant C3H/RV mice injected with sheep anti-interferon globulin and then infected with either West Nile or yellow fever virus survived and displayed no disease symptoms. Also, treatment of embryo fibroblast cultures prepared from C3H/RV or congenic susceptible C3H/HE mice with anti-interferon serum resulted in an increased yield of West Nile virus from both types of cultures, but the amount of infectious virus produced by resistant cultures remained 1 to 1.5 logs lower than that produced by susceptible cell cultures. These results indicate that the mode of expression of the flavivirus resistance gene differs significantly from that of the *Mx* gene conferring resistance to influenza virus-induced disease in A2G mice.

A murine gene has been demonstrated which codes for resistance to flavivirus-induced encephalitis. Resistance is inherited as a simple autosomal dominant allele (13, 15, 18). The resistance allele has been identified in the BRVR, BSVR (18), and PRI (15) mouse strains and was introduced into C3H/HE mice from PRI to form the congenic C3H/RV strain (8). Mice that inherit the resistance allele support the replication of flaviviruses, but virus yields from their tissues are 2 or more logs lower, and the spread of the infection is slower than in congenic susceptible mice (5, 6, 11). Also, cell cultures derived from various tissues obtained from resistant (C3H/RV) mice produce lower yields of flaviviruses than do comparable cultures of cells from congenic susceptible (C3H/HE) animals (4, 19). Flavivirus adsorption and penetration apparently occur equally well in resistant and susceptible cells, since the same percentage of cells show virus-positive immunofluorescence in both types of cultures by 6 to 8 h after infection (4). A previous report by Hanson et al. (11) indicated that mouse interferon produced an enhanced inhibition of flavivirus replication in both C3H/RV animals and cell cultures.

A number of other murine genes have been identified which specifically affect the expression of disease caused by different types of RNA and DNA viruses (2, 3a, 14). One of these, the autosomal, dominant locus, *Mx*, determines resistance of adult A2G mice to orthomyxoviruses (12). Recently, it was shown that treatment of adult A2G mice with sheep anti-mouse interferon globulin caused these mice to become phenotypically susceptible to influenza infection (9). In support of a specific involvement of interferon in the expression of resistance to influenza infection, interferon treatment of newborn A2G mice that are susceptible to the lethal effect of an influenza infection was found to protect these mice more efficiently than comparable A/J mice that lack the *Mx* allele. Also, cells isolated from A2G mice are permissive for orthomyxovirus replication, and less interferon was required to render these cell cultures resistant to influenza infection than was required for susceptible A/J cultures (1, 10). Both in vitro and in vivo, the enhanced effect of interferon was observed only against influenza infections. These data indicate that resistance to orthomyxoviruses correlates with a virus-specific genetic control of sensitivity to interferon action (10).

Since treatment of flavivirus-resistant C3H/RV animals or cell cultures with mouse interferon had similarly been reported to cause a greater suppression in the yield of flaviviruses (5, 7), it was of interest to further investigate the specific involvement of interferon in the expression of flavivirus resistance through the use of antibody to interferon.

MATERIALS AND METHODS

The congenic mouse strains of flavivirus-resistant C3H/RV and flavivirus-susceptible C3H/HE are maintained as inbred colonies at the Wistar Institute. Adult animals of both sexes were used for experiments. Embryo fibroblast (EFB) cultures were prepared from 14- to 18-day-old embryos obtained from pregnant C3H/RV or C3H/HE mice. EFB cultures were propagated in Eagle minimal essential medium supplemented with glutamine (0.29 mg/ml), gentamicin (50 mg/ml), and 8% (vol/vol) fetal calf serum and incubated in a

humidified atmosphere of 5% CO_2 in air at 37°C. Cells were used for experiments after the first or second subculture. On one occasion, C3H/RV and C3H/HE EFB cultures were infected with simian virus 40, and transformed cells were selected during subsequent subculture and cloned. The C3H/HE and C3H/RV cell lines thus generated have been continuously subcultured for 3 years, are morphologically similar, and have retained their differing capacities to produce infectious flaviviruses (4).

A 10% (wt/vol) homogenate of suckling mouse brains infected with plaque-purified West Nile virus (WNV), strain E101, was prepared. The titer of this pool was 2.5×10^8 PFU/ml. The 17D vaccine strain of yellow fever virus (YFV), propagated in chicken embryos, was obtained from Connaught Laboratories, Inc., Swiftwater, Pa., and contained 2×10^5 PFU/ml. Infectivity titers of WNV and YFV were determined by plaque assay on confluent monolayers of BHK-21/WI2 (16) cells in six-well Linbro plates as described previously (3). Plates were incubated at 37°C, and plaques were visualized 2 to 3 days later after addition of a second overlay of an agarose solution supplemented with neutral red (0.1 mg/ml). Infectivity titers were calculated as the average number of plaques from duplicate endpoint wells.

Sheep anti-mouse interferon globulin (from sheep no. 1) was kindly provided by Ion Gresser (7). The neutralizing titer of this antibody preparation was 1.2×10^6 against 8 U of mouse interferon (type 1) induced by Newcastle disease virus in mouse C-243 cells. Normal sheep serum globulin was also provided by Ion Gresser. For in vivo experiments, globulin preparations were diluted 1:3 in phosphate-buffered saline. Mice were injected with 0.1 ml intravenously just before virus challenge. For tissue culture experiments, the globulin preparations were diluted 10^{-3} in culture media. Rabbit anti-mouse interferon globulin was obtained from the National Institute of Allergy and Infectious Diseases Antiviral Substances Program. The neutralizing titer of this antibody was 6,000 against 10 U of mouse interferon. For tissue culture experiments, the rabbit antibody was diluted 1:500 with culture media. Mouse reference interferon was provided by the Research Resources Branch of the National Institute of Allergy and Infectious Diseases. Interferon titers were assayed by a cytopathic effect inhibition assay, using encephalomyocarditis virus and LF-2 cells.

RESULTS

C3H/RV mice developed no disease symptoms after an intracerebral injection of YFV (6×10^3 PFU), whereas 100% of C3H/HE mice developed hind limb paralysis and died after an intracerebral injection of 600 PFU of YFV. The effect of antibody to mouse interferon on the induction of disease by YFV was investigated in C3H/HE and C3H/RV mice. Groups of 10 C3H/RV and 10 C3H/HE mice were injected intravenously with sheep antibody to mouse interferon or normal sheep serum globulin. Immediately thereafter, all mice received an intracerebral injection of YFV (6×10^3 PFU). Four mice in each group were observed daily during the next 3 weeks for disease symptoms (Table 1). Antibody to mouse interferon was found not to alter the phenotypic expression of flavivirus resistance in the C3H/RV mice.

The replication of YFV in brain tissue was monitored to determine whether the presence of antibody to mouse interferon allowed an increased level of virus replication. Two YFV-infected C3H/HE mice injected with normal sheep globulin and two infected C3H/HE mice injected with antibody to mouse interferon were killed on the 3rd, 6th, and 9th days after infection. Likewise, two infected C3H/RV mice treated with normal serum globulin and two treated with antibody to mouse interferon were killed on the 6th, 9th, and 12th days after virus infection. The brains of these mice were removed immediately and frozen at −70°C, and subsequently infectivity titers were determined (Table 2). Virus titers in brain tissues obtained from C3H/RV mice were consistently 4 to 5 logs lower than those from the brain tissues obtained from C3H/HE mice, whether or not antibody to mouse interferon had been given. However, titers in brain tissues from both C3H/HE and C3H/RV mice treated with antibody to mouse interferon were slightly higher than those for control animals treated with normal sheep globulin.

Although the 17D strain of YFV caused no disease in susceptible C3H/HE mice when injected by the intraperitoneal route, the more virulent flavivirus, WNV (strain E101), killed C3H/HE mice after inoculation by this route. The intraperitoneal 50% lethal dose of WNV for C3H/HE mice was found to be $10^{7.5}$ PFU. In contrast, none of the C3H/RV mice developed disease symptoms after an intraperitoneal injection of $10^{8.5}$ PFU of WNV. The ability of antibody to mouse interferon to enhance disease induction after a sublethal peripheral WNV in-

TABLE 1. Effect of antibody to mouse interferon on an intracerebral YFV infection in C3H/HE and C3H/RV mice

Mouse strain	Treatment[a]	Mortality	
		No.[b]	Mean day of death
C3H/HE	NSG	4	12
	AIF	4	13.5
C3H/RV	NSG	0	
	AIF	0	

[a] Sheep normal serum globulin (NSG) or sheep anti-mouse interferon globulin (AIF) was diluted 1:3 with phosphate-buffered saline, and 0.1 ml was injected intravenously just before virus was injected.

[b] Mice (four per group) were injected intracerebrally with 6×10^3 PFU of YFV (strain 17D) reconstituted in phosphate-buffered saline. Mice were observed daily for 21 days.

TABLE 2. YFV titer in brains of C3H/HE and C3H/RV mice

Mouse strain	Treatment[a]	Virus titer (PFU/brain) on given day after infection[b]			
		3	6	9	12
C3H/HE	NSG	2×10^2	3.6×10^5	2.5×10^5	
		3×10^2	4.3×10^5	4.1×10^5	
	AIF	6.5×10^2	5.4×10^5	2.2×10^6	
		8.6×10^2	7.5×10^5	5.2×10^6	
C3H/RV	NSG		4.0×10^1	1.0×10^1	1.5×10^2
			5.0×10^1	2.0×10^1	3.1×10^2
	AIF		7.0×10^1	1.5×10^1	5.5×10^2
			6.0×10^1	4.0×10^1	7.8×10^2

[a] See Table 1.

[b] Two mice were killed at each time indicated, and their brains were removed and frozen at −70°C. Subsequently, each brain was homogenized separately, and a 10% (wt/vol) clarified suspension was titrated for YFV infectivity by plaque assay.

fection was investigated. Groups of four C3H/HE and four C3H/RV mice received either normal sheep globulin or antibody to mouse interferon by intravenous injection. All mice were then infected with 10^7 PFU of WNV by the intraperitoneal route. These mice were observed daily for the development of disease symptoms. No disease symptoms were observed to develop in the C3H/RV mice given either normal sheep globulin or sheep antibody to mouse interferon before virus infection. In contrast, although C3H/HE mice given normal sheep globulin did not become sick, three of the four C3H/HE mice given antibody to interferon developed paralysis and died. The mean day of death of these mice was 14 days after infection.

Cell cultures prepared from tissues obtained from C3H/RV mice produce 1 to 2 logs less virus when infected with a flavivirus than do comparable cultures of cells from congenic C3H/HE mice (4). The effect of antibody to mouse interferon on WNV replication in these cells was investigated. The media of confluent cultures of simian virus 40-transformed C3H/HE and C3H/RV EFB were supplemented with either normal sheep serum globulin or sheep antibody to mouse interferon. Once added, these globulins were maintained in the culture fluids through the remainder of the experiment. Twenty-four hours later, the cultures were infected with WNV at a multiplicity of infection of 20. Samples (0.3 ml) were removed at the indicated times after infection for titration of infectivity, and an equal volume of fresh medium was replaced. Virus titers were observed to be approximately 1 to 1.5 logs higher in cultures treated with antibody to interferon (Fig. 1). However, C3H/RV cultures treated with antibody to interferon produced less WNV than did antibody-treated C3H/HE cultures.

The effect of antibody to mouse interferon produced in rabbits on the replication of WNV in primary cultures of C3H/RV and C3H/HE EFB was examined. Confluent cultures were incubated with the rabbit antibody beginning 24 h before infection with WNV at a multiplicity of infection of 10. At 36 h, infectivity titers in C3H/RV culture fluids were 1.8×10^5 PFU/ml if the culture had been treated with antibody to interferon and 3.6×10^4 PFU/ml in control C3H/RV cultures. At the same time, the titers in compa-

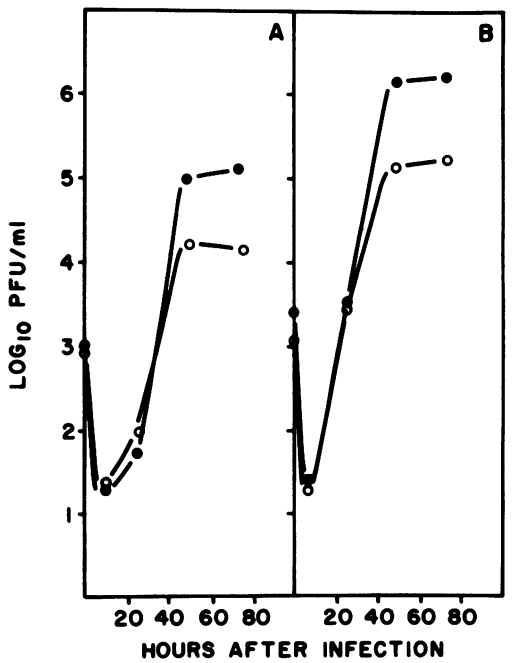

FIG. 1. Effect of sheep anti-mouse interferon globulin on the growth of WNV in (A) resistant C3H/RV and (B) susceptible C3H/HE cultures. Anti-interferon globulin (●) or normal serum globulin (○) was added to culture fluids 24 h before infection with WNV at a multiplicity of infection of 10.

rable C3H/HE culture fluids were 8.2×10^6 PFU/ml after antibody to interferon treatment and 2.5×10^6 PFU/ml in control C3H/HE cultures.

Culture fluids harvested 72 h after virus infection were dialyzed against 0.1 M NaCl (pH 2.0) for 5 days at 4°C, neutralized to pH 7.2, and assayed for interferon levels by an encephalomyocarditis virus cytopathic effect inhibition assay. The interferon titer in the fluid from the infected C3H/RV cultures was 20 U/ml, whereas that in the fluid from the C3H/HE culture was 80 U/ml. In fluids from C3H/RV and C3H/HE cultures treated with rabbit antibody to mouse interferon, interferon levels were found to be less than 10 U/ml.

Analysis of intracellular WNV RNA synthesis has indicated that the incorporation of [^{3}H]uridine into 40S genome RNA is less efficient in C3H/RV cells than in C3H/HE cells (3). The effect of antibody to mouse interferon on WNV RNA synthesis was assessed. Confluent cultures of primary C3H/RV and C3H/HE EFB were supplemented with rabbit antibody to mouse interferon 24 h before infection with WNV at a multiplicity of infection of 10. Once added, the rabbit antibody was maintained in the cultures throughout the experiment. Actinomycin D (2 μg/ml) was added to culture fluids at 23 h after infection, and [^{3}H]uridine (20 μCi/ml) was added at 24 h. After a labeling period of 1.5 h, control and antibody-treated infected cells were lysed with 10 mM Tris-hydrochloride (pH 7.2) containing 1% (wt/vol) sodium dodecyl sulfate, vanadyl ribonucleoside complex (2 mM; Bethesda Research Laboratories, Rockville, Md.), and 20 μg of self-digested pronase per ml (Sigma Chemical Co., St. Louis, Mo.). The extracts were then phenol extracted, ethanol precipitated, and centrifuged on linear 15 to 30% sucrose density gradients. After treatment with rabbit antibody to mouse interferon, incorporation of [^{3}H]uridine into 40S RNA was observed to increase by about 2- to 2.5-fold in C3H/RV cells and 1.2- to 1.5-fold in C3H/HE cultures. However, the total amount of label incorporated into 40S genome RNA in C3H/RV cultures remained three- to sixfold lower than in C3H/HE cultures (data not shown).

DISCUSSION

The identification of a number of genes controlling specific virus infections in laboratory mice (2, 3a, 14), as well as the finding of similar genes in some wild mouse populations (5), indicates the importance of such genetic effects. Since different classes of viruses vary considerably in their modes of replication, it would be expected a priori that the gene products of the various resistance genes would also differ in their mechanisms of action. Host resistance genes may act on a particular virus infection at the level of intrinsic susceptibility of the target cell, interferon inhibition, or the immune response. Although a host resistance gene is an important factor in determining the outcome of a particular virus infection, the severity of a virus-induced disease is the result of the interaction between host resistance genes, viral virulence genes, and the host defense systems.

The mechanism involved in the expression of the flavivirus resistance gene is not understood. It had been suggested that interferon might be specifically involved in the limitation of flavivirus replication in resistant C3H/RV animals and cell cultures (11). To determine whether interferon was a necessary component in the expression of flavivirus genetic resistance, the effect of pretreatment of resistant mice and cell cultures with anti-interferon globulin was investigated. In the case of genetically determined resistance to orthomyxoviruses, anti-interferon globulin rendered resistant A2G mice fully susceptible to the lethal effects of an influenza infection (9). Also, A2G mice treated with anti-interferon antibody displayed an increased production of virus, so that virus titers produced in resistant animals were comparable to those observed in susceptible A/J mice. The same preparation of sheep anti-interferon globulin and protocol for injection as used in the A2G studies (9) were also used for the experiments described here. In contrast to what was observed with myxovirus-resistant A2G mice, anti-interferon globulin did not cause flavivirus-resistant C3H/RV mice to develop paralysis or die after either a YFV or a WNV infection. Furthermore, a slight increase in virus production was observed in both resistant C3H/RV and susceptible C3H/HE animals and cell cultures after treatment with anti-interferon serum, but virus titers produced by resistant cells and animals remained lower than those from susceptible ones.

The dosage of sheep antibody to mouse interferon used in this study was identical to that used to successfully abrogate the expression of resistance to influenza virus challenge in A2G mice (9). This small amount of antibody was effective after intracerebral challenge with neurotropic influenza, intranasal challenge with pneumotropic influenza, and intraperitoneal challenge with hepatotropic influenza virus. In contrast, flavivirus-resistant C3H/RV mice after treatment with this antibody continued to express resistance to WNV and YFV to the same degree as untreated control mice. The enhancement of the lethality of an intraperitoneal WNV infection in C3H/HE mice by antibody to interferon indicated that this antibody could affect

flavivirus-induced disease. The number of animals used in these in vivo studies was small due to the paucity of the sheep (no. 1) antibody preparation. Nevertheless, treatment of C3H/RV mice with antibody to mouse interferon was consistently found to have no effect on the expression of resistance in vivo. Similarly, neither sheep nor rabbit anti-mouse interferon globulin preparations were capable of stimulating WNV production in C3H/RV cells to the extent that it became comparable to that produced by C3H/HE cultures.

These data indicate that virus-induced interferon is not an essential factor in the expression of flavivirus genetic resistance. However, our results provide no data on other types of interferon that are not neutralized by the anti-interferon sera used, such as immune or gamma interferon. The effect of interferon on flavivirus replication in resistant C3H/RV cells appears to be similar to that in C3H/HE cells. The apparent differential interferon inhibition of flavivirus replication in resistant and susceptible cells (4, 7) may occur because the interferon-mediated inhibition synergizes with the restriction already imposed on an intracellular step in flavivirus replication by the resistance gene product. Recent data (3) indicate that resistant cells alter the amount and size of the progeny flavivirus RNA synthesized, which results in the release of less infectious virus. Also, resistant cells appear to be more resistant to cytopathic effects caused by flavivirus replication (3).

Adult A/J mice display inborn resistance to infection with mouse hepatitis virus, type 3. As in the case of flavivirus resistance, the injection of anti-interferon globulin did not affect the expression of resistance to virus infection in A/J mice (17). It therefore appears that the specific involvement of interferon in the expression of genetically controlled resistance to orthomyxovirus infection is unique and that interferon serves only as a contributory defense mechanism in the expression of the genes controlling resistance to flavivirus- and type 3 mouse hepatitis virus-induced lesions.

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