

# GENETICALLY DETERMINED, INTERFERON-DEPENDENT RESISTANCE TO INFLUENZA VIRUS IN MICE\*

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We have previously described the genetically determined resistance to several orthomyxoviruses in mice carrying a dominant gene, *Mx* (1). Resistance was also expressed in in vitro cultures of the resistant mouse peritoneal macrophages (2). Several attempts to overcome resistance in vivo or in vitro have failed; these included whole body x-irradiation, neonatal thymectomy, treatments with cortisone, cyclophosphamide, methotrexate, thorotrast, silica (3, 4), and radioactive strontium (O. Haller, unpublished observations), as well as introduction of the gene *Mx* into athymic mice (5). The availability of potent antiserum prepared against partially purified mouse interferon (6) permitted us to determine the role of interferon in this resistance. A priori, this did not seem likely because resistance was highly specific for a group of closely related viruses, whereas interferon is not thought to be virus specific. Nevertheless, the use of anti-interferon serum had proven useful in showing that interferon was part of the resistance of C3H and A/J mice to mouse hepatitis virus-3 (7). In the experiments to be presented herein, it was found that small amounts of sheep anti-mouse interferon globulin (AIF)<sup>1</sup> rendered genetically resistant mice fully susceptible to the lethal effect of influenza virus, and virus titers in such mice reached levels similar to those observed in genetically susceptible animals. We conclude therefore, that interferon is an important component of the genetically determined resistance to influenza virus in some strains of mice.

## Materials and Methods

*Mice.* A/J mice were purchased from Gl. Bomholtgård Ltd., Ry, Denmark. Inbred A2G mice (genetically *Mx/Mx*) (1) and crosses (A2G × A/J)F<sub>1</sub> were bred locally as described (2). Adult animals of both sexes were used.

*Viruses.* The following avian influenza A virus strains derived from A/Turkey/England/63 (Hav1, Nav3; Langham strain) (8) were used: (a) *M-TUR* was adapted to grow in mouse

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<sup>1</sup> *Abbreviations used in this paper:* ACN, globulin directed against contaminants of mouse interferon; AHI, anti-human interferon globulin; AIF, anti-mouse interferon globulin; CPE, cytopathic effect; FCS, fetal calf serum; HA, hemagglutination; HAI, hemagglutination inhibition; HKH, influenza virus A/Hong Kong/1/68; i.c., intracerebral; i.n., intranasal; i.p., intraperitoneal; i.v., intravenous; LD<sub>50</sub>, mean lethal dose; M-TUR, macrophage adapted influenza virus A/Turkey/England/63; NSG, normal sheep serum globulin; PBS, phosphate buffered saline; TCID<sub>50</sub>, mean tissue culture infective dose; TURH, hepatotropic influenza virus A/Turkey/England/63.

peritoneal macrophages in vitro (2). Stock virus grown in 10-d-old embryonated eggs had a hemagglutination (HA) titer of 1:640 and contained  $10^8$  mean tissue culture infective doses (TCID<sub>50</sub>)/ml as determined by titrations in susceptible A/J macrophages. (b) *TURH* was a hepatotropic variant causing acute liver necrosis and death within 3–4 d in normal adult mice (9). Egg-grown stocks with a HA titer of 1:16 contained  $10^{6.7}$  mean lethal doses (LD<sub>50</sub>)/ml as estimated by intraperitoneal (i.p.) titration in susceptible A/J mice. The Stuart-Harris strain of neurotropic influenza A virus, *NWS* (H<sub>0</sub> N<sub>1</sub>) (10), was used as a 10% A/J brain extract (2). The human influenza strain *HKH*, A/Hong-Kong/1/68 (H<sub>3</sub> N<sub>2</sub>), had undergone 132 passages in mouse lung and was kept as 10% A/J lung extract (11).

*Virus Titrations.* These were done by standard procedures (1). Infectivity of *TURH* was titrated by intra-allantoic inoculation of serial 10-fold virus dilutions into 10-d-old embryonated eggs or by i.p. titrations in adult A/J mice. M-TUR was titrated in cultured A/J macrophages. *HKH* was assayed by intranasal (i.n.), *NWS* by intracerebral (i.c.) titrations in adult A/J mice. Hemagglutinin and hemagglutination inhibition (HAI) titers were measured by the pattern method in World Health Organization hemagglutination trays. Standard rabbit antisera prepared several years earlier against *HKH*, *NWS*, or A/Turkey/England/63 were repeatedly used in HAI tests to check the identity of the viruses.

*Media.* Culture medium consisted of RPMI 1640 with L-glutamine (Gibco, Biocult, Glasgow, Scotland) supplemented with 30% or 15% fetal calf serum (FCS) (Gibco, batch selected for low content of virus inhibitors), penicillin (100 U/ml), and streptomycin (100 µg/ml). Phosphate-buffered saline (PBS) contained 8.0 g NaCl, 2.7 g Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O and 0.4 g K<sub>2</sub>PO<sub>4</sub>/liter.

*Macrophage Cultures.* These were prepared as previously described in detail (2). Briefly, peritoneal macrophages from mice stimulated 3 d previously by 2-ml i.p. injections of thioglycollate medium (Difco Laboratories, Detroit, Mich.) were harvested, washed, and counted in a hemocytometer.  $5 \times 10^6$  viable cells in 2 ml of culture medium containing 30% FCS were plated into 35-mm diameter wells of FB-6-TC disposable six-well tissue culture trays (Linbro Chemical Co., Hamden, Conn.). After 3–4 h of incubation at 37°C in a moist atmosphere of 5% CO<sub>2</sub> in air, all cells not firmly attached to the plastic bottom of the wells were resuspended by repeated pipetting and were discarded. 2 ml of fresh culture medium supplemented with 30% FCS were added to the adherent cells which were further incubated for 16–18 h. Adherent cells were then washed again and refed with 1.8 ml of fresh medium containing 15% FCS; they were now ready for treatment with globulin preparations and/or for virus challenge.

*Virus Challenge of Macrophages.* 0.2 ml of 10-fold dilutions of M-TUR in culture medium containing 15% FCS were added to each well to give the desired virus input dose as stated in the text. At various times thereafter, the wells were inspected with an inverse phase-contrast microscope (× 40 objective). The occurrence of a cytopathic effect (CPE) was scored by comparison with uninfected control wells. At the same time, 0.25 ml or 0.5 ml of culture supernate were removed from individual wells and titrated for hemagglutinin. In general, no measurable hemagglutinin was detected in cultures not showing CPE.

*Virus Challenge of Experimental Animals.* *TURH:* 0.5 ml of virus dilutions in PBS were inoculated i.p. Deaths due to liver necrosis (9) were recorded. Blood and livers from infected animals were collected and titrated for *TURH* infectivity as previously described (9). *NWS:* 0.03 ml of A/J brain extract diluted in PBS to contain  $10^3$  LD<sub>50</sub> were inoculated i.c. into mice under ether anesthesia. Animals were observed for 14 d. Deaths occurring from the 3rd to the 8th d after inoculation were recorded as the result of neurotropic influenza infection (2). *HKH:* 0.1 ml of serial 10-fold virus dilutions in PBS were inoculated dropwise i.n. into mice, anesthetized with ether as described (1). Deaths occurring within 24 h of inoculation were discounted. Inspection was carried out daily thereafter. On day 14, all surviving mice were killed and the degree of gross pulmonary consolidation was scored.

*Histology.* Liver sections were fixed in 10% neutral formalin. Routine paraffin sections were prepared and stained with hematoxylin and eosin (HE).

*Serum Globulin Preparations.* The methods of preparation, partial purification and assay have been previously described (6). The following globulin preparations were used: (a) AIF from sheep No. 1. Its neutralizing titer was  $1.2 \times 10^{-6}$  against 8 U of mouse interferon induced by Newcastle disease virus in mouse C-243 cells. (b) Normal sheep serum globulin (NSG). (c) Anti-human interferon globulin (AHI) from a sheep immunized with human leukocyte interferon

(12). The human-interferon neutralizing titer was  $3 \times 10^{-6}$  and its mouse-interferon neutralizing titer was  $1 \times 10^{-1}$ . (d) The globulin fraction (ACN) from a sheep No. 11 immunized with contaminants present in the partially purified mouse interferon preparations. It did not neutralize mouse interferon at  $1 \times 10^{-1}$ . For the in vivo experiments, globulin preparations were diluted 1:3 in PBS. Mice were injected once i.v. with 0.1 ml immediately before virus challenge unless stated otherwise in the text. For the in vitro experiments, the various globulin preparations were diluted in culture medium containing 15% FCS to give the desired concentrations as stated in the text.

*Interferon.* Mouse interferon was assayed by inhibition of CPE on mouse L cells infected with vesicular stomatitis virus (13).

## Results

*Effect of AIF on the Course of Myxovirus Hepatitis in Mx-Bearing Mice.* TURH is a hepatotropic influenza virus strain causing liver necrosis and death within 3–4 d in susceptible adult mice (9). We have previously shown that inborn resistance of *Mx*-carrying mice towards lethal infection with TURH virus could not be abrogated by various treatments leading to immunosuppression or macrophage damage (3). To check the possible involvement of interferon in the resistance mechanism, groups of susceptible A/J mice or resistant animals homozygous (A2G) or heterozygous (A/J  $\times$  A2G) $F_1$  for the resistance gene *Mx* were injected i.v. with various globulin preparations before i.p. challenge with graded doses of TURH virus. The globulin fractions were (a) NSG, (b) AHI, (c) ACN, and (d) AIF. Virus infected, untreated mice were included as further controls.

*Mortality.* Mortality in AIF-treated *Mx*-carriers was 100% for all challenge doses used and equaled that of treated or untreated susceptible A/J mice (Table I). Untreated resistant mice and resistant mice inoculated with the other globulin fractions all survived. Onset of death in AIF-treated *Mx*-bearing animals (especially in heterozygous  $F_1$  hybrids) was comparable to that in susceptible control A/J mice. AIF, but not the other globulin preparations, further decreased the short life span of A/J mice injected with 100 lethal virus doses.

*Virus Replication.* TURH grows to high titers in the liver of infected susceptible animals and causes viremia (9). Infectivity titers of whole blood samples and of liver homogenates from susceptible or resistant donors infected and treated with NSG or AIF 48 h previously were compared (Table II). Equal amounts of infective virus were found in organs from both groups of A/J mice irrespective of globulin treatment. Virus content in samples from AIF treated *Mx*-carriers was similarly high and was  $10^3$ – $10^6$  times greater than in organs from NSG-treated resistant mice.

*Comparative Interferon Production.* Interferon titers in sera from susceptible or resistant mice injected i.v. with infectious TURH virus were determined (Table III). Injection of  $10^3$  LLD<sub>50</sub> led to a measurable response in A/J but not in A2G mice. Serum interferon levels most likely reflected rapid viral multiplication in the susceptible host in contrast to decreased viral multiplication in *Mx*-carriers. These findings are in agreement with previous data showing that interferon production in organs of infected A2G mice was much lower than in the organs of A/J mice (4).

*Pathology.* The livers from viral-infected A2G mice treated with NSG, ACN, or AHI were macroscopically normal. There were a few small foci of mononuclear cell infiltration (Fig. 1 A). In contrast, the livers from infected AIF-treated A2G mice were enlarged and friable as a result of widespread liver cell necrosis (Fig. 1 B). Immunofluorescent staining showed viral antigens in these livers to have the same

TABLE I  
Abrogation of Inborn Resistance to Hepatotropic Influenza A/Turkey/England 63 by AIF

Exp. No.	Mouse			Virus dose*	Mortality‡	Death§		
	Strain	Genotype	Treatment					
1	A/J	(+/+)	None	10 <sup>4</sup>	4/4	2 <sup>(4)</sup>		
			AIF	10 <sup>4</sup>	4/4	2 <sup>(4)</sup>		
			NSG	10 <sup>2</sup>	4/4	3 <sup>(4)</sup>		
			AIF	10 <sup>2</sup>	4/4	2 <sup>(4)</sup>		
			(A × A2G)F <sub>1</sub>	(M <sub>x</sub> /+)	None	10 <sup>4</sup>	0/4	—
					AIF	10 <sup>4</sup>	4/4	2 <sup>(4)</sup>
					NSG	10 <sup>2</sup>	0/4	—
			A2G	(M <sub>x</sub> /M <sub>x</sub> )	AIF	10 <sup>2</sup>	4/4	3 <sup>(4)</sup>
					NSG	10 <sup>4</sup>	0/4	—
	AIF	10 <sup>4</sup>			4/4	2 <sup>(1)</sup> , 3 <sup>(2)</sup> , 5 <sup>(1)</sup>		
	2	A/J	(+/+)	None	10 <sup>2</sup>	0/4	—	
				AIF	10 <sup>2</sup>	4/4	3 <sup>(3)</sup> , 6 <sup>(1)</sup>	
				NSG	10 <sup>3</sup>	4/4	2 <sup>(3)</sup> , 3 <sup>(1)</sup>	
				AHI	10 <sup>3</sup>	4/4	2 <sup>(2)</sup> , 3 <sup>(2)</sup>	
				ACN	10 <sup>3</sup>	4/4	2 <sup>(1)</sup> , 3 <sup>(3)</sup>	
AIF				10 <sup>3</sup>	4/4	2 <sup>(4)</sup>		
(AxA2G)F <sub>1</sub>				(M <sub>x</sub> /+)	NSG	10 <sup>3</sup>	0/4	—
					AHI	10 <sup>3</sup>	0/4	—
					ACN	10 <sup>3</sup>	0/4	—
A2G		(M <sub>x</sub> /M <sub>x</sub> )	AIF	10 <sup>3</sup>	4/4	2 <sup>(1)</sup> , 3 <sup>(3)</sup>		
			NSG	10 <sup>3</sup>	0/4	—		
			AHI	10 <sup>3</sup>	0/4	—		
A2G		(M <sub>x</sub> /M <sub>x</sub> )	ACN	10 <sup>3</sup>	0/4	—		
			AIF	10 <sup>3</sup>	4/4	3 <sup>(3)</sup> , 4 <sup>(1)</sup>		

\* 0.5 ml TURH diluted in PBS was inoculated i.p.

‡ No. of dead animals on day 7/total No. of mice inoculated.

§ Number indicates day of death after infection. Exponent is No. of mice dying on that day.

|| Sera were given i.v. immediately before virus challenge (Materials and Methods).

TABLE II  
TURH Replication in AIF-Treated Mice\*

Sample	Mouse strain	EID <sub>50</sub> (log 10/ml)			
		NSG-treated mice		AIF-treated mice	
		10 <sup>2</sup> LD <sub>50</sub>	10 <sup>4</sup> LD <sub>50</sub>	10 <sup>2</sup> LD <sub>50</sub>	10 <sup>4</sup> LD <sub>50</sub>
Blood‡	A/J	6.1	6.0	6.6	6.0
	(A × A2G)F <sub>1</sub>	3.5	3.5	6.4	6.5
	A2G	3.0	3.7	6.5	6.3
Liver homogenate§	A/J	9.5	ND	8.5	ND
	(A × A2G)F <sub>1</sub>	5.0	5.3	9.0	9.5
	A2G	2.0	4.0	8.0	7.7

\* Injected i.v. with NSG or AIF immediately before i.p. challenge with 10<sup>2</sup> or 10<sup>4</sup> LD<sub>50</sub> of TURH.

‡ Heparinized blood pooled from 5 mice per group 48 h after infection.

§ Prepared from individual livers 48 h after infection.

|| ND = not done.

TABLE III  
*Comparative Serum Interferon Titers in TURH-Infected Mx-Bearing and Control Mice\**

Virus dose i.v.	Serum sample	Mouse strain and genotype		
		A/J (+/+)	(A × A2G)F <sub>1</sub> (Mx/+)	A2G (Mx/Mx)
	<i>h</i>			
10 <sup>5</sup> LD <sub>50</sub>	6	1:40	1:30	1:20
	18	1:320	1:120	1:160
10 <sup>3</sup> LD <sub>50</sub>	6	<1:20	<1:40	<1:20
	18	1:480	<1:20	<1:20

\* Pools of serum from four mice (7- to 9-wk-old males).

distribution as in livers from treated or untreated A/J mice (not shown). As a consequence of liver cell destruction, liver-specific F antigen (9) could be detected in the sera of these mice (data not shown). No pathological changes were found in AIF treated, uninfected control mice.

*Abrogation of Inborn Resistance towards Neurotropic and Pneumotropic Strains of Influenza Virus by AIF.* Genetically determined resistance towards myxoviruses has been shown to manifest itself in brain and lung to a similar extent as in the liver (1-5). If AIF treatment interfered with an element of general importance for the resistance mechanism this treatment should also overcome in vivo resistance to neurotropic and pneumotropic virus strains.

**INTRACEREBRAL CHALLENGE.** A/J mice usually die between the 3rd and 8th d after NWS-virus challenge (2). AIF-treated Mx-bearing mice (together with treated or untreated A/J controls) infected i.c. with NWS virus succumbed within that period. NWS virus was not lethal for untreated homozygous resistant mice (Table IV). One untreated heterozygous F<sub>1</sub> mouse died on day 9.

**INTRANASAL CHALLENGE.** AIF given i.v. severely impaired resistance of A2G mice towards i.n. infection with pneumotropic HKH virus (Fig. 2). Death was accelerated in AIF-treated susceptible mice in comparison to controls after i.n. infection with 10<sup>4</sup> LD<sub>50</sub> (Fig. 2) or 10<sup>2</sup> LD<sub>50</sub> of HKH (mean survival time 7.6 d in AIF-treated mice as compared to 9 d in untreated controls). On day 14, surviving animals were sacrificed, the degree of lung consolidation was scored, and serum antibody titers against HKH hemagglutinin were determined. Pulmonary lesions were much more pronounced and HAI titers were approximately four times higher in AIF-treated mice (both A2G and A/J) than in control mice suggesting increased virus replication in these animals.

In conclusion, the influence of AIF on resistance against i.n. infection was clear-cut but less dramatic than that observed on resistance towards infection with neurotropic or hepatotropic virus strains, presumably because of the relative inability of the globulin to reach the tracheobronchial epithelium as previously discussed (14).

*Resistance of Peritoneal Macrophages Obtained from Mx-Bearing Mice and Infected in the Presence of AIF In Vitro.* It has previously been shown that peritoneal macrophages from genetically resistant mice express resistance towards infection in vitro with the macrophage-adapted influenza virus strain M-TUR (2). Attempts to increase the growth potential of M-TUR in resistant macrophages by serial passages have so far failed (2). We therefore determined whether addition of AIF to resistant macrophage

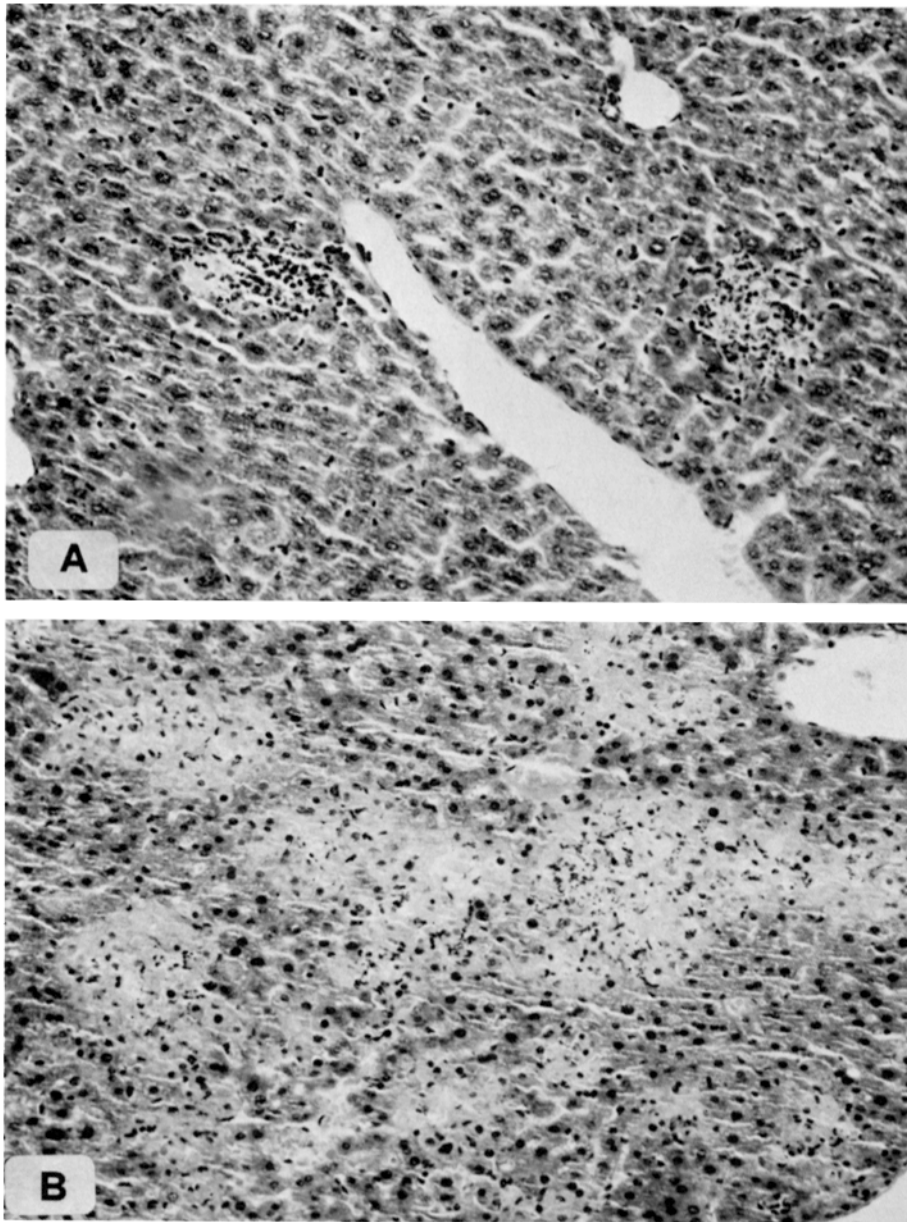


FIG. 1. Liver sections from resistant A2G mice 48 h after i.p. inoculation of  $10^6$  LD<sub>50</sub> (as estimated in A/J mice) of TURH virus. (A) NSG treatment: focal lesions with marked inflammatory infiltrates. (B) AIF treatment: widespread liver cell necrosis. Hematoxylin and eosin,  $\times 160$ .

cultures at the time of virus challenge would render these cells susceptible. One representative experiment (out of 4) is summarized in Table V, showing that virus resistance of *Mx*-carrying macrophages was preserved despite the presence of AIF. Even 10 times higher AIF concentrations did not abolish resistance. No cytopathic effect was ever seen in such cultures during the usual 1-wk observation period. The

TABLE IV  
Abrogation of Inborn Resistance to Neurotropic Influenza A Virus NWS by AIF

Strain	Mouse		Mortality*	Death‡
	Genotype	Treatment§		
A/J	(+/+)	None	6/6	<i>d</i> 3 <sup>(1)</sup> , 4 <sup>(4)</sup> , 7 <sup>(1)</sup>
		AIF	4/4	3 <sup>(4)</sup>
(A × A2G)F <sub>1</sub>	(Mx/+)	None	1/5	9 <sup>(1)</sup>
		AIF	4/4	5 <sup>(2)</sup> , 6 <sup>(1)</sup> , 8 <sup>(1)</sup>
A2G	(Mx/Mx)	None	0/4	—
		AIF	4/4	4 <sup>(1)</sup> , 6 <sup>(2)</sup> , 7 <sup>(1)</sup>

\* No. of dead animals on day 14/total No. of mice inoculated.

‡ Number indicates day of death after infection. Exponent is No. of mice dying on that day.

§ AIF was given i.v. 15 min before i.c. challenge with 10<sup>2</sup> i.c. LD<sub>50</sub> of NWS.

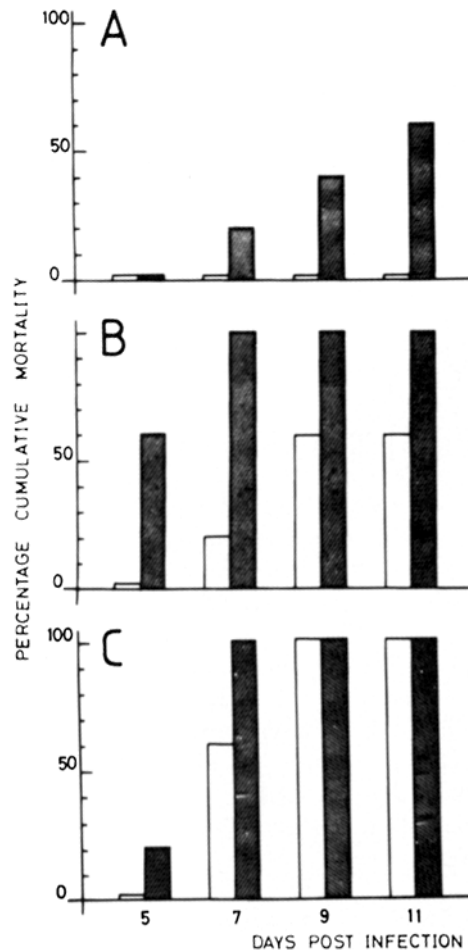


FIG. 2. Effect of AIF treatment on intranasal infection with pneumotropic influenza A/Hong Kong/1/68. Groups of five mice were challenged i.n. with virus and were injected i.v. with AIF (solid bars) or left untreated (open bars). A = A2G, 10<sup>4</sup> LD<sub>50</sub> i.n., B = A2G, 10<sup>5</sup> LD<sub>50</sub> i.n., C = A/J, 10<sup>4</sup> LD<sub>50</sub> i.n.

TABLE V  
Resistance of Peritoneal Macrophages from Mx-Bearing Mice to Infection with M-TUR in the Presence of AIF In Vitro

Macrophage*		M-TUR‡ TCID <sub>50</sub> (log 10)	Hemagglutination titers of culture supernate§					
Origin	Culture No.		AIF-treated cultures			NSG-treated cultures¶		
			Time after infection			Time after infection		
			48	72	168	48	72	168
				<i>h</i>			<i>h</i>	
A/J	1	6	1:512	>1:1024	ND	1:512	>1:1024	ND
	2	5	1:256	>1:1024	ND	1:128	>1:1024	ND
	3	4	1:16	>1:1024	1:1024	1:8	>1:1024	1:512
	4	4	1:8	>1:1024	1:1024		ND	
	5	3	0	>1:1024	ND	0	>1:1024	ND
(A × A2G)F <sub>1</sub>	6	6	1:16	1:16	1:8	1:8	1:4	1:4
	7	5	1:2	1:8	1:4	0	0	0
	8	4	0	1:8	1:2	0	0	0
	9	4	0	0	1:2		ND	
	10	3	0	0	0	0	0	0
A2G	11	5	1:8	1:8	1:2	0	0	0
	12	4	0	1:4	1:2		ND	

\* Pooled from five mice per group and kept for 22 h in culture before virus challenge.

‡ 0.2 ml of 10-fold virus dilutions in culture medium were added to 1.8 ml of culture medium containing AIF or NSG. Input virus was not removed.

§ 0.25 ml/well of culture medium was removed and titrated for hemagglutinin using twofold dilution steps. 0 indicates no hemagglutination of 1:2 dilutions. 0.5 ml of fresh medium (representing ¼ of total volume) was added to each well on day 4.

|| AIF was added before virus infection and was present during the entire experiment at a dilution of 10<sup>-3</sup> corresponding to a neutralizing titer of 1.2 × 10<sup>-3</sup> against 8 U of interferon.

¶ NSG was added before virus infection and was present during the whole experiment at a dilution of 10<sup>-3</sup>.

small amounts of hemagglutinin in the supernates of AIF-treated resistant cultures may reflect in part the input virus.

*Virus Susceptibility of Macrophages Taken from AIF Treated Mx-Carrying Donors and Maintained in the Presence of AIF.* Resistance of macrophages and in vivo resistance of the whole animal have previously been shown to be correlated (2). Because treatment of mice with AIF abolished resistance to influenza virus, we determined the virus susceptibility of macrophages obtained from AIF-treated donors (Table VI). As expected, macrophages from NSG injected or untreated A2G mice were resistant even in the presence of AIF. In contrast, M-TUR grew well in macrophages from AIF-treated A2G mice, provided AIF was also present in vitro at the time of virus infection. Virus multiplying in such A2G cultures was identified as M-TUR by hemagglutination inhibition tests using antisera of known specificity. It was noninfectious for ordinary A2G macrophages but attained high titers (10<sup>7</sup> TCID<sub>50</sub>/ml) in susceptible A/J macrophage cultures.

### Discussion

It has long been assumed that interferon plays an important role in host defense in viral infections. Thus, Isaacs and Hitchcock (15) stated that interferon was formed at the right place and at the right time to influence virus infection in vivo. Nevertheless,



TABLE VI  
*Susceptibility of Peritoneal Macrophages from AIF-Treated A2G Mice to Infection with M-TUR*

Exp. No.	Mouse		Macro- phage culture No.	M- TUR <sup>‡</sup> 10 <sup>6</sup> TCID <sub>50</sub>	Type of§ globulin added in vitro	Virus growth				
	Strain	In vivo* treat- ment				48 h		72 h		
						CPE	HA titer	CPE	HA titer	
1	A2G	AIF	1	+	NSG	±	1:16	±	1:8	
			2	+	NSG	±	1:32	±	1:4	
			3	+	AIF	+++	1:512¶	+++	1:256	
			4	+	AIF	+++	1:256	+++	1:64	
			5	+	AIF	+++	1:256	+++	1:128	
			6	-	AIF	-	0	-	0	
2	A2G	NSG	7	+	NSG	-	0	-	0	
			8	+	AIF	-	0	-	0	
			9	+	AIF	-	0	-	0	
			AIF	10	+	NSG	-	0	-	0
				11	+	NSG	-	0	-	0
				12	+	AIF	+	1:160	++	1:160
		None	13	+	AIF	+	1:80	++	1:160	
			14	+	AIF	-	1:2	-	1:2	
			15	+	None	-	0	-	0	
		A/J	None	16	+	NSG	++	1:160	+++	1:320
				17	+	None	++	1:160	+++	1:160

\* Three mice per group were injected i.v. with 0.2 ml (exp. 1) or 0.1 ml (exp. 2) of a 1:3 dilution of AIF or NSG 24 h (exp. 1) or 3 h (exp. 2) before stimulation by i.p. injections of thioglycollate medium.

‡ Macrophages were infected after a 20-h culture period. +, infected cultures; -, uninfected control cultures. Input virus was not removed.

§ Sheep anti-mouse interferon globulin (AIF) or normal sheep globulin (NSG) at 10<sup>-3</sup> dilutions giving neutralizing titers of AIF of 1.2 × 10<sup>-3</sup> were added to culture wells either immediately (exp. 1) or 16 h (exp. 2) before virus challenge and were kept present throughout the experiments.

|| Assessed by cytopathic effects (CPE: -, none; ±, tiny; + moderate; ++ pronounced; +++ total) and hemagglutinin (HA) titers of culture supernatants. 0.5 ml/well were removed at the times after infection indicated and replaced by fresh medium (representing ¼ of total volume). 0 indicates no hemagglutination at a dilution of 1:2.

¶ Hemagglutination inhibition with a standard rabbit antiserum directed against A/Turkey/England/63 was positive. This A2G culture supernate was infectious for susceptible A/J macrophages but not for A2G macrophages.

the evidence for the role of interferon has been indirect, based for the most part on temporal associations between the presence of virus and interferon in different tissues. Recent work, however, using antibody to mouse interferon has provided more direct evidence for the beneficial role of interferon in the initial response to viral infection (6, 7, 14, 16, 17). We have shown herein that interferon is an integral and important part of the inborn resistance to influenza virus in mice determined by the gene *Mx*. Thus, anti-interferon globulin (AIF) increased susceptibility of A2G mice to the lethal action of influenza A virus > 100-fold. This abrogation of resistance in AIF treated mice was manifested in varying degrees with all virus strains tested, neurotropic, pneumotropic, and hepatotropic.

We had previously shown that macrophages from mice bearing the *Mx* gene expressed resistance in vitro (2), and that a direct correlation existed between the resistance of an individual mouse to influenza virus and the in vitro resistance of that mouse's macrophages (2). In the experiments reported herein, it was found that when macrophages were taken from mice treated with AIF, and then cultivated in vitro

with AIF, they were fully susceptible to influenza virus, and capable of producing as much virus as macrophages from susceptible mice (Table VI). Treatment of mice with AIF followed by omission of AIF *in vitro* (Table VI) or, conversely, cultivation of resistant macrophages with AIF (from a mouse not treated with AIF) (Table V) failed to convert resistant macrophages into susceptible macrophages. Although virtually all work on interferon to date suggests that interferon is an induced protein, the findings just mentioned imply the possibility of the existence of endogenous interferon in mice. In support of this possibility it may be mentioned that rabbit (18) and mouse macrophages (19) and human leucocytes (20) have been shown to liberate interferon spontaneously into the culture medium. If macrophages for example produced small amounts of interferon *in vivo* and *in vitro*, this would explain the necessity of treatment of mice with AIF as well as the maintenance of AIF in the culture medium. More recently, however, we have found that macrophages from resistant mice can be converted to susceptibility even in the absence of AIF, after cultivation *in vitro* for 2 wk. Perhaps endogenous interferon acts on peritoneal macrophage *in vivo* and this effect gradually wanes *in vitro*.

Whether we suggest greater amounts of interferon before or after infection with influenza virus, or greater sensitivity to interferon in A2G mice than in A/J mice, we are nevertheless obliged to explain the specificity of this resistance for influenza virus. Therefore, if interferon is implicated as the experiments with AIF indicate, why are A2G mice selectively resistant to influenza virus and not also resistant to other viruses? In fact, A2G mice are as sensitive to several other viruses as are control mice (21). Likewise, other strains of mice show varying levels of specific resistance to flaviviruses (22, 23) and mouse hepatitis virus (7). We should like to suggest the following hypothesis to explain the resistance of A2G mice to influenza virus. The interferon produced (either spontaneously or upon induction) is more effective in protecting A2G macrophages (and possibly also other cells) against influenza virus than it is in protecting A/J mouse cells against influenza virus. In other words, we are suggesting that there is a genetically determined difference between strains of mice in this sensitivity to interferon which is specific for a given group of viruses. In the case of A2G mice, we would suggest that a threshold amount of interferon renders certain A2G mouse cells resistant to influenza virus, whereas the same amount is insufficient to protect A/J mouse cells against the same challenge. Interferon would be equally effective in protecting A2G and A/J mouse cells against infection with some other viruses. Although we have no clear cut evidence to support this hypothesis yet, which has also been discussed with regard to flaviviruses (22), it is of interest that a similar finding is obtained for cells derived from different species. Thus, the relative sensitivities of viruses to interferons were found to be characteristic of the species tested. For example, vaccinia virus was the most sensitive of five viruses when tested in mouse and hamster cells, but the least sensitive when tested in human, rabbit, and bat cells (24). In another study, pseudorabies and vaccinia viruses were as susceptible as vesicular stomatitis virus to inhibition by interferon when tested in chicken or mouse cells but were refractory to inhibition in interferon-treated rabbit cells (25). The authors suggested that separate resistance factors might exist in interferon treated cells for DNA and RNA viruses, and that certain interferon-treated rabbit cells could not generate resistance factors for some DNA viruses (25). Our hypothesis implies that a similar hierarchy of response to interferon's effect as regards a given virus varies

within the species (i.e., the mouse in our experiments). If this phenomenon existed it would imply not only that individuals varied in their sensitivity to interferon (which might be expected) but that the enhanced or decreased sensitivity might also be manifested for a specific virus. Furthermore, because genes like *Mx* could, in certain tissues, be temporarily or permanently switched off, this might explain some puzzling tissue tropisms.

Rather than viewing interferon as the primary factor in the anti-viral state and assuming its modulation by host genes, we could also consider interferon as an auxiliary necessary for the expression of a genetically determined cellular resistance. According to this hypothesis, the inherent resistance of *Mx*-bearing cells, whatever its mechanism, would be the major component of resistance. Removal of interferon however creates an artificial situation in which resistance can no longer be expressed efficiently. In any case, the intimate cooperation of interferon with the genetic make up of the host as revealed by our experiments promises deeper insights into the complexities of antiviral defense mechanisms.

### Summary

The genetically determined resistance towards orthomyxoviruses exhibited by mice homozygous (A2G) or heterozygous (A2G × A/J) for the gene *Mx* was abolished or greatly diminished by treatment with anti-interferon globulin (AIF). AIF induced increased susceptibility to challenge with hepatotropic, neurotropic, and pneumotropic strains of influenza A virus. Hepatotropic virus titers in blood and livers of AIF-treated, *Mx*-bearing mice were higher by a factor of  $10^3$ – $10^6$  than those in untreated mice of the same genotype, and were comparable to those in genetically susceptible (untreated or AIF-treated) mice.

Peritoneal macrophages from *Mx*-bearing untreated mice were resistant to challenge with a macrophage-adapted strain of influenza A virus even in the presence of AIF. However, when macrophages were taken from resistant mice injected with AIF and also cultivated in the presence of AIF, they were as susceptible to the virus as macrophages taken from susceptible mice. We conclude that interferon is an important factor in resistance to orthomyxoviruses governed by the gene *Mx*.

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