JOURNAL OF VIROLOGY, Dec. 1986, p. 1062–1067
0022-538X/86/121062-06$02.00/0

Vol. 60, No. 3

Influenza Virus-Induced Encephalopathy in Mice: Interferon Production and Natural Killer Cell Activity During Acute Infection

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Received 17 March 1986/Accepted 15 August 1986

Mice injected intracerebrally with infectious influenza virus (60 hemagglutinin units) developed lethargy, seizures, comas, and died 2 to 5 days postinfection. As early as 6 h after infection, the cerebrospinal fluid (CSF) in these animals was infiltrated with polymorphonuclear cells, mononuclear leukocytes, and large granular lymphocytes. Potent natural killer (NK) cell activity was observed for both CSF and spleen cell populations over the same period. This NK cell activity correlated with interferon (IFN) levels in the CSF and serum. Treatment of lethally infected mice with either anti-IFNα-IFNβ or anti-ganglio-*n*-tetraoglyceramide antiserum ameliorated the disease, reduced mortality, and effected changes in the relative proportions of inflammatory cell populations infiltrating the CSF. The possible significance of IFN and NK cell activity in the development of this influenza virus-induced encephalopathy is discussed.

Viral encephalitis is a common disease (20, 26), but the specific virus involved often cannot be isolated and remains unidentified (5, 20, 21). Disease is often manifested after the virus is detectable (4, 7, 16, 22, 24). Mice infected intracerebrally (i.c.) with lymphocytic choriomeningitis virus (10, 35), ectromelia virus (13), vaccinia virus (8, 9), and influenza virus (1, 14, 15, 23) can develop encephalitis and fatal virally induced lesions. Mims (23) found that ependymal and meningeal cells were infected by both neuroadapted and nonneuroadapted strains of influenza virus inoculated i.c., but that the nonneuroadapted virus strains were only capable of a single cycle of growth. Inoculation of a 10^6 50% infectious dose or greater was lethal to the mice, and death was attributed directly to the number of infectious particles in the inoculum. Experiments in our laboratory have shown that after i.c. inoculation with influenza virus, low titers of infectious virus and the hybridizable viral genome do persist for several days postinfection (p.i.) in the brain (1).

Influenza virus-induced encephalopathy in mice is marked by brain edema, local inflammation, lesions in the ependymal and meningeal cells (14, 23), histological damage of the liver accompanied by fatty infiltration (6), and biochemical dysfunction including hyperammonemia (6). The appearance of lethargy, convulsions, coma, and high morbidity within 2 to 5 days p.i. (1) suggests that death occurs before any protective immune response is mounted (1, 8). Recent studies, however, have shown that infections with lymphocytic choriomeningitis virus (30, 32, 33), vaccinia virus (8), and influenza virus (1) elicit cytotoxic cells with the properties of natural killer (NK) cells, both systemically and locally, before initiating a specific T-cell response.

In the present studies we examined some immunopathological effects in mice of an influenza virus-induced encephalitis in which virus replication was limited. The cerebrospinal fluid (CSF) of mice infected i.c. with influenza virus A/PR/8/34 was analyzed for the presence and phenotype of inflammatory cells, in particular NK cells, and for the presence of interferon (IFN), which is known to potentiate NK cell activity. Local IFN levels and NK cell activity in the CSF were compared with those found in serum.

MATERIALS AND METHODS

Animals. Male BALB/c mice (8 to 10 weeks old) were obtained from Jackson Laboratories, Bar Harbor, Maine, and Dominion Laboratories, Dublin, Va.

Viruses. Influenza virus A/PR/8/34 (A/PR/8; H1N1) was grown in 10-day-old embryonated eggs (SPAFAS, Inc., Norwich, Conn.). The infectious allantoic fluid was harvested 36 to 40 h later and titrated as previously reported (1). Virus inactivation was accomplished by subjecting 2 to 3 ml of infectious allantoic fluid in a petri dish (60 by 15 mm) to 6-W of UV light at a distance of 15 cm for 10 min. The virus suspension was stirred every 2 min by swirling the petri dish. The inactivated viral preparation contained no residual infectious virus when assayed as previously described (1).

Inoculation of mice with virus. Mice were inoculated i.c. with virus at either a lethal (60 to 100 hemaglutinating units [HAU]; 30 to 50 μl) or a sublethal dose (30 HAU). In experiments to analyze the role of virus infectivity in the observed mortality, mice were inoculated i.c. with 100 HAU of UV-inactivated virus. This dose was the equivalent of the maximum lethal dose of live virus administered to any of the mice. Control mice were inoculated with 50 μl of normal egg allantoic fluid or left untreated. If animals died within 6 h p.i., their deaths were attributed to trauma and not to influenza virus-induced encephalopathy. For the kinetic studies, inoculation times were established; harvesting of CSF and ^{51}Cr-release assays were performed simulatenously for all groups.

Treatment of mice with antiserum. Immediately after i.c. inoculation with a lethal dose of infectious virus, mice were injected intravenously with 0.5 mg of anti-ganglio-*n*-tetraoglyceramide (anti-asialo-GM1) antiserum (Wako Pure Chemicals, Dallas, Tex.). This antiserum reacts with mouse and rat NK cells and immature mouse thymocytes and depletes NK activity in the spleens of mice (19). Another group of lethally infected mice was similarly treated with 10^6 neutralizing units (100 μl) of sheep anti-mouse IFN antiserum (kindly provided by D. Murasko, Medical College of

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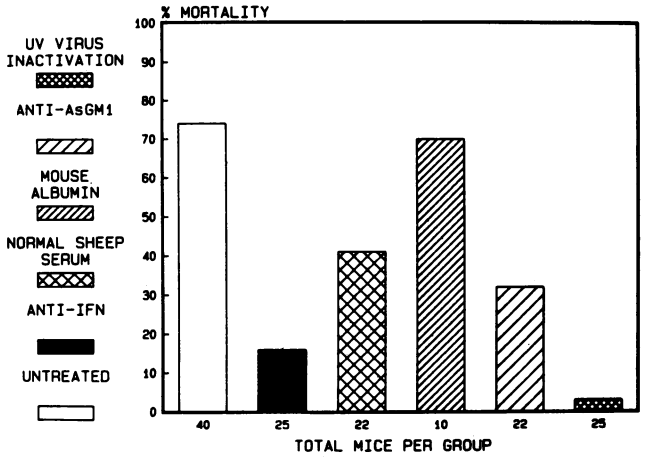

FIG. 1. Mortality of mice inoculated i.c. with influenza virus A/PR/8. Eight-week-old male BALB/c mice inoculated with a lethal dose (60 HAU) of infectious virus exhibited >70% mortality within 2 to 5 days p.i. Treatment of mice with UV-inactivated virus reduced the mortality to <5%. Mice receiving a sublethal dose of 30 HAU survived (data not shown). Decreased mortality was also observed in mice treated with either anti-IFN or anti-asialo-GM1 (anti-AsGM1) antiserum immediately after i.c. inoculation of a lethal dose of virus.

Pennsylvania, Philadelphia). Normal rabbit serum, normal sheep serum (obtained through the University of Pennsylvania Veterinary Hospital, Philadelphia), or mouse albumin (0.5 mg) were used as respective controls. When tested for the ability to inhibit the infectivity of egg-grown A/PR/8 on Madin-Darby canine kidney cells in vitro, these normal sera did not reduce the titer of infectious A/PR/8 virus (data not shown).

Collection of CSF and preparation of cytolytic effector cells. Mice were anesthetized with 2-bromo-2-chloro-1,1-trifluoroethane (Halocarbon Laboratories, Hackensack, N.J.). The skin was removed to expose the dura covering the cisterna magna. A small opening was made in the dura by using a 26-gauge needle, and the CSF was aspirated with a 25-μl micro-cap pipette (Drummond Scientific Co., Broomall, Pa.). The CSF was aseptically harvested, pooled, and stored on ice until assayed for NK and IFN activities.

Effector cytolytic cells were prepared by centrifugation of CSF samples pooled from 10 to 30 mice per experimental group, whereas splenocyte effectors were prepared by pooling spleens from 4 to 5 mice. Cell suspensions from the spleens of experimental animals or appropriate controls were obtained by using Ten Broeck tissue grinders (A. H. Thomas, Philadelphia, Pa.). Splenocytes were washed three times and suspended in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS).

NK cytotoxicity assay. NK lytic activity was tested in a 4-h ^{51}Cr-release assay by incubating effector cells serially diluted in DMEM supplemented with 10% FCS in 96-well microtiter plates at 37°C with 10^4 ^{51}Cr-labeled YAC-1 lymphoma target cells. For maximal ^{51}Cr release, 100 μl of 0.1% sodium dodecyl sulfate was added to 100 μl of 10^4 target cells. Spontaneous release, determined by using 10^4 target cells in 200 μl of medium without effector cells, was always less than 10% of the maximum detergent release.

Percent cytotoxicity was calculated according to the formula: % specific ^{51}Cr release = (cpm test sample − cpm spontaneous)/(cpm maximum − cpm spontaneous) × 100.

IFN assay. IFN was assayed by testing the ability of mouse serum and cell-free CSF to protect monolayers of L929 (H-2K) adherent cells from the cytopathic effect (CPE) of encephalomyocarditis virus as previously described (25). Briefly, twofold serial dilutions of test samples in triplicate were made in DMEM (0.1 ml per well) supplemented with 10% FCS in 96-well microtiter plates. A reference sample of IFNα-IFNβ was tested in parallel, and normal mouse serum and culture medium served as negative controls. Sample-containing plates were irradiated (5,000 rads) and incubated with 2×10^4 L929 cells in 0.1 ml of DMEM supplemented with 10% FCS for 24 h at 37°C in a 5 to 10% CO_2 atmosphere. At confluence, cells were inoculated with 5,000 PFU of encephalomyocarditis virus in 50 μl of DMEM. Cultures were then incubated under the same conditions until the control cultures showed 100% CPE. The medium was aspirated from each well, and the monolayers were stained with 0.5% crystal violet in 70% methanol for 5 min, washed in tap water, and air dried. The IFN titer was read as the reciprocal of the dilution that protected 50% of the cell monolayer. Values were corrected against the mouse IFNα-IFNβ reference standard and expressed in units per milliliter of fluid.

Histological preparations. Inflammatory cells from the CSF were washed twice and suspended at 10^6 cells per ml in DMEM supplemented with 10% FCS. Histological slides were prepared by cytocentrifugation of 100 μl (10^6 cells) of the suspension in a Shandon-Elliot Cytospin (Shandon Southern Instruments, Sewickley, Pa.) and by Wright staining. Cell forms were analyzed by light microscopy, and relative proportions of cell types were evaluated.

RESULTS

Mortality. Consistent with previous reports (1, 14, 23), mice infected i.c. with a lethal dose of infectious virus exhibited high morbidity (>70% mortality) by 2 to 5 days p.i. (data not shown). In contrast, UV-inactivated virus was not a potent inducer of mortality (Fig. 1). Lethally infected mice treated with either anti-IFN or anti-asialo-GM1 exhibited reduced mortality (Fig. 1). Administration of mouse albumin did not alter the percent mortality, whereas treatment with normal sheep serum significantly reduced the percent mortality for reasons which are still unclear.

Kinetics of NK cell activity. Mice were inoculated i.c. with an infectious lethal dose, a sublethal dose, or a UV-inactivated dose of influenza virus A/PR/8. At various times CSF pooled from groups of 10 to 30 mice and splenocytes from infected and control mice were collected and assayed for NK activity on ^{51}Cr-labeled YAC-1 lymphoma target cells. There was an increase in splenic NK cell activity as early as 6 h p.i. in all three experimental groups, with the highest activity in mice that received a lethal dose of infectious virus (Table 1). By contrast, CSF-derived effectors from mice inoculated with UV-inactivated virus exhibited a higher cytotoxicity than those obtained from infectious virus-inoculated mice. Mice that received a lethal dose of infectious virus showed peak NK cell activity in the CSF at 24 h p.i. Because mice in this group were dying, insufficient CSF was available for evaluation at additional times. Mice receiving either a sublethal dose or UV-inactivated virus, however, exhibited NK activity in the CSF even at 48 h p.i. Splenocyte NK activity was more transient, declining quickly after 24 h compared with CSF NK activity.

Role of NK cell activity in influenza virus-induced encephalopathy. To investigate the role of NK cell cytotoxicity in the development of influenza virus-induced encephalopathy,

TABLE 1. Kinetics of NK activity in BALB/c mice inoculated i.c. with influenza virus A/PR/8

Treatment	NK activity (% specific ^{51}Cr release) in[a]:					
	CSF			Spleen		
	10:1[b]	3:1	1:1	100:1	33:1	11:1
Lethal dose (60 HAU)						
6 h	6	1	1	32	20	9
12 h	11	4	3	44	25	14
24 h	15	2	1	17	15	7
48 h	ND[c]	ND	ND	7	3	2
Control				14	8	8
Sublethal dose (30 HAU)						
6 h	12	2	0	22	10	4
12 h	20	7	2	31	13	6
24 h	18	9	0	34	22	9
48 h	23	10	4	7	1	0
Control				13	4	0
UV-inactivated dose						
6 h	31	13	11	9	9	5
12 h	28	10	5	17	13	9
24 h	13	11	8	15	12	5
48 h	22	14	8	4	5	9
Control				6	7	3

[a] Assayed as described in Materials and Methods, using pooled CSF from 10 to 30 mice and spleens from four mice.
[b] Effector/target ratios.
[c] ND, Not determined due to insufficient cell numbers.

mice were inoculated i.c. with a lethal dose of infectious virus followed immediately by an intravenous injection of anti-asialo-GM1. Control mice were given normal rabbit serum. In vivo inoculation of anti-asialo-GM1 abolished NK activity in both the spleen and the CSF (Table 2). Normal rabbit serum did not affect splenocyte NK activity but seemed to exert a minor suppressive effect on CSF-derived NK cell activity. These findings correlated with the observed decrease in mortality (Fig. 1).

Kinetics of IFN production. To determine the correlation between NK cell activity and the level of IFN, CSF and sera from the same groups of mice used in the preceding experiment were collected and tested for IFN activity. The data (Table 3) indicate IFN production as early as 6 h p.i., with peak activity observed at 12 h, except in the case of CSF from animals inoculated with UV-inactivated virus, in which peak IFN levels occurred at 6 h p.i. In general, the CSF showed higher levels of IFN activity per milliliter than did the serum.

TABLE 2. Effect of in vivo administration of anti-asialo-GM1 (0.5 mg) on NK cell activity in BALB/c male mice inoculated i.c. with a lethal dose of influenza virus A/PR/8

Treatment	Time (h)	NK activity (% specific ^{51}Cr release) in[a]:			
		CSF		Spleen	
		10:1[b]	3:1	100:1	33:1
A/PR/8 alone	12	12	7	21	16
	24	23	10	17	14
A/PR/8 + anti-asialo-GM1	12	4	3	2	4
	24	2	2	2	1
A/PR/8 + rabbit serum	12	9	5	30	13
	24	13	8	18	11

[a] Pooled CSF and pooled spleen were as described in Table 1, footnote *a*.
[b] Effector/target ratios.

TABLE 3. Kinetics of IFN production in BALB/c mice inoculated i.c. with influenza virus A/PR/8

Time (h)	IFN titer (U/ml) in[a]:					
	Serum			CSF		
	Lethal dose	Sublethal dose	UV-treated	Lethal dose	Sublethal dose	UV-treated
6	138	138	275	344	344	2,750
12	275	275	550	688	688	688
24	69	69	275	344	344	344
48	9	9	9	ND[b]	344	344

[a] Based on ability of IFN to protect monolayers of L929-adherent cells from the CPE of encephalomyocarditis virus as described in Materials and Methods; IFN titers in serum and CSF were 0 in normal uninfected mice.
[b] ND, Not determined.

Effect of anti-IFN antiserum on NK activity. Mice were inoculated i.c. with a lethal dose of infectious virus followed immediately by the intravenous administration of 100 μl (10^6 neutralizing units) of sheep anti-mouse IFN. Control mice received normal sheep serum. The data (Table 4) indicate that anti-IFN treatment abolished NK cell activity in the spleen and reduced the NK activity of CSF-derived effectors three- to fourfold. Normal sheep serum had no suppressive effect on splenocyte NK activity, but the CSF-derived effectors exhibited a twofold reduction in cytotoxicity. These findings also correlated with the reduced data (Fig. 1).

Effect of anti-IFN and anti-asialo-GM1 on CSF inflammatory cells. To investigate the relative frequency of different types of inflammatory cells in mice lethally inoculated with virus, slides containing CSF cells were prepared and examined by light microscopy. Figure 2 shows the distribution of different cell types. There was a predominantly polymorphonuclear pleocytosis in the CSF of influenza virus-inoculated mice. Segmented polymorphonuclear cells constituted 68% of total cells at 12 h p.i. and 44% of cells at 24 h p.i., whereas mononuclear leukocytes made up only 15 to 20% of the cellular composition at both 12 and 24 h p.i. A major shift in the inflammatory cells infiltrating the CSF was observed in the proportion of large granular lymphocytes (LGLs) which showed an increase from 12% (at 12 h) to 37% (at 24 h). This finding correlated with NK cell activity in the CSF-derived

TABLE 4. Effect of in vivo administration of anti-IFN on NK activity in mice injected i.c. with a lethal dose of influenza virus A/PR/8

Treatment	Time (h)	NK activity (% specific ^{51}Cr release) in[a]:			
		CSF		Spleen	
		30:1[b]	10:1	100:1	33:1
A/PR/8 alone	12	24	5	30	11
	24	43	11	23	14
A/PR/8 + anti-IFN[c]	12	10	1	4	1
	24	12	3	4	1
A/PR/8 + normal sheep serum	12	13	3	37	13
	24	17	6	11	7

[a] Pooled CSF and pooled spleen were as described in Table 1, footnote *a*.
[b] Effector/target ratios.
[c] Sheep anti-mouse serum containing 10^6 neutralizing units of anti-IFN.

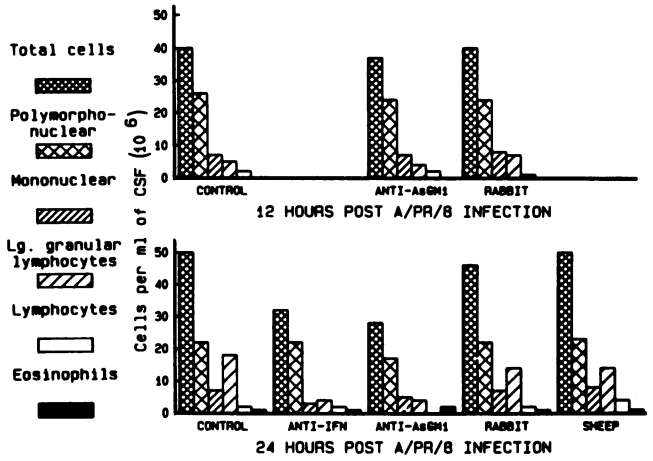

FIG. 2. The relative frequency of different cell types within the CSF exudate was determined by microscopic examination and enumeration. Polymorphonuclear leukocytes were predominant at both 12 and 24 h. A major increase (from 12 to 37%) was observed in the number of infiltrating LGLs between 12 and 24 h. The infiltration of LGL and mononuclear leukocytes was diminished by anti-IFN treatment. Similarly, anti-asialo-GM1 (anti-AsGM1) administration reduced the numbers of LGLs and mononuclear leukocytes (to a lesser degree) and completely abolished mature lymphocytes from the CSF exudate. Control mice were A/PR/8 infected but not serum treated.

effectors. Treatment of infected mice with anti-asialo-GM1 reduced the population of LGLs at 24 h p.i. from 37 to 12%, completely obliterating the population of mature lymphocytes but not significantly affecting the proportions of polymorphonuclear or mononuclear cells. Similarly, administration of anti-IFN reduced the population of LGLs at 24 h p.i. from 37 to 10%. Anti-IFN-treated mice, however, exhibited a twofold reduction in the number of mononuclear cells infiltrating the CSF without altering the proportion of mature lymphocytes. Neither rabbit nor sheep control serum altered the proportions of cells entering the CSF.

Effect of in vivo administration of anti-asialo-GM1 and anti-IFN on IFN concentration in CSF and serum. In vivo administration of anti-IFN to lethally infected mice completely abolished NK cell activity of splenocyte-derived effectors but failed to abolish all the NK activity mediated by the CSF-derived effectors. To determine whether this residual NK activity correlated with small amounts of IFN, serum and CSF from infected mice that had also been treated with anti-IFN were tested for IFN activity. The effect of anti-asialo-GM1 on IFN concentration was similarly investigated. There was no detectable IFN in either sera or CSF after treatment of infected mice with anti-IFN (Table 5). Mice receiving anti-asialo-GM1, however, produced normal levels of IFN, despite the reduction in mortality.

DISCUSSION

The pathogenesis of influenza virus-induced encephalopathy requires an interaction between infectious virus and the host immune response. We previously showed that the titer of virus in the brains of mice inoculated with large doses of influenza virus declined rapidly with no indication of a complete replicative cycle (1). Nevertheless, an abortive cycle of replication (23) may be necessary, as injection of UV-inactivated virus was not lethal.

Intracerebral inoculation of the non-mouse brain-adapted strain of influenza virus A/PR/8 induced a strong inflammatory response with a predominantly polymorphonuclear pleocytosis. The infiltration of in excess of 40×10^6 cells per ml into the CSF far exceeds the number (3×10^4 cells per ml) reported for Reye's syndrome patients (7) and the number (5×10^3 cells per ml) observed in mice infected intravenously with a lethal dose of influenza B virus (6). As reported previously for lymphocytic choriomeningitis virus (10, 35), vaccinia virus (8), and ectromelia virus (13), many of the cells infiltrating the CSF are either actively or passively involved in the inflammatory process, since there is normally no resident lymphoid tissue in the brain.

The inflammatory cells present during viral encephalitis were analyzed in ^{51}Cr-release assays for NK cytotoxic activity in the CSF and spleens of virus-infected mice. Potent nonspecific cytotoxic effector populations were detected within the CSF and spleens as early as 6 h p.i., but maximum cytotoxicity was observed at 12 and 24 h p.i. for spleens and for CSF-derived effectors, respectively. The relative level of lytic activity was generally higher for CSF effectors than for splenocytes, a finding consistent with previous observations for vaccinia virus-induced meningitis (8).

The possibility that NK cells were responsible for immunopathology in virus-induced encephalitis was studied by administration in vivo of anti-asialo-GM1 after i.c. inoculation of the lethal dose of infectious influenza virus. NK cells bear the differentiation antigen asialo-GM1 (2, 11, 18, 34), and treatment of mice with anti-asialo-GM1 in vivo has been shown to significantly reduced NK cell activity without affecting the lytic functions of cytotoxic macrophages (12) or cells that exert natural cytotoxicity (3, 17, 31). Cytotoxic T cells, however, have been shown to be affected (28). Such treatments resulted in a total abrogation of lytic activity (assessed in vitro) in both the spleen and the CSF exudate and in amelioration of the disease, with improved survival rate in lethally infected mice. The obliteration of nonspecific cytotoxicity was correlated with the decline in LGLs and mature lymphocytes. The anti-asialo-GM1 treatment had no significant detectable effect on the level of induced IFN in either the CSF or serum. In contrast to our observation of an increased survival of mice treated with anti-asialo-GM1 antiserum, others have shown that mice depleted of NK cells

TABLE 5. Effect of in vivo administration of anti-asialo-GM1 and anti-IFN on serum and CSF levels of IFN in BALB/c mice inoculated with a lethal dose of influenza virus A/PR/8

Group	IFN (U/ml)[a]			
	12 h		24 h	
	CSF	Serum	CSF	Serum
A/PR/8-infected				
Untreated	1,104	276	276	276
Anti-asialo-GM1	1,104	552	276	276
Anti-IFN	0	0	0	0
Sheep serum	1,104	276	552	276
Rabbit serum	552	552	276	276
Uninfected				
Mouse serum	0	0	0	0
Sheep serum	0	35	ND[b]	35
Rabbit serum	ND	0	ND	0

[a] Assayed in serum and CSF from mice in Tables 2 and 4; values were corrected to a reference standard and expressed as units per milliliter of test fluid.

[b] ND, Not determined.

by using anti-asialo-GM1 after intranasal infection with influenza virus A/PR/8 exhibit increased morbidity and mortality (27). One possible explanation is that this reflected differences in the site of infection (i.e., lungs versus nervous system), which could affect the ability of the virus to continue to replicate at the site of infection.

IFN is an important regulator of immune function and has been shown to augment the kinetics of lysis and the recycling ability of NK cells (2, 29). Treatment of lethally infected mice with anti-IFN abolished all NK activity in the spleen and suppressed the infiltration of LGLs into the CSF but did not abrogate all the NK activity of the CSF-derived effectors. This also correlated with an increase in survival of the mice.

An unexpected finding was the reduction of CSF NK activity, but not splenocyte NK activity, by treatment with normal sheep and rabbit sera. Normal sheep serum also reduced mortality in lethally infected mice. The results in Table 5 suggest that normal serum did not contain sufficient anti-IFN to neutralize IFN in infected animals, and uninfected control mice that received normal sheep serum produced low levels of IFN. The mechanism for the compartmentalized effect on NK cell activity and the factors within normal serum that mediate modulation of the disease remain unknown.

Whereas both the UV-treated virus and the sublethal doses of virus induced NK cell activity and IFN production in mice, they were not lethal. This suggests that a large dose of infectious virus is essential to the development of experimental viral encephalitis. Since there is a rapid decline of IFN and NK cell activity to normal levels by 48 h p.i. and the i.c. A/PR/8 infection is abortive (14, 15, 23), fatal lesions appear to be inflicted in the early phase of infection. The data appear to implicate both the virus and the host immune response in the pathogenesis of viral encephalitis, but the situation may be different for a wild-type strain of influenza virus which might be capable of complete replication.

Our results suggest a strong correlation between IFN and NK cell activity and pathogenesis and mortality in mice lethally infected i.c. with influenza virus. Although the application of this finding to other virus infections is unknown, it is possible that other demyelinating and pathogenic effects of viral encephalitis may be augmented by NK and IFN.

ACKNOWLEDGMENTS

We thank Marina Hoffman for editorial assistance.

This research was supported by Public Health Service grants NS-17752, AI-14162, and AI-20338 from the National Institutes of Health.

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