

Effect of Immunosuppression on the Genetic Resistance of A2G Mice to Neurovirulent Influenza Virus¹

R. A. FISKE² AND P. A. KLEIN*

Department of Pathology, College of Medicine, University of Florida, Gainesville, Florida 32610

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A2G mice are genetically resistant to lethal infection with neurotropic and pneumotropic influenza viruses. A possible immunological explanation for this resistance was sought by assessing the effect of cyclophosphamide and X irradiation immunosuppression on the infection of A2G mice with lethal doses of neurovirulent virus. Immunosuppressed A2G mice survived lethal infection even though rendered unable to produce specific antiviral antibody or to generate cell-mediated delayed-type hypersensitivity responses. Measurement of infectious virus replication and detailed observation of the infection by immunofluorescence microscopy show that immunosuppression does not potentiate or allow spread of the virus in A2G brains. Interferon levels were essentially the same in normal and immunosuppressed A2G brains but were 3 to 5 times lower than in the brains of susceptible mice dying of the infection. The results strongly suggest that the genetic resistance of A2G mice to the acute lethal effects of neurovirulent influenza virus infection does not depend on the induction of primary immune mechanisms as we currently understand them. Other possible explanations for this resistance are considered.

Some inbred mouse strains manifest striking inherited differences in susceptibility to certain virus infections (1, 21). Experiments designed to explain such "natural resistance" to infection have revealed involvement of a T-cell-mediated mechanism in polyoma infections (3), an innate resistance of macrophages to infection with mouse hepatitis virus (37), and a putatively selective action of interferon in resistance to group B arboviruses (14).

Inbred A2G mice are genetically resistant to infection with lethal doses of diverse strains of pneumotropic and neurotropic influenza viruses (20, 23). Although the mechanism responsible for this resistance is unknown, it seems to be controlled by a single dominant autosomal gene, *Mx* (19), and is thus far unique among over 20 strains of inbred mice tested (7).

Immunosuppression has been used extensively to assess the contribution of host immunological mechanisms to recovery from virus infections (1, 31, 33). Because cyclophosphamide can potentiate infections with neurovirulent influenza viruses in susceptible animals (26, 29), we tested whether or not immunosuppression would abrogate the unique natural resistance of A2G mice to influenza virus.

The results of this study suggest that the genetic resistance of A2G mice to the acute lethal effects of neurovirulent influenza infection does not depend on the induction of primary immune mechanisms as we currently understand them.

MATERIALS AND METHODS

Animals. A2G mice were reared in our own breeding colony in the Research Animal Department, J. Hillis Miller Health Center. A/J mice were purchased from the Jackson Laboratory, Bar Harbor, Me. Age-matched male and female mice of both strains were employed. White random-bred rabbits were purchased from approved local dealers.

Viruses. The A₀/WSN strain of neurotropic influenza virus (WSN) was obtained from Irene Schulze, Department of Microbiology, St. Louis University School of Medicine, St. Louis, Mo. After several passages in brains of susceptible mice, stock virus was prepared in MDBK cell cultures and stored in ampoules at -85 C. The stock WSN contained 2.3×10^8 mean intracerebral lethal doses (ICLD₅₀) per ml as determined by intracerebral (i.c.) inoculation into adult A/J mice and was free of bacteria and mycoplasma.

Vesicular stomatitis virus (VSV; Indiana serotype) and B/Lee influenza virus were maintained as egg-grown stocks at -85 C.

Intracerebral inoculation of virus. Stock WSN was diluted in sterile phosphate-buffered saline (PBS) as required by the protocol. A 0.03-ml portion was injected directly into the left cerebral hemisphere

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² Present address: Department of Veterinary Pathology, College of Veterinary Medicine, Texas A & M University, College Station, Tex. 77843.

of each lightly anesthetized mouse with a 0.25-ml syringe and a 27-gauge, $\frac{3}{8}$ -inch (ca. 0.95 cm) needle. Rare deaths occurring within the first 24 h postinfection (PI) were considered to be due to injection trauma and were discounted in experimental results.

Tissue culture. MDBK cells were maintained by twice weekly passage in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, 150 U of penicillin per ml, 150 mg of streptomycin per ml, and 5 ml of a 2.52% arginine hydrochloride solution per liter to make the medium equivalent to that used by Choppin to support high yield replication of WSN (7).

L-929 cells were grown in minimum essential medium supplemented with 10% fetal calf serum and antibiotics as described above.

Preparation of mouse brain homogenates. Whole brains aseptically removed from mice were homogenized individually in cold minimum essential medium with antibiotics using prechilled glass tissue grinders to make 10% (wt/vol) suspensions. The homogenates were centrifuged for 15 min at 1,500 rpm in a refrigerated centrifuge, and the resultant supernatants were stored at -85°C until they could be assayed for virus content.

Brain homogenates to be assayed for interferon (IFN) were prepared exactly as described above. The supernatants, however, were dialyzed in the cold for 24 h against normal saline adjusted to pH 2.0 with HCl and then against Hanks balanced salt solution for 24 h more. After centrifuging for 15 min at 1,500 rpm, these neutralized supernatants were stored at 85°C until assayed for IFN content.

Virus assays. Total hemagglutinin was measured in a standard microtiter system using 0.5% chick erythrocytes as indicator cells. Infectious WSN influenza virus in brain homogenates was assayed by the end point dilution method on MDBK cells grown in 24-well plastic multidishes (Linbro Chemical Company, New Haven, Conn.). Four to six cultures were infected with 0.2 ml of each 10-fold dilution prepared in Dulbecco's modified Eagle medium. After a 2-h incubation period at 37°C , each monolayer was overlaid with 0.8 ml of Dulbecco's modified Eagle medium supplemented with 2.4% agamma calf serum. The end point was determined by scoring cytopathic effect at each dilution 48 and 72 h PI. The titer of each preparation was expressed as 50% tissue culture infectious doses per milliliter. Comparative assays of infected brain homogenates in 10-day-old embryonated chick eggs and on MDBK cells yielded the same titer.

IFN assay. IFN levels in brain homogenates were assayed by a conventional plaque inhibition assay utilizing a large-plaque variant of VSV on L-929 monolayers (11). Interferon in 10% (wt/vol) brain homogenates was expressed as 50% plaque-depressing doses per milliliter.

Delayed hypersensitivity assay. Normal or immunosuppressed adult mice were sensitized to oxazalone (OX) (2-phenyl-4-ethoxymethylene oxazalone; BDH Ltd., London, England) by applying 0.1 ml of a 3% solution of OX to their shaved abdomens (3). Seven to ten days later, contact sensitivity was assessed quantitatively by measuring the 24-h in-

crease in ear thickness of mice challenged on the ear with 0.025 ml of 3% OX in olive oil. Controls consisted of normal mice challenged with OX in olive oil and sensitized mice challenged with olive oil alone. Ear thickness was measured by using an automatic micrometric dial gauge (Starrett Instrument Company, Athol, Mass.) to insure that the force applied to the ear each time would be independent of the observer.

Assays for serum antibody activity. When required, hemagglutination-inhibiting antibodies were assayed in a conventional microtiter test against 4 hemagglutinin units of WSN. Serum was first treated with trypsin-periodate and incubated at 56°C for 30 min to destroy nonspecific serum inhibitors (18).

Serum neutralizing antibody to WSN was assayed on MDBK monolayer cultures against 100 mean tissue culture infectious doses of WSN. Virus-antiserum mixtures were incubated together for 1 h at 37°C before applying them to cell monolayers. Controls including normal serum from both unimmunized normal mice and immunosuppressed mice showed no neutralizing activity by this method. Final cytopathic effect readings were made 72 h PI.

The hemolytic antibody response to sheep erythrocytes was determined in a conventional microtiter test. A 0.025-ml portion of a 1% suspension of washed sheep erythrocytes in PBS was added to serial twofold dilutions of serum. This was followed immediately with 0.025 ml of a 1:5 dilution of guinea pig complement. The plates were placed in a 37°C incubator and read 45 min later.

Preparation of rabbit antiviral antisera. White rabbits (2 kg) were immunized with egg-grown WSN, VSV, and B/Lee viruses using complete Freund adjuvant for the primary intramuscular and subcutaneous injections. Individuals were boosted without adjuvant until satisfactory antiviral titers were achieved.

Preparation of fluorescein-labeled anti-WSN antibody. Rabbit antisera to WSN was fractionated and conjugated with fluorescein isothiocyanate by the method of Cebra and Goldstein (6). The fluorescein-labeled rabbit anti-WSN gamma globulin (FA) had a 280 m/495 m ratio of 3.5 and was used at a protein concentration of 0.25 to 0.50 mg/ml.

Immunofluorescent staining procedures. Brains and other organs removed from mice killed in an ether jar were placed immediately into a -85°C freezer in airtight containers. Coronal brain sections ($6\ \mu\text{m}$) were cut in a cryostat at -18°C and immediately plunged into 95% ethanol for 10 min. After fixation sections were washed in PBS and allowed to air dry. Fixed sections were overlaid with FA at 0.25 to 0.50 mg of protein per ml, incubated at room temperature for 30 min, washed five times with PBS, and mounted under cover slips with PBS-glycerin (50% vol/vol). Sections were observed in a Leitz research microscope equipped with a fluorescence Ploem vertical illuminator. Selective excitation of FA-stained specimens by the xenon source was achieved using a KP490 interference filter in the illuminator and K510/K515 edge filters in the filter holder slide bar. A KG1 heat filter and BG38 red suppression filter were used in the lamp housing. The fluorescence observed in FA-stained sections was specific for WSN. It could be blocked by prestaining sections with specific unlabeled rabbit

anti-WSN antibody but not by normal rabbit serum, rabbit anti-VSV, or rabbit anti-B/Lee.

Immunosuppressive procedures. Cyclophosphamide (CY, Cytoxan; Mead Johnson & Co., Evansville, Ind.) was prepared fresh in sterile distilled water and diluted to 10 mg/ml with PBS. Mice received CY at the rate of 150 mg/kg 1 day before and 1 day after antigen administration or virus infection.

Total body irradiation was achieved by using a Gammator (model B; Radiation Machinery Corp., Parsippany, N.J.) delivering a dose of 110 R/0.1 min. Each mouse was irradiated separately in a perforated plastic restraining tube. The 770-R dose used for A2G mice is approximately a 25% lethal dose over a 30-day observation period for this strain.

RESULTS

Neurovirulence and growth of WSN in normal A/J and A2G brains. A/J mice are representative of those mouse strains which invariably succumb to the rapidly progressive encephalomyelitis which follows i.c. inoculation of neurotropic influenza virus (20; Fig. 1). In addition, the close genetic similarity of A/J to A2G makes them the most appropriate susceptible control in the current absence of mice congenic with A2G at the virus resistance locus, *Mx* (20). WSN-infected A/J mice begin to develop a hunched posture with ruffled fur as early as 2 to 3 days PI. This is followed in the next few days by increased lethargy with extension and paresis of the hind limbs. Deaths occur not usually later than day 7 PI (Fig. 1). In marked contrast, A2G mice neither succumb to nor show any clinically apparent signs of central nervous system (CNS) disturbance at any virus dose short of one that is toxic (23).

Figure 1 reveals that both A2G and A/J

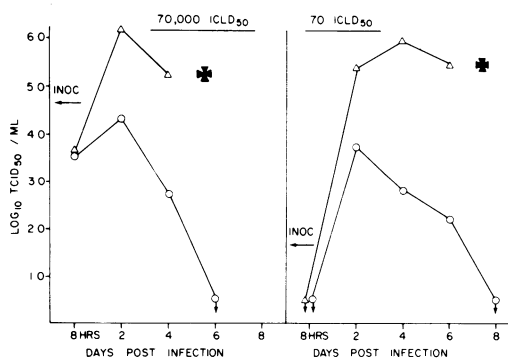


FIG. 1. Growth of neurotropic influenza virus (A_o/WSN) in the brains of normal adult A/J and A2G mice. Symbols: Δ , A/J mice; \circ , A2G mice; \ddagger , time to death of all mice in group. INOC, Inoculum dose injected i.c. at start of experiment. Each point represents the mean titer of three individual brain homogenates assayed separately.

brains supported the growth of WSN, but that maximum titers reached in A2G were at least 100-fold lower than those in A/J, which is in complete accord with earlier observations (23). The virus growth curves reflect the fact that only 5% of the i.c. inoculum is probably retained within the brain, the remainder being disseminated intravenously (5, 28). Thus, in Fig. 1, whereas the high dose inoculum (70,000 ICLD₅₀) is correctly indicated, the actual amount of virus retained at primary sites of infection in the brain is considerably less.

Effect of immunosuppression on primary immune responses and resistance to lethal infection in A2G mice. X irradiation and CY are potent immunosuppressants which have been utilized previously to elucidate mechanisms of host recovery from virus infections (31-33). These experimental modalities effectively suppressed primary serum antibody responses to both influenza virus and sheep erythrocytes by A2G mice (Fig. 2). Furthermore, CY and X irradiation prevented sensitization of A2G mice to OX, a potent inducer of cell-mediated, delayed-type cutaneous hypersensitivity in mice (3; Fig. 3). A2G mice rendered unresponsive to topical OX challenge were nevertheless able to resist concurrent challenge with a lethal dose of WSN administered i.c. (Fig. 3). Under all of these conditions, A2G mice exhibited no outward signs of CNS infection throughout the 40- to 60-day observation periods.

Table 1 (suppression of A2G with CY) and Table 2 (suppression of A2G with X irradiation) present this phenomenon in more detail. A few deaths occurred in CY-suppressed A2G mice challenged with virus (Table 1, groups C and F). These, however, occurred later and were unaccompanied by any of the signs of CNS disturbance seen in A/J mice. Necropsy revealed that A2G mice dying under these conditions had severe *Pseudomonas* sp. pneumonia. Deaths and pneumonia could be sharply reduced and even prevented in immunosuppressed WSN-infected A2G by keeping the mice on acidified drinking water (13, 15).

Attempts to isolate or to observe WSN growing in the lungs or livers of i.c. infected normal or immunosuppressed A2G mice were unsuccessful. The failure of CY immunosuppression to permit the extension of WSN replication in A2G (Fig. 4, 5, and 6) reinforces our conclusion that the occasional A2G deaths observed were not due to a direct potentiation of the WSN infection by CY. Similarly, the few deaths observed among WSN-infected A2G mice during irradiation experiments coincided in time

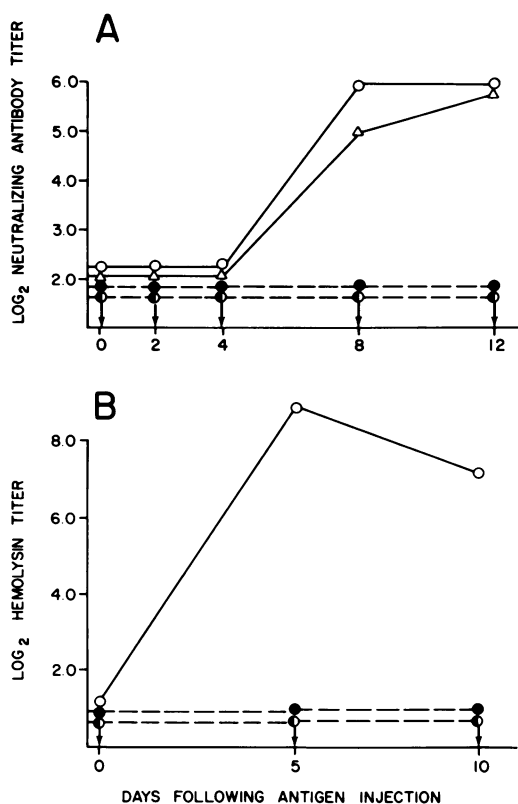


FIG. 2. Serum antibody responses to various antigens by normal and immunosuppressed A2G mice. Symbols: —, normal mice; ----, immunosuppressed mice; O, normal A2G; Δ, normal A/J; ●, A2G immunosuppressed with 770R; ▲, A2G immunosuppressed with CY. (A) neutralizing antibody response to live influenza virus (A₁/WSN) injected intraperitoneally (70,000 ICLD₅₀). (B) Hemolytic antibody response to sheep erythrocytes injected intraperitoneally (0.5 ml of a 2% suspension). Each point represents the mean titer of a serum pool from six mice.

with deaths caused by the effects of irradiation alone (Table 2, group F) and were also unaccompanied by evidence of CNS disturbance.

CY was tested for its ability to spare A/J mice from the lethal effects of infection as has been demonstrated with pneumotropic influenza infection by the intranasal route (Table 1, group H) (39). Although our experience with this situation is more limited, we have not observed any sparing effect of CY in susceptible mice infected i.c. with WSN.

Growth of WSN in brains of immunosuppressed A2G mice. Immunosuppression failed to potentiate the growth of low and high inocula of WSN in A2G to levels equal to or greater than those in normal A/J, but seemed rather to delay the clearance of virus from A2G brains (Fig. 5

and 6). In some instances, virus could be detected in brains as late as 19 days PI (Fig. 5). The slightly elevated levels of WSN in im-

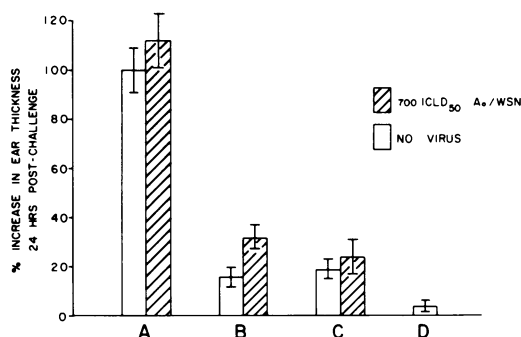


FIG. 3. Contact hypersensitivity to OX in normal and immunosuppressed A2G mice simultaneously resisting a lethal dose of neurotropic influenza virus (A₁/WSN). (A) Mice sensitized with OX, no immunosuppression. (B) CY-immunosuppressed mice sensitized with OX. (C) 770R-immunosuppressed mice sensitized with OX. (D) Normal A2G mice, not sensitized with OX, not immunosuppressed, but challenged with an eliciting dose of OX. Bar heights represent the mean 24-h ear swelling responses of six mice assayed individually. Statistic shown is the standard error of the mean.

TABLE 1. Effect of cyclophosphamide immunosuppression on mortality of A/J and A2G mice after infection with high and low doses of WSN influenza virus^a

Group	Mouse strain	Virus dose ^b	Immunosuppression ^c	Mortality ^d	Day of death ^e
A	A/J	Low	None	27/27	4 ⁷ , 5 ¹⁷ , 6 ³
B	A2G	Low	None	0/27	None
C	A2G	Low	CY	1/27	24 ¹
D	A/J	High	None	27/27	3 ⁹ , 4 ¹⁶ , 5 ²
E	A2G	High	None	0/27	None
F	A2G	High	CY	4/27	8 ² , 12 ¹ , 20 ¹
G	A2G	None: PBS, i.c.	CY	1/27	19 ¹
H	A/J	Low	CY	7/7	4 ⁴ , 5 ¹ , 6 ⁶ , 7 ²

^a Cumulative data from three independent experiments. All deaths in CY-immunosuppressed A2G mice occurred in one experiment in which mice were not given acidified water to drink (see text).

^b Low virus dose equals 70 ICLD₅₀; high virus dose equals 70,000 ICLD₅₀. All virus injections were given i.c.

^c CY (150 mg/kg) was given i.p. 1 day before and 1 day after virus inoculation.

^d Number of mice dying/number of mice injected i.c. with virus. Final readings were taken on day 55 PI.

^e Number is day of death PI. Exponent is number of mice dying on that day. Example: 3⁹ means nine mice died on day 3 PI.

TABLE 2. Effect of immunosuppression with X irradiation on mortality of A/J and A2G mice after infection with high and low doses of WSN influenza virus^a

Group	Mouse strain	Virus dose ^b	Immunosuppression ^c	Mortality ^d	Day of death ^e
A	A/J	Low	None	17/17	4 ⁵ , 5 ¹¹ , 6 ¹
B	A2G	Low	None	0/17	None
C	A2G	Low	770R	0/17	None
D	A/J	High	None	17/17	3 ⁶ , 4 ¹⁰ , 5 ¹
E	A2G	High	None	0/17	None
F	A2G	High	770R	2/17	16 ¹ , 24 ¹
G	A2G	None; PBS, i.c.	770R	1/17	16 ¹

^a Cumulative data from two independent experiments.

^b Low virus dose equals 70 ICLD₅₀; high virus dose equals 70,000 ICLD₅₀. All injections given i.c.

^c 770R, Mice irradiated individually and inoculated i.c. within 2 h of irradiation. 770R is approximately a 25% lethal dose over a 50-day period for A2G mice (see Materials and Methods).

^d Number of mice dying/number of mice injected i.c. with virus. Final readings were taken day 55 PI.

^e Number is day of death PI. Exponent is number of mice dying on that day.

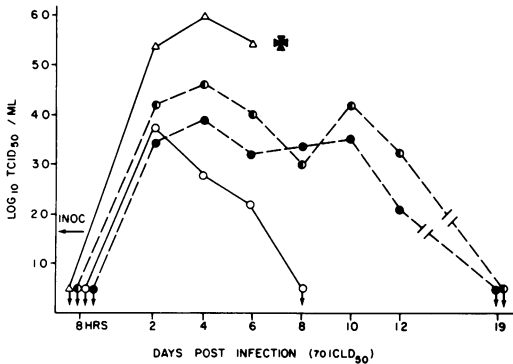


FIG. 4. Growth of a low-dose inoculum (70 ICLD₅₀) of neurotropic influenza virus (A₀/WSN) in the brains of normal A/J mice compared with growth in brains of normal and immunosuppressed A2G mice. Symbols: —, normal mice; ----, immunosuppressed mice; Δ, normal A/J mice; ○, normal A2G mice; ●, A2G immunosuppressed with 770R; ●, A2G immunosuppressed with CY; †, time to death of all mice in group. INOC, Inoculum dose injected i.c. at start of experiment. Each point represents the mean titer of three individual brain homogenates assayed separately.

munosuppressed A2G brains 8 h PI with the high virus dose (Fig. 5) may be in part due to this impairment of virus clearance, since under

normal conditions 95% of the i.c. inoculum escapes from the brain (13, 15). The major portion of WSN recovered from immunosuppressed A2G brains beyond day 8 most probably represents retained virus rather than new virus being continuously synthesized by large numbers of reinfected susceptible cells in A2G. This conclusion is based primarily on our observation that beyond day 8 PI, FA-stained sections of immunosuppressed A2G brain revealed only occasional small foci of cells producing WSN virus (Fig. 6; see below).

IFN levels in WSN-infected brains. IFN levels in the brains of nonsuppressed A/J dying from the WSN infection were three to five times higher than those in comparable A2G brains (Table 3). This undoubtedly reflected the higher levels of virus replication in A/J brains 24 to 48 h PI. Immunosuppression of A2G with CY or X irradiation did not abrogate the A2G brain IFN response, although IFN levels in CY-suppressed A2G at both 24 and 48 h PI were diminished. An inhibitory effect of CY on IFN responses has been observed in other systems (39). Although slightly reduced at 24 h PI, IFN levels in X-irradiated brains were generally not different from those in nonsuppressed A2G brains. This is in contrast to the reported marked inhibition of serum IFN induction by influenza virus after 125-R whole-body irradiation (9).

Light microscopy. The histopathology of murine influenza virus encephalomyelitis in

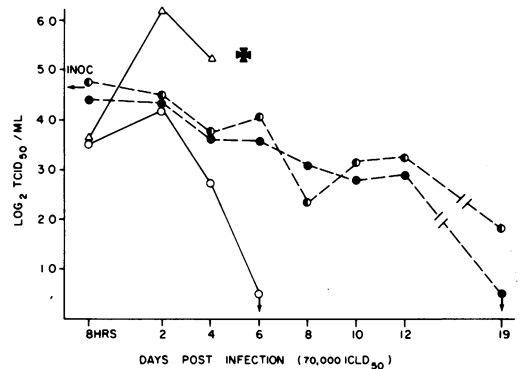


FIG. 5. Growth of a high-dose inoculum (70,000 ICLD₅₀) of neurotropic influenza virus (A₀/WSN) in the brains of normal and immunosuppressed A2G mice. Symbols: —, normal mice; ----, immunosuppressed mice; Δ, normal A/J mice; ○, normal A2G mice; ●, A2G immunosuppressed with 770R; ●, A2G immunosuppressed with CY; †, time to death of all mice in group. INOC, Inoculum dose injected i.c. at start of experiment. Each point represents the mean titer of three individual brain homogenates assayed separately.

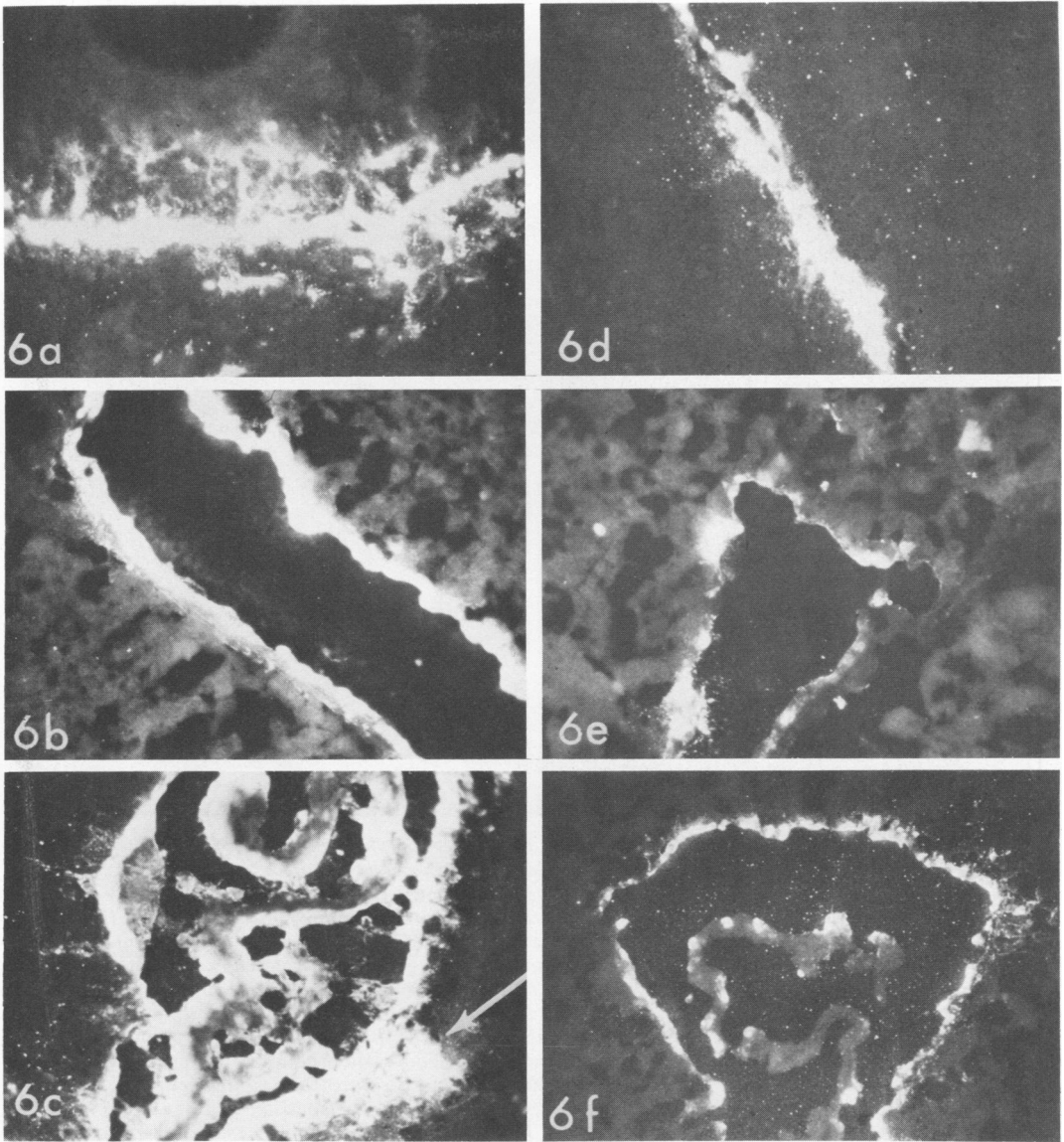


FIG. 6a-f. (a) FA-stained WSN-infected normal A/J brain section; 3rd ventricle, 24 h PI with 700 ICLD₅₀. Immunofluorescence of the ependymal lining extends irregularly to isolated cells in the subependymal areas. Individual filaments appear to connect the ependymal and subependymal regions. $\times 440$. (b) FA-stained WSN-infected normal A/J brain; 4th ventricle, 24 h PI with 700 ICLD₅₀. Continuous fluorescence of the ventricular lining is shown. Compare with the irregular, less intense staining seen in this area of A2G brain infected with the same virus dose (Fig. 6e). $\times 440$. (c) FA-stained WSN-infected A/J brain section; lateral ventricle, 48 h PI with 700 ICLD₅₀. Fluorescence involving the ependymal lining and most cells of the choroid plexus can be observed extending into the subependymal region from one segment of the ventricle (arrow). For comparison, see Fig. 6f showing the same stained region of an A2G brain infected with the same virus dose. $\times 440$. (d) FA-stained WSN-infected A2G brain section from a mouse immunosuppressed with CY; 3rd ventricle, 24 h PI with 700 ICLD₅₀. Fluorescence involves cells of the ependymal lining and is not observed extending to the parenchyma (see Fig. 6a for comparison). $\times 440$. (e) FA-stained WSN-infected A2G brain section from a mouse immunosuppressed with CY; 4th ventricle, 24 h PI with 700 ICLD₅₀. Specific fluorescence involves few cells of the ependymal lining. A rare, isolated fluorescent parenchymal cell could be seen in some brain sections of both normal and CY-suppressed A2G mice at this time. For comparison, see Fig. 6b. $\times 440$. (f) FA-stained WSN-infected A2G brain section from a mouse immunosuppressed with CY; lateral ventricle, 48 h PI with 700 ICLD₅₀. Specific fluorescence involves 50 to 75% of the cells of the ependymal lining and choroid plexus. Extension of the infection to the subependymal areas was not observed. For comparison see Fig. 6c. $\times 250$.

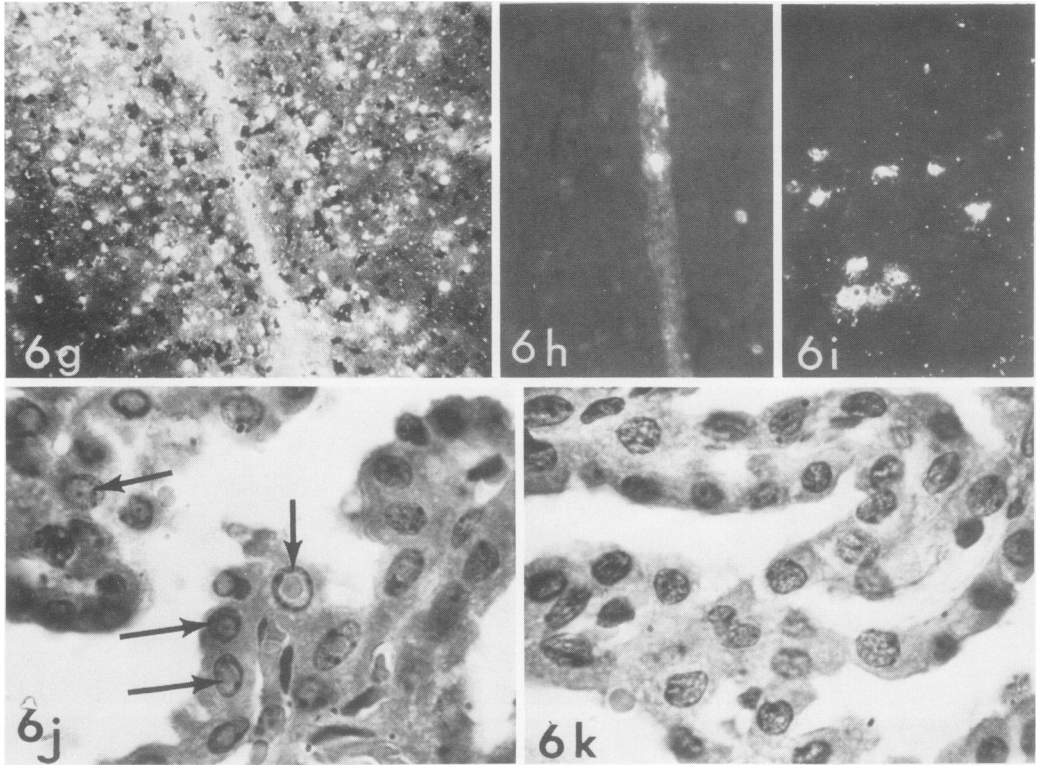


FIG. 6g-k. (g) FA-stained WSN-infected normal A/J brain section, 3rd ventricle, 96 h PI with 700 ICLD₅₀. Specific intense fluorescence extends from the ventricle to the subependymal areas and into the parenchyma. Multiple diverse cellular elements are stained but difficult to identify individually. Many cells appeared to be disintegrating when viewed in an adjacent H and E-stained brain section (not shown). Compare this picture with Fig. 6a, h and i. $\times 200$. (h) FA-stained WSN-infected A2G brain section from a mouse immunosuppressed with CY; 3rd ventricle, 96 h PI with 700 ICLD₅₀. Staining is restricted to a few isolated ependymal cells. $\times 440$. (i) FA-stained WSN-infected A2G brain section from a mouse immunosuppressed with CY; zone outside the 4th ventricle, 144 h PI with 700 ICLD₅₀. Staining of isolated parenchymal cells. $\times 440$. (j) H and E-stained WSN-infected A2G brain section from a mouse immunosuppressed with CY; choroid plexus, 4th ventricle, 144 h PI with 700 ICLD₅₀. Numerous intranuclear inclusion bodies may be seen (arrows) in otherwise morphologically intact cells of the choroid plexus. $\times 200$. (k) H and E-stained A2G brain section from a mouse immunosuppressed with CY but injected i.c. with PBS only; choroid plexus, 4th ventricle, 144 h PI of PBS. Normal morphology of choroid plexus cells is evident. Inclusion bodies are absent from cells. $\times 200$.

susceptible mice as described by others was essentially the same in this study (30). The infection in A/J mice was characterized by a severe ventriculitis with a necrotizing ependymitis and progression of inflammatory and degenerative lesions into the paraventricular regions. Extensive necrosis of A/J choroid plexus was encountered by 4 to 5 days PI in contrast to the less severe choroidal changes observed previously by others (30). In A2G mice, the ventricular inflammation was almost as intense as in A/J mice, but there was less ependymal necrosis. Inflammation in A2G extended no further than the immediate subependymal regions, and degenerative lesions of neuronal and glial elements were not observed. The

choroid plexus of infected A2G mice remained morphologically intact. Perivascular inflammatory infiltrates, though not prominent, were observed in both A/J and A2G mice. WSN-infected A2G mice immunosuppressed with CY presented the same neuropathological picture as normal A2G mice except that inflammatory infiltrates were entirely absent or reduced to a few isolated polymorphonuclear leukocytes.

A most striking and regular finding in both normal and CY-suppressed A2G was the occurrence of numerous intranuclear inclusion bodies in epithelial cells of the choroid plexuses from days 2 to 10 PI (Fig. 6j). These inclusions were not evident in normal or sham-inoculated A2G mice (Fig. 6k) and were never seen in the

TABLE 3. Effect of immunosuppression on IFN levels in brains of A/J and A2G mice infected with WSN influenza virus

Mouse strain	Immuno-suppression	Time PI ^a	
		24 h (PDD ₅₀ /ml) ^b	48 h (PDD ₅₀ /ml)
A2G	None	9.45 ± 0.66	5.95 ± 0.52
A2G	CY ^c	7.35 ± 0.52	2.66 ± 0.14
A2G	770R ^d	6.66 ± 0.73	7.09 ± 0.28
A/J	None	31.64 ± 1.26	27.88 ± 1.61

^a All mice were injected i.c. with 70,000 ICLD₅₀ of WSN influenza virus.

^b PDD₅₀, 50% plaque-depressing doses of brain homogenate per ml as assayed in VSV/L929 plaque system (see Materials and Methods). Result presented is mean PDD₅₀ of a pool of three brains assayed in triplicate ± the standard error of the mean.

^c CY was given at a dose of 150 mg/kg 1 day before and 1 day after virus infection.

^d 770R, Mice irradiated individually and then injected i.c. within 2 h of irradiation.

choroid plexuses of A/J mice. They have not been previously reported as present in the choroid plexus during murine influenzal encephalomyelitis (30). Attempts to stain these inclusions with FA were unsuccessful. Electron microscopically numerous intranuclear helical structures were evident in choroid epithelial cells of infected A2G mice (data not shown). These resembled the helical ribonucleoproteins of myxo- and paramyxoviruses (8).

Fluorescent microscopy. Our impressions concerning the extent of WSN infection obtained by virus growth measurements and by light microscopy were substantiated utilizing specific antiviral immunofluorescence (Fig. 6a through i). In A/J and in normal and CY-treated A2G, specific fluorescence could not be found in liver or lung sections at days 1, 3, or 5 PI.

Immunofluorescent observations in A/J brain. At 24 h PI, fluorescence of ependyma and choroid plexus varied from specific staining of individual cells or short segments of cells to fluorescence of entire ventricular profiles, depending on the level examined (Fig. 6a and b). In some sections isolated fluorescent cells were seen in the immediate subependymal region; these cells appeared to be connected to the ependymal lining by short fluorescent filaments (Fig. 6a). By day 2 PI the entire ependyma and most of the choroid plexus of the lateral, 3rd, and 5th subependymal cells had increased in number (Fig. 6c). On day 3 parenchymal staining took the form of definite periventricular zones of fairly dull diffuse fluorescence sur-

rounding the lateral and 3rd ventricles. By day 4, the zones of fluorescence surrounding the lateral and 3rd ventricles had increased in size (Fig. 6g). Fluorescent foci were seen in the meninges of the ventral surface of the brain, and fluorescence often extended into the adjacent parenchyma. Isolated fluorescent foci were noted deep within the septal area and the thalamus. The corpus colosum contained many fluorescent cells, which formed a band connecting the paraventricular fluorescent zones of the lateral ventricles. By day 5, paraventricular fluorescent zones were larger, and deep parenchymal fluorescent foci had increased in number and size. The pyramidal layer of the hippocampus could be seen as a band of fluorescent cells paralleling the lateral ventricular arc. These findings are summarized schematically in Fig. 7.

Immunofluorescent observations in brains of normal and CY-treated A2G mice. The immunofluorescent findings were essentially identical in normal and CY-suppressed A2G. At 24 h PI, fluorescence in both groups was observed only in isolated ependymal cells or short segments of ependymal cells and rarely in single or small clusters of choroidal epithelial cells (Fig. 6d and e). On day 2, there were increased

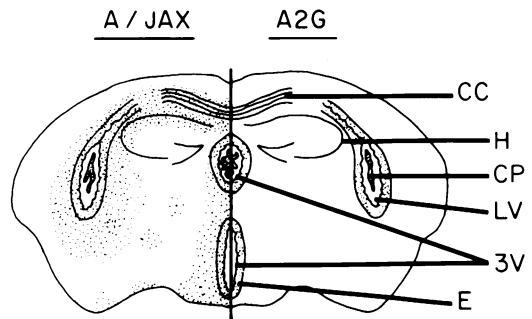


FIG. 7. Composite sketch summarizing the immunofluorescent observations of FA-stained brain sections of normal A/J mice and normal and CY-immunosuppressed A2G mice infected i.c. with 700 ICLD₅₀ of WSN influenza virus. Left side, A/J brain; right side, A2G brain. Stippling is used to define the location and maximum extent of fluorescence observed in each mouse strain. For A/J mice the maximum extension of fluorescence was reached 4 to 5 days PI, which coincided with the onset of deaths in these mice. For A2G this maximum was reached 2 days PI. The localization and extent of immunofluorescence observed in A2G mice was the same whether the virus-infected mice were normal or CY immunosuppressed. For comparison and details see Results and Fig. 6a through 6i. Abbreviations: CC, corpus callosum; H, hippocampus; LV, lateral ventricle; 3V, third ventricle; CP, choroid plexus; E, ependyma.

numbers of fluorescent ependymal and choroidal cells in both groups, but in any one section specific staining never involved more than 50 to 75% of the ependyma or choroid plexus (Fig. 6f). Isolated fluorescent cells were occasionally seen in the immediate subependymal regions in both intact and suppressed mice. Ependymal and choroidal fluorescence on day 3 was reduced in both groups to approximately the level seen on day 1. A few isolated fluorescent subependymal cells were again noted. On days 4 and 5, specific staining of the ependymal surfaces was further reduced, and fluorescence was not detected in the choroid plexus in either intact or CY-treated mice (Fig. 6h). Fluorescent subependymal cells were rarely seen in intact mice at this time, whereas the number of such cells appeared slightly increased in immunosuppressed mice. On day 6 fluorescence was undetectable in the nonsuppressed A2G group except for a single focus of several fluorescent subependymal cells in one brain. In suppressed A2G, on days 6 and 8 PI, a few isolated fluorescent ependymal and parenchymal cells could be seen (Fig. 6i). By day 12 PI only a rare paraventricular fluorescent focus was evident in a few suppressed A2G brains. The above findings are summarized schematically in Fig. 7.

DISCUSSION

Although first described over 11 years ago (23), the natural resistance of the A2G mouse to lethal infection with neurotropic and pneumotropic influenza viruses has not been critically analyzed. Our previous studies utilized A2G extensively because of this innate resistance but focused primarily on tumor immunity following viral oncolysis in this strain (6, 28). This study represents an attempt to shed light on the genetic resistance mechanism involved.

Immunological mechanisms have been shown to play an important role in resistance to and recovery from different virus infections in the mouse, although the relative contribution of specific antibodies and sensitized lymphocytes can vary markedly (1, 33). Thus, recovery from generalized ectromelia (mouse pox) seems to depend on intact cell-mediated immunity (4). Alternatively, an important role for antibody has been demonstrated in the recovery of mice from Coxsackie virus (35) and vaccinia virus infections (41).

The data show that immunosuppression with CY or X irradiation neither renders A2G mice susceptible to lethal i.c. infection with neurotropic WSN nor results in increased levels of virus replication in A2G brains. Furthermore, the location and course of the infection in

immunosuppressed and normal A2G brains is identical as revealed by specific antiviral immunofluorescence. These facts lead us to the conclusion that the natural resistance of the A2G mouse to lethal i.c. infection with neurotropic influenza virus does not depend on PI humoral or cell-mediated immune responses.

This conclusion is at odds with an observation that CY abolished the genetic resistance of A2G to lethal infection with neurotropic influenza strain A₂/NWS (26). We are unable to provide any rational explanation for this discrepancy. However, major differences may exist between methods used in the two sets of experiments.

Evidence other than the data presented here argues that the primary immune response has no critical role in A2G resistance to i.c. infection. We have, in A2G mice, corroborated findings (27) that influenza virus is a thymus-dependent antigen (9). Thus, A2G mice rendered immunologically unresponsive to neurotropic influenza virus by newborn or adult thymectomy nevertheless resist lethal i.c. infection (P. A. Klein, manuscript in preparation). In addition, Haller and Lindenmann have shown that the genetic resistance of A2G mice is fully expressed in 75% of the F₂ progeny of a cross between A2G (mx/mx) and nude (nu/nu) mice despite immunological unresponsiveness to the virus (12).

If not immunological, what then is the mechanism of A2G genetic resistance to influenza virus? We know that the resistance appears to be highly specific. A2G mice are fully susceptible to lethal i.c. infection with herpes simplex, Newcastle disease, rabies, vesicular stomatitis, yellow fever, West Nile, encephalomyocarditis, poliomyelitis, and Echo type 9 viruses (20). One pneumotropic strain (A₂/Singapore/1/57) proved lethal to A2G by the intranasal route, but this has not been confirmed (20).

A2G mice do have receptors for influenza viruses on cells derived from the brain parenchyma (36; P. A. Klein, unpublished observations). In addition, no difference was found in the ability of A2G brain parenchymal cells to absorb varying numbers of WSN particles from suspension as compared to cells from susceptible A/J brains.

No differences have been observed in the neurovirulence of progeny virus grown from high dilution in A2G brains (23). Thus, A2G mouse brain does not preferentially support the synthesis of nonvirulent virus.

While IFN production is not abolished by immunosuppression in A2G brains, IFN levels are always three to five times lower in A2G brains than in the brains of A/J dying of the

infection. As has been discussed by others (33), it becomes difficult to reconcile this type of result with a positive role for IFN in the recovery of A2G mice from i.c. infection unless IFN has specificity attributes in A2G. In that case IFN, although present in smaller quantities, would specifically protect vulnerable A2G cells more effectively against influenza virus replication than it does similar cells in susceptible mouse strains. One example of such virus-specific IFN activity has been reported in mice naturally resistant to arboviruses (33), but has neither been observed elsewhere nor confirmed.

We have considered the possibility that CY and X irradiation fail to suppress the activity of a unique brain-based immunological system in A2G which is refractory to immunosuppression. This idea is untenable for several reasons. First, the immunosuppression was effective at the level of the brain tissues because of the reproducible and striking absence of inflammatory infiltrates in the brains of CY-treated and X-irradiated A2G concurrently resisting infection (25). Furthermore, no cellular lymphoid elements in A2G brains are missing from A/J brains. Finally, A2G brains do not contain a preexisting inhibitor capable of stopping virus replication in susceptible brains (23).

Although the majority of the i.c. inoculum spills over into the bloodstream (5, 28), the distribution of viral antigen in A/J brain sections early in the infection does not suggest a reentry of virus from blood into the parenchyma or growth from the edges of the i.c. stab wound (16). On the contrary, the data suggest a direct spread of the virus from the ventricular surfaces into the brain in A/J mice. Electron microscope studies of this infection in susceptible mice by others have revealed that virion formation is limited to the free surface of the ependymal cells lining the ventricles (10). This, coupled with the fact that the intercellular spaces in normal mammalian nervous tissues are probably no larger than 20 nm (10, 16), means that influenza virions which are about 100 nm in diameter must have great difficulty infecting parenchymal cells by free diffusion from the ependymal lining into the parenchyma. This suggests that spread of the virus from the ventricles into the parenchyma could involve actual cell-to-cell transfer of infectious virus. If this were so, antibody and cell-mediated effector mechanisms might have to be augmented by nonspecific inflammatory cell activities for the infection to be arrested, as has been demonstrated with herpesvirus infections (24, 34). As an explanation for the clear-cut resistance of

immunosuppressed A2G this possibility seems remote, since both specific immune and nonspecific inflammatory activity are not evident in such mice.

Finally, the possibility exists that the resistance is explicable in terms of the failure of A2G cells to support neurotropic influenza replication. This putative replication defect, however, must be highly selective, i.e., must occur in some but not all cells of the A2G mouse, since WSN can undergo complete cycles of replication in ependymal cells of the ventricles and in at least some choroid epithelial cells (Fig. 6). In addition, the virus can grow in cultured A2G kidney cells (21) and complete replication must certainly occur in some cells of the lung (23). Hepatotropic influenza virus can also replicate in cells of A2G liver (J. Lindenmann, personal communication).

Recent results by Allison and co-workers have suggested that cultured A2G peritoneal macrophages fail to support replication of fowl plague influenza A virus (A. C. Allison, personal communication). This finding is analogous to that of Shif and Bang in studies on the genetic resistance of C3H mice to mouse hepatitis virus (37, 38). There are, however, several noteworthy differences between the C3H and A2G systems. While thymectomy of newborn C3H mice prevents development of their macrophage-based resistance to mouse hepatitis virus (1, 40), thymectomy of newborn A2G mice has no effect on the development of their resistance to influenza. Furthermore, whereas CY immunosuppression leaves A2G resistance to influenza intact, it effectively abolishes the genetic resistance of C3H mice to mouse hepatitis virus (40).

It is difficult to conceive how A2G macrophage resistance could account for the failure of virus to invade the parenchyma of normal A2G from the ependymal lining, since few macrophages are evident at primary infection sites early in infection (less than 24 h PI) and a mononuclear infiltrate is never seen in immunosuppressed A2G mice resisting infection (26). Nevertheless, the intrinsic resistance of wandering A2G macrophages to WSN infection could be important to the fate of the animal if these cells are involved in transport of infectious virus from the ventricles to the deep parenchyma where intrinsically susceptible cells could then be infected by virus.

Any mechanism ultimately elucidated to explain resistance to lethal intracerebral influenza infection must then be examined to test its applicability to the known but yet unstudied resistance of A2G lung to pneumotropic influenza (23). Although it seems unlikely that

diverse resistance mechanisms exist and function in different organs of the same A2G mouse, only future experiments can provide an answer to this and other questions raised by this study.

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