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Role of Immunity in Age-Related Resistance to Paralysis After Murine Leukemia Virus Infection

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Resistance to the paralytic effects of a wild mouse (Cas-Br-M) murine leukemia virus infection develops with age and is complete by 10 days of age in susceptible NFS mice. The possibility that cell-mediated immunity plays a significant role in this resistance was suggested by the observation that treatment of 10-day-old mice with antithymocyte serum rendered them susceptible to paralysis. By comparison, mice rendered incapable of generating a humoral immune response by treatment from birth to 1 month of age with anti-immunoglobulin M serum did not develop paralysis after challenge with virus at day 10. Transfer of unseparated and T-cell-enriched populations of Cas-Br-M murine leukemia virus-immune spleen cells protected neonatally infected NFS recipients from paralysis; transfer of Cas-Br-M murine leukemia virus-immune populations enriched for B cells delayed the onset but did not ultimately protect neonatally infected NFS mice from paralysis. Transfer of naive adult spleen cells had no protective effect in neonatally infected NFS mice. High-level virus replication occurred in the spleens and brains of all mice that developed paralysis regardless of treatment; low-level virus replication in spleen and barely detectable replication in brain occurred in mice that remained clinically normal. These studies suggest that the age-acquired resistance to the paralytic effect of Cas-Br-M murine leukemia virus infection is immunologically mediated and that T cells may play a major role.

Wild mouse ecotropic retrovirus infection of feral (8, 9) and susceptible neonatal laboratory (22, 23) mice can result in paralysis and lymphoma, both of which occur in adult life. The incidence and latency of paralysis are both age and virus dose dependent (4, 12), and resistance to paralysis is complete by 10 days of age, even in the most susceptible laboratory strains (12). To determine if maturation of the immune response played a role in this age-related resistance to paralysis, we performed immune ablation and syngeneic spleen cell transfer experiments in susceptible NFS mice. Our results suggest that maturation of the immune response, particularly the T-cell component, may play an important role in resistance to wild mouse ecotropic murine leukemia virus (Cas-Br-M MuLV)-induced paralysis.

MATERIALS AND METHODS

Virus. The origin of Cas-Br-M MuLV (10) and its propagation on mouse embryo (SC-1) cells in our laboratory have been described previously (12). Cloned Cas-Br-M MuLV was grown on cells and titrated by the XC plaque assay (24). Pooled supernatants from Cas-Br-M MuLV-infected SC-1 cells were free of amphotropic, xenotropic, and recombinant mink cell focus-inducing viruses before inoculation.

Mice. Pregnant NFS/N mice were obtained from the Small Animal Section, Veterinary Resources Branch, National Institutes of Health, Bethesda, Md. This inbred strain is *Fv-1^n*, *H-2^{sq4}*, expresses low levels of endogenous xenotropic but no ecotropic MuLV (21), and has shown marked susceptibility to the neurological disease associated with Cas-Br-M MuLV infection (12). Observations for neurological disease as evidenced by tremulousness, inability to extend the hind limbs, hind limb weakness, or paralysis were made every 3 to 4 days after inoculation with Cas-Br-M MuLV. Mice were bled by orbital venipuncture and perfused or sacrificed by cervical dislocation when moribund or at 4 or 9 months of age. Spleens, brains, and thymuses were removed and fixed for light microscopy or homogenized and frozen at −70°C for virological studies.

Viral protein determination. A competition radioimmunoassay for MuLV p30 was performed on spleen and brain homogenates prepared in 0.01 M potassium phosphate buffer containing 1% Triton X-100. The assay utilizing ^{125}I-labeled Rauscher MuLV p30 and goat anti-wild mouse ecotropic MuLV (a gift from Murray Gardner) has been previously described (11). Results were expressed as nanograms of MuLV p30 per milligram of tissue protein.

Antithymocyte and anti-mu serum treatment. Rabbit anti-mouse thymocyte serum (ATS) was purchased from M. A, Bioproducts, Walkersville, Md. Before use, the serum was heat inactivated (56°C, 30 min) and absorbed exhaustively with a mixture of mouse erythrocytes and bone marrow cells. Nine-day-old NFS mice were inoculated intraperitoneally (i.p) with 0.1 ml of ATS at 2-day intervals for 10 days. In some groups of mice, inoculations were continued twice weekly for an additional 2 weeks. Previous studies of mice treated by this procedure showed them to be markedly depleted of T cells; less than 5% of spleen cells from treated mice were Thy-1$^+$, proliferative responses to stimulation with phytohemagglutinin were absent, and no antibody-secreting plaque-forming cells (PFC) were observed 5 days after immunization with sheep erythrocytes (H. C. Morse, unpublished data). However, the frequency of surface immunoglobulin-positive B cells in the spleens of treated mice was normal or increased, and their function measured by the PFC response 4 days after immunization with type III

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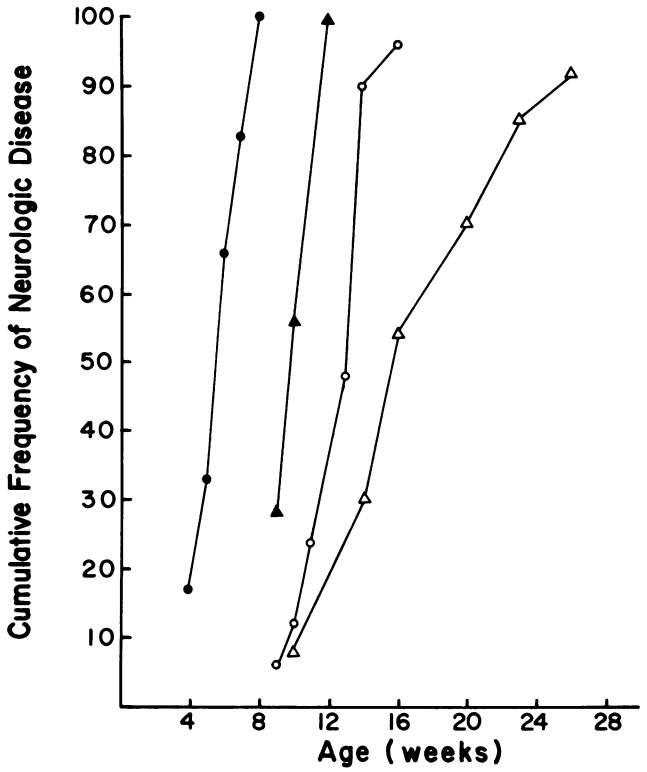

FIG. 1. Neurological disease occurring after Cas-Br-M MuLV inoculation and ATS treatment of NFS mice. Neurological signs occurred in all NFS mice inoculated i.c. before 2 days of age with Cas-Br-M MuLV ($10^{5.6}$ PFU/ml, $n = 12$ [●], or $10^{4.9}$ PFU/ml, $n = 14$ [▲]). NFS mice treated with ATS for 10 days or 3.5 weeks beginning at 9 days of age and inoculated with Cas-Br-M MuLV i.c. at 10 days of age also developed neurological disease (16 of 17 mice receiving $10^{5.6}$ PFU/ml [○] and 11 of 12 mice receiving $10^{4.9}$ PFU/ml [△]). None of 20 mice inoculated i.c. with either dose of Cas-Br-M MuLV at 10 days of age developed neurological disease in the absence of ATS treatment.

pneumococcal polysaccharide was equivalent to that of nude mice of the same strain (20; Morse, unpublished data).

Newborn NFS mice were given 30 daily doses (0.05 to 0.10 ml i.p) of goat anti-mouse immunoglobulin M heavy-chain sera (AMS) prepared as described previously (18). Sera from AMS-treated mice showed no detectable immunoglobulin M by double diffusion in agar. Additional analysis of 10 of these sera showed that 9 also contained antibodies to immunoglobulin M. Mice with circulating anti-immunoglobulin M antibodies were previously shown to lack splenic immunoglobulin-positive cells and to be incapable of producing PFC after immunization with sheep erythrocytes or type III pneumococcal polysaccharide (20; Morse, unpublished data).

Cas-Br-M MuLV (0.03 ml) was inoculated intracerebrally (i.c.) into untreated and AMS- or ATS-treated mice on day 10. Eighty percent of inoculated mice survived the AMS treatment, 95% survived the ATS treatment, and none in either group died of intercurrent infection.

Spleen cell preparation and transfer. Normal adult and Cas-Br-M MuLV-immune adult NFS mice served as spleen cell donors. Twelve- to 14-week-old NFS mice received three injections of Cas-Br-M MuLV (10^4 PFU i.p or intramuscularly) over 8 weeks. Mice with high Cas-Br-M MuLV antibody levels in sera 2 weeks after the last injection (determined by indirect immunofluorescence, using Cas-Br-M MuLV-infected SC-1 cells) were chosen as donors. Single spleen cell suspensions were prepared by gentle flushing through a 25-gauge needle, and erythrocytes were lysed by the addition of ammonium chloride lysing buffer. The spleen cell suspensions were washed twice before suspension in phosphate-buffered saline at a concentration of 2×10^8 cells per ml for direct transfer to neonatal NFS recipients. Recipients were injected with 0.05 ml (i.p.) of unseparated spleen cell suspension (10^7 cells) or suspensions enriched for T or B cells (see below). Recipient mice were given suspensions containing 3.5×10^6 T cells or 6.5×10^6 B cells, which corresponded to the numbers of T and B cells in the unseparated spleen cell preparations. Mice were inoculated with Cas-Br-M MuLV (0.03 ml i.c.) from pools with titers of $10^{5.6}$ or $10^{4.9}$ PFU/ml 8 h after transfer of the separated or unseparated spleen cells.

Spleen cell separations. Immune and nonimmune spleen cells were separated into T- and B-cell-enriched populations by adherence to antibody-coated tissue culture dishes as described previously in detail (5, 19). The adherent population contained 85 to 89% B cells, whereas the nonadherent T-cell-enriched population contained 3 to 5% B cells as determined by direct immunofluorescence, using fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin (Cappel Laboratories, Cochranville, Pa.).

A total of 1 to 8% of the cells in unseparated spleen cell suspensions were stained with a nonspecific esterase stain for macrophages (13). In some experiments, macrophages were depleted from unseparated spleen cells and from enriched T- and B-cell populations by using a previously described adherence technique (17). After a 15-min incubation period on fetal calf serum-coated tissue culture dishes, 1 to 2% of nonadherent cells were stained with a nonspecific esterase stain.

Antibody determination. Antibodies specific for Cas-Br-M MuLV were quantitated by flow microfluorometry. Normal SC-1 cells or SC-1 cells chronically infected with Cas-Br-M MuLV were treated with 20 μl of sera for 30 min at 4°C, washed twice, and then treated with 10 μl of purified fluorescein isothiocyanate-labeled goat anti-mouse Fab that detects all classes of mouse immunoglobulin (goat 123; 6). After 30 min at 4°C and two more washes, the cells were

TABLE 1. Neurological disease in Cas-Br-M MuLV-infected NFS mice

Treatment[a]	Neurological disease	
	Incidence	Onset (wk post-inoculation)[b]
None	12/12	4–8
Immune spleen cells	1/12[c]	10
Immune T cells	2/12[c]	8–9
Immune B cells	15/16	5–13
Nonimmune spleen cells	10/10	5–8
Nonimmune T cells	14/14	4–6
Nonimmune B cells	17/17	4–8

[a] Newborn to 2-day-old NFS mice received 10^7 spleen or equivalent T or B cells i.p. followed 8 h later by Cas-Br-M MuLV at $10^{5.6}$ PFU/ml i.c. T-cell-enriched populations used for transfer contained 2 to 5% immunoglobulin-positive cells and 1% macrophages; B-cell-enriched populations contained 90 to 94% immunoglobulin-positive cells, 1 to 2% macrophages, and 4 to 8% non-immunoglobulin-positive cells.

[b] Onset of neurological signs.

[c] Significantly different from Cas-Br-M MuLV without treatment, $P < 0.001$.

activated cell sorter (FACS II; Becton Dickinson and Co., Mountain View, Calif.) under conditions previously described (21). For each serum sample, specific mean fluorescence values were calculated by subtracting the mean fluorescence value obtained with uninfected cells from that obtained with infected cells. This method for antibody determinations was chosen over virus neutralization or antibody-dependent cell-mediated cytoxicity assays because of its high sensitivity and because it detects all virus-specific antibodies, regardless of immunoglobulin class (6).

RESULTS

Neonatal NFS mice inoculated i.c. at 2 days of age with pools of Cas-Br-M MuLV containing $10^{5.6}$ or $10^{4.9}$ PFU/ml developed paralysis with mean onsets of 6 and 9 weeks, respectively (Fig. 1). By comparison, none of the 20 mice inoculated at 10 days of age with the same Cas-Br-M MuLV pools developed neurological signs.

A series of experiments were undertaken to determine whether this age-related resistance to paralysis was immunologically mediated. NFS mice were treated for 10 days or 3.5 weeks with a preparation of ATS that depletes almost all T cells but leaves B cells functionally intact. Mice treated with ATS i.p. beginning on day 9 and Cas-Br-M MuLV ($10^{5.6}$ or $10^{4.9}$ PFU/ml i.c.) on day 10 had mean onsets of neurological disease at 13 or 17 weeks (Fig. 1). ATS treatment for 3.5 weeks produced no additional effects from those seen after ATS treatment for 10 days. In contrast, none of 10 NFS mice inoculated i.c. at 10 days of age with Cas-Br-M MuLV ($10^{5.6}$ PFU/ml) but rendered incapable of producing antibodies by treatment with AMS from birth to 30 days of age developed neurological signs during a 9-month observation period.

Syngeneic spleen cell transfer experiments were performed in neonatal NFS mice to further evaluate the roles of humoral and cellular immunity in resistance to MuLV-induced paralysis. Three different preparations of immune or normal adult spleen cells were given i.p 8 h before challenge with Cas-Br-M MuLV. These preparations included unfractionated spleen cells and spleen cells enriched for or depleted of B cells by "panning" on anti-immunoglobulin-coated plates. Eleven of 12 recipients of unfractionated immune spleen cells and 10 of 12 recipients of a B-cell-depleted, T-cell-enriched preparation failed to develop neurological signs over a 16-week observation period (Table 1). In contrast, 15 of 16 recipients of a B-cell-enriched, T-cell-depleted preparation of immune spleen cells developed paralysis within 13 weeks of Cas-Br-M MuLV inoculation (Table 1). Newborn NFS recipients of unfractionated, T-cell-enriched, or B-cell-enriched normal adult spleen cells all developed neurological disease by 8 weeks post-Cas-Br-M MuLV inoculation (Table 1).

TABLE 2. Cas-Br-M MuLV antibody levels in sera from Cas-Br-M MuLV-infected NFS mice

Group	Clinical status	Treatment	Antibody levels[a]
A (n = 22)	Normal	Cas-Br-M MuLV (day 10), AMS, immune spleen, immune T cells	560 ± 45
B (n = 30)	Paralysis	Cas-Br-M MuLV (day 2), nonimmune spleen, non-immune T or B cells	464 ± 50
C (n = 9)	Normal	Immune T cells	416 ± 54
D (n = 13)	Paralysis	Immune B cells	702 ± 90[b]

[a] Mean fluorescence units ± standard error determined with a FACS II.
[b] Significantly different from group B or C, $P < 0.05$.

Sera obtained from mice used in these immune ablation and syngeneic transfer experiments were tested by flow microfluorometry for antibodies to Cas-Br-M MuLV (Table 2). No virus-specific antibodies were detected in sera obtained from mice during the course of treatment with AMS (data not shown). However, after termination of AMS treatment (Table 2), various levels of antibody were detected in sera. The levels of circulating antibodies to Cas-Br-M MuLV in sera from paralyzed recipients of unfractionated or T- and B-cell-enriched normal spleen cells did not differ significantly from the levels in sera from clinically normal mice treated with AMS or recipients of unfractionated or immune T-cell-enriched populations (Table 2). By comparison, antibody levels in sera from paralyzed recipients of immune B-cell-enriched populations were significantly higher than those in sera from clinically normal recipients of immune T-cell-enriched populations (Table 2).

To determine the effects of immune ablation and syngeneic spleen cell transfer on virus replication, homogenates of brains and spleens from mice in the different treatment groups were assayed for levels of MuLV p30 expression (Fig. 2). The levels of MuLV p30 in tissues from ATS-treated mice infected with Cas-Br-M MuLV at 10 days of age increased during the 4 to 8 weeks after infection and, at the onset of paralysis, were significantly higher than in tissues of untreated mice infected at the same age (Fig. 2A). The MuLV p30 levels in brains of paralyzed ATS-treated mice infected at day 10 were comparable to those in brains of paralyzed mice infected with Cas-Br-M MuLV at 2 days of age (Fig. 2B). In contrast, MuLV p30 levels in tissues of AMS-treated mice infected at day 10 were comparable to those in tissues of normal mice inoculated with Cas-Br-M MuLV at the same age; however, both groups of mice infected at day 10 had significantly lower levels than mice infected at 2 days of age (Fig. 2A and B). NFS recipients of immune T-cell-enriched spleen cell preparations had MuLV p30 levels equivalent to those of NFS mice infected at day 10 (Fig. 2A) but significantly lower than those of NFS mice infected at 2 days of age.

DISCUSSION

Previous studies demonstrated that mice infected from birth to 6 days of age with Cas-Br-M MuLV developed clinical neurological disease whereas mice inoculated at 10 days of age exhibited no neurological abnormalities (12). This study confirms our previous results (Fig. 1). To determine if the age-related resistance to Cas-Br-M MuLV-induced paralysis was immunologically mediated, immune ablation and syngeneic transfer experiments were undertaken.

Mice treated with AMS from birth to 30 days of age were devoid of functional B cells but developed resistance to paralysis when inoculated with Cas-Br-M MuLV at 10 days of age (Table 1). The transfer of B-cell-enriched populations of syngeneic immune spleen cells to neonatal mice failed to protect the recipients from paralysis (Table 2). Cas-Br-M MuLV-inoculated mice developed similar levels of Cas-Br-M MuLV antibody in serum, regardless of neurological status (Table 2). Taken together, these data strongly suggest that B cells and their products do not contribute in a major way to the development of resistance to paralysis after Cas-Br-M MuLV infection. These findings should not be considered contradictory to other studies that showed that MuLV-associated paralysis could be inhibited by treatment at birth with antivirus antibodies (7). The amount of antibody transferred to those mice was apparently sufficient to inhibit

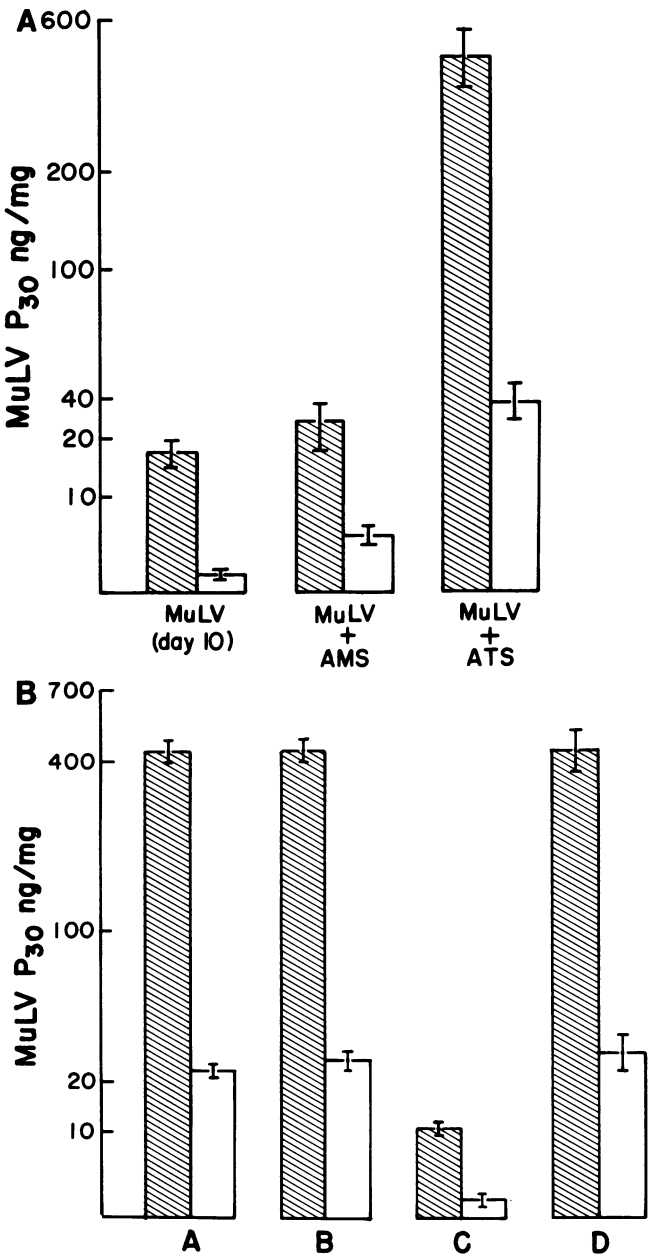

FIG. 2. Effect of ATS and AMS treatment on MuLV p30 expression (A) and effect of syngeneic adult spleen cell transfer on MuLV p30 levels (B). (A) MuLV p30 expression in the spleen (shaded bar) and brain (white bar) of Cas-Br-M MuLV-inoculated NFS/N mice. ATS treatment was begun at 9 days of age and continued for 10 or 24 days. AMS treatment was begun at birth and continued for 30 days. All mice were inoculated with Cas-Br-M MuLV ($10^{5.6}$ PFU/ml i.c.) at 10 days of age. MuLV p30 was measured at 16 to 24 weeks of age by competition radioimmunoassay, utilizing ^{125}I-labeled Rauscher MuLV p30 and goat anti-wild mouse MuLV antisera. The results from eight spleens and five brains (MuLV only), six spleens and three brains (MuLV plus AMS), and five spleens and five brains (MuLV plus ATS) were expressed as mean ± standard error (I) in nanograms per milligram of tissue protein. Uninfected NFS mice had spleen p30 levels of ≤6.0 ng/mg and brain p30 levels of ≤2.0 ng/mg. (B) Eight hours before Cas-Br-M MuLV ($10^{5.6}$ PFU/ml i.c.) inoculation, 2-day-old NFS/N mice received no cells (group A), 10^7 nonimmune spleen cells (group B), or equivalent numbers of immune T (group C) or B (group D) cells by i.p. transfer. Results represent mean ± standard error from 7 spleens and 10 brains (group A), 8 spleens and 9 brains (group B), and 4 brains and 4 spleens (groups C and D).

viremia (16) and reduced the level of replicating virus below that necessary to induce neurological disease. In the present study Cas-Br-M MuLV antibody sufficient to prevent neurological disease could not be generated by the transfer of immune B cells. Cas-Br-M MuLV, which rapidly replicates to high levels in neonatal tissues, was therefore capable of overwhelming the limited capacity of neonatal NFS mice to make antigen-specific T- and B-cell responses 4 to 6 days after infection. Once the infection has been established, antibody and immune cells are no longer capable of altering neurological disease expression (23). The finding that paralyzed adult NFS/N mice neonatally inoculated with Cas-Br-M MuLV have detectable Cas-Br-M MuLV antibodies in their sera differs from studies of congenitally infected paralyzed wild mice in which serum antibody against endogenous MuLV could not be detected by indirect immunofluorescence (16). Wild mice are exposed to a mixture of amphotropic and ecotropic MuLV from mother's milk (7–9). An oral exposure to virus that causes tolerance or the possibility that wild mice have a genetically determined low-level or absent antibody response to endogenous MuLV may account for this difference (14). Alternatively, by using flow microfluorometry, a highly sensitive method for detecting virus-specific antibodies regardless of class (6), we may have detected an antibody response not previously demonstrable by other techniques (12, 16).

The view that resistance to Cas-Br-M MuLV-induced paralysis is dependent on the activity of T cells is supported by the observations that mice depleted of functional T cells by treatment with ATS developed paralysis after inoculation with virus at day 10 (Fig. 1) and that adoptive transfer of T-cell-enriched populations of immune spleen cells inhibited the development of paralysis in mice inoculated with virus at 2 days of age (Table 1). The effect of these immune T cells transferred to neonatal NFS mice and that of the natural resistance which develops in 10-day-old NFS mice are similar and appear to affect the replication and dissemination of infectious Cas-Br-M MuLV (Fig. 2A and B).

Age-dependent T-cell-mediated resistance to the disease-producing effects of viral infection have been demonstrated for polyoma virus-induced tumors (1), lactate dehydrogenase virus-induced polioencephalomyelitis (3), and the oncogenic effects of an ecotropic MuLV infection in congenic strains of C57BL/10 mice (2). The genes controlling the T-cell-mediated antiviral response in C57BL/10 mice were linked to the I/E-C subregion of the H-2 complex (2). Using congenic strains of C3H mice, we were unable to show a linkage of the H-2 complex with genes mediating age-related resistance to Cas-Br-M MuLV neurological disease (P. M. Hoffman and H. C. Morse III, J. Virol., in press).

The observation that mice without functional B cells are as resistant to neurological disease at day 10 as untreated mice (Fig. 1) suggests that those T cells responsible for protection do not produce this effect through a T-cell–B-cell interaction. Immune interferon could not be implicated in resistance since interferon levels in spleens and brains of both paralyzed and immune protected mice infected with Cas-Br-M MuLV were very low and did not differ significantly (J. A. Bilello and P. M. Hoffman, unpublished data). Other possible mechanisms for the observed T-cell effect including the induction of cytotoxic T cells and the interaction of T cells with macrophages or NK cells are currently being investigated.

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