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Wild Mouse Ecotropic Murine Leukemia Virus Infection of Inbred Mice: Dual-Tropic Virus Expression Precedes the Onset of Paralysis and Lymphoma

PAUL M. HOFFMAN,[1]* WENDY F. DAVIDSON,[2] SANDRA K. RUSCETTI,[3] THOMAS M. CHUSED,[3] AND HERBERT C. MORSE III[3]

Veterans Administration Medical Center and Departments of Neurology and Basic and Clinical Immunology and Microbiology, Medical University of South Carolina, Charleston, South Carolina 29545;[1] Laboratory of Microbial Immunity, National Institute of Allergy and Infectious Diseases,[2] and Laboratory of Tumor Virus Genetics, National Cancer Institute,[3] Bethesda, Maryland 20205

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NFS/N mice inoculated at birth with an ecotropic murine leukemia virus (Cas-Br-M MuLV) obtained from wild mice developed hind limb paralysis beginning at 7 weeks of age and nonthymic lymphomas beginning at more than 20 weeks of age. Studies of 1- to 7-week-old Cas-Br-M MuLV-infected mice showed the following: (i) a marked increase in nonecotropic MuLV-related antigens on spleen cells but not thymocytes beginning at 2 weeks; (ii) the appearance of dual-tropic mink cell focus-forming (MCF) MuLV-related gp70 in spleen but not thymus or brain cells at 4 weeks; and (iii) the isolation of infectious MCF MuLV from spleen cells of 7-week-old mice. A role for MCF MuLV in Cas-Br-M MuLV-induced nonthymic lymphomas is indicated by these studies, and a role for recombinant MuLV in neurological disease is considered.

Type C RNA viruses (retroviruses) have long been implicated as the etiological agents in the development of lymphomas in mice (17), cats (4), nonhuman primates, and other species (21). Recent work has also suggested that retroviruses may be associated with other chronic conditions such as neurological disease (8, 10) and arthritis (2) in mice and goats. Neurogenic hind limb paralysis and lymphoma associated with the expression of high levels of infectious murine leukemia viruses (MuLV's) were described in wild mice trapped in Southern California (7, 8). Virus isolates from some of these wild mice consisted of a mixture of ecotropic and amphotropic MuLV's (6). Cloned ecotropic MuLV induced both paralysis and lymphoma after inoculation of susceptible inbred strains, whereas inoculation of cloned amphotropic MuLV resulted in the development of lymphomas alone (9). This work clearly defined wild mouse ecotropic MuLV as a necessary factor in the paralytic and lymphomagenic processes but left open the question as to whether ecotropic virus alone is sufficient for the development of either of these diseases.

Recent studies of the late preleukemic phase of AKR mice have implicated recombinant MuLV in lymphomagenesis (12). AKR mice express high levels of ecotropic MuLV from birth, and this virus was considered to be the transforming agent in this strain (17). Subsequent evaluations of preleukemic mice showed that their thymocytes produced amounts of infectious ecotropic MuLV similar to those of young mice but differed in that they had a markedly increased expression of cell surface MuLV-related antigens (13). The increased expression of cell surface antigens correlated with the appearance of a novel class of MuLV in the thymocytes. MuLV's of this class grow in both mouse and xenogeneic cells and induce characteristic foci in mink lung cells; hence, they are called dual-tropic or mink cell focus-forming (MCF) MuLV's (5, 12). These viruses have been shown to be genetic envelope region recombinants between ecotropic MuLV- and xenotropic MuLV-related sequences (3). The demonstration that AKR mouse MCF MuLV's accelerate the development of lymphomas in young AKR mice strongly suggests that these agents play an etiological role in AKR mouse leukemogenesis (1). Viruses with similar characteristics have been isolated from erythroleukemia induced by Friend MuLV (22), from lymphomas induced by Moloney MuLV (5), and from other tumors (12).

This study was undertaken to determine whether MCF or related recombinant viruses might play a role in the development of paralytic disease and lymphoma after inoculation of a cloned isolate (Cas-Br-M) of wild mouse eco-

tropic MuLV. The results demonstrate that the latent period before the development of either paralysis or lymphoma is characterized by the following: (1) the rapid appearance of xenotropic MuLV-related cell surface antigens (XenCSA) on spleen cells but not on thymocytes; (ii) the presence of material that is cross-reactive with MCF gp70 in spleen but not thymus or brain cells; and (iii) the subsequent emergence of MCF viruses in prelymphomatous and lymphomatous spleens. These findings suggest that the production of recombinant MuLV may be intrinsic to the development of splenic lymphomas in Cas-Br-M-infected animals.

MATERIALS AND METHODS

Mice. Pregnant NFS/N mice were obtained from the Small Animal Section, Verterinary Resources Branch, National Institutes of Health, Bethesda, Md. Mice were inoculated intraperitoneally or intracerebrally with Cas-Br-M MuLV (10^3 to 10^4 PFU) when less than 24 h old. NFS/N mice have never been found to produce ecotropic MuLV either spontaneously or after induction with iododeoxyuridine (J. Hartley, personal communication) and lack complete sequences for integrated ecotropic MuLV (16).

Virus and virus assays. The origin and characteristics of Cas-Br-M MuLV are as previously described (11). The virus was cloned by limiting dilution in SC-1 cells. Pools of cloned virus used for mouse inoculation were found to be free of detectable xenotropic, amphotropic, and MCF MuLV's as well as lactate dehydrogenase virus. Cas-Br-M virus titers were determined on SC-1 cells by the XC plaque assay (17).

Antisera. Antibodies reactive with XenCSA were prepared by immunizing a rabbit (R283) with rabbit corneal (SIRC) cells infected with NZB xenotropic MuLV as previously described (15). XenCSA expression on lymphocytes of inbred mouse strains and on cells lines exogenously infected with ecotropic, xenotropic, and MCF MULV's has been quantitated by using fluorescein-labeled $F(ab')_2$ fragments of immunoglobulin G prepared from R283 serum (Fl-R283) (15). These and unpublished studies showed that R283 antibodies have predominant specificity for the gp70 of xenotropic MuLV but also react strongly with the gp70 of all MCF viruses tested, including viruses from tumors of AKR and *Akv*-congenic mice and Moloney MuLV-induced tumors. The antibodies show some limited reactivity with cell lines exogenously infected with ecotropic MuLV (15) but they do not neutralize AKR or Cas-Br-M ecotropic MuLV (P. Hoffman, J. Hartley, and H. Morse, unpublished data). In addition, lymphocytes from NFS mice congenic for *Akv-1* stain no more intensely for XenCSA than do cells from normal NFS mice. These results indicate that staining for XenCSA cannot be attributed to expression of ecotropic MuLV.

Goat antiserum to Rauscher MuLV gp70 was provided by the Division of Cancer Cause and Prevention, National Cancer Institute, Bethesda, Md. An antiserum specific for MCF viral gp70 was obtained by absorbing goat antiserum to Moloney MCF gp70 with ecotropic Moloney MuLV as previously described (18). Rat antiserum containing MCF gp70 reactivity was prepared by immunizing Osborne-Mendel rats with SFFV-NRK clone 1 cells (18). These antibodies appear to be MCF specific in that they do not react with AKR, Friend, or Moloney ecotropic MuLV or with BALB/c, AKR, or NFS xenotropic MuLV (18; S. Ruscetti, unpublished data). Biotin-labeled anti-Thy-1.2 was purchased from Becton Dickinson, Mountain View, Calif.; Biotin-labeled anti-mouse Fab and X-RITC avidin were generously provided by Thomas M. Chused.

Flow microfluorometry. The methods used in preparation and staining of lymphocytes for analysis by flow microfluorometry, using an FACS II system (Becton Dickinson), were as previously described (15). Mean fluorescence values (15) were used to compare the levels of staining detected on different preparations of lymphocytes.

Radioimmunoassay. Tissue extracts were analyzed for the presence of MCF gp70 by competition radioimmunoassay, using iodinated Moloney MCF gp70 and rat antiserum to SFFV-NRK clone 1 cells, as previously described (18).

Metabolic labeling of cells, immunoprecipitation, and sodium dodecyl sulfate-polyacrylamide electrophoresis. Metabolic labeling of cells and analysis of immune precipitates were carried out as previously described (19). Briefly, subconfluent monolayers of cells grown in 100-mm petri dishes were labeled for 30 min with 300 Ci of [^{35}S]methionine. A total of 5×10^6 trichloroacetic acid-precipitable counts were then immune precipitated with 5 μl of the appropriate immune serum and *Staphylococcus aureus*. The washed precipitates were electrophoresed on 7% sodium dodecyl sulfate-polyacrylamide gels, which were then fixed, fluorographed, and exposed to X-ray film at −70°C.

RESULTS

Ecotropic virus and disease in Cas-Br-M MuLV-infected mice. NFS/N mice inoculated with Cas-Br-M MuLV at birth began to show tremulousness and hind limb weakness at 7 weeks of age, and by 12 weeks of age, 100% (37/37) of mice were symptomatic. Nonthymic lymphomas were detected in mice 20 weeks of age or older in 16% (6/37) of the animals.

Virus-infected mice were sacrificed at 1, 3, 4, and 7 weeks of age, and brain, spleen, and thymus extracts were assayed for ecotropic MuLV (Fig. 1). All inoculated mice were found to have significant titers of ecotropic MuLV in thymus and spleen cells by 1 week after inoculation; lower titers of virus were detected in brain extracts of 1-week-old mice. Ecotropic MuLV titers reached peak levels in all three tissues at 3 weeks after inoculation and remained relatively constant thereafter (Fig. 1).

Expression of XenCSA on lymphocytes of Cas-Br-M MuLV-infected mice. Single-cell suspensions from thymuses and spleens of mice inoculated at birth with Cas-Br-M MuLV were

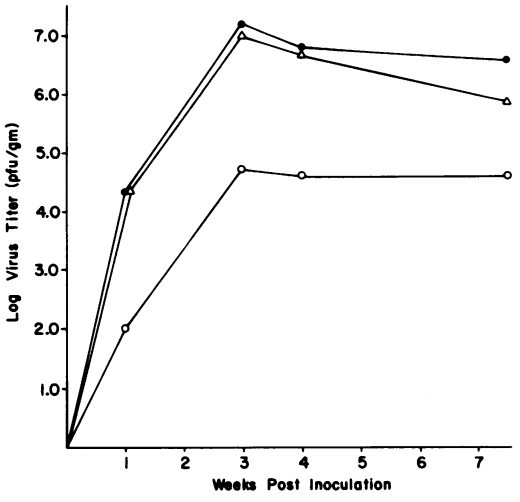

FIG. 1. *Titers of ecotropic MuLV obtained from tissue homogenates of spleen (●), thymus (△), and brain (○) of NFS/N mice inoculated at birth with Cas-Br-M MuLV. Points indicate the mean titers for four mice.*

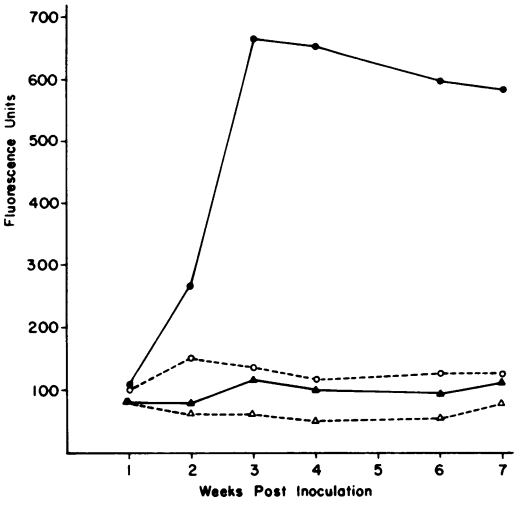

FIG. 2. *Expression of XenCSA on thymocytes (△, ▲) and spleen cells (○, ●) of uninoculated mice (open symbols) or mice inoculated at birth with Cas-Br-M MuLV (closed symbols). Points indicate the mean fluorescence values obtained in assays of tissues from four mice.*

incubated with Fl-R283 and analyzed on the FACS II flow microfluorometer for XenCSA expression. As shown in Fig. 2, thymocytes and spleen cells from mice tested 1 week after inoculation of Cas-Br-M MuLV showed no increase in expression of XenCSA as compared with uninoculated mice or mice inoculated with supernatants from uninfected SC-1 cells (data not shown). XenCSA levels on spleen cells, but not on thymocytes, increased significantly 2 to 3 weeks after inoculation and remained relatively constant thereafter. Lymph node cells from NFS/N mice at 4 and 7 weeks after infection also showed increased XenCSA expression (data not shown). At no time was XenCSA expression on thymocytes of infected mice significantly higher than that on thymocytes of control mice.

A comparison of the fluorescence profiles for XenCSA staining in 4-week-old infected and control mice showed that thymocytes from Cas-Br-M MuLV-infected mice had slightly increased staining as compared with uninfected mice (Fig. 3). However, spleen cells from Cas-Br-M MuLV-infected mice showed a marked increase in cells with high levels of fluorescent staining.

To determine whether the spleen cells expressing high XenCSA levels were predominantly T cells or B cells, we examined cells from 7-week-old infected mice by two-color immunofluorescence for coexpression of XenCSA and Thy-1.2 or XenCSA and cell surface immunoglobulin (Fig. 4). The contour maps shown in

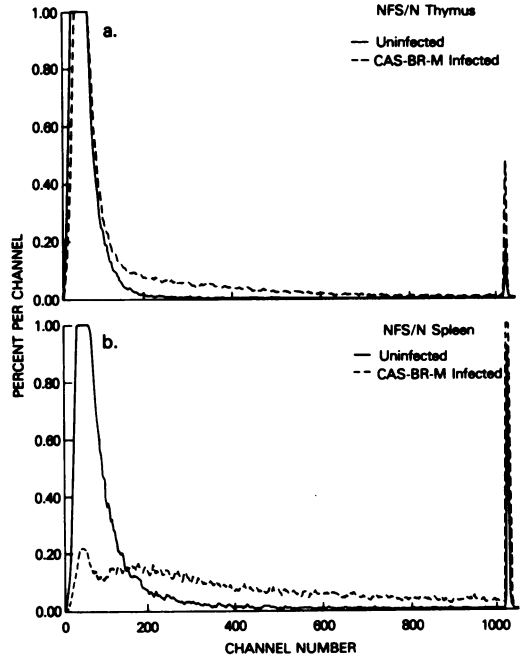

FIG. 3. *Fluorescence profiles of thymocytes (a) and spleen cells (b) from 4-week-old uninfected (——) or Cas-Br-M MuLV-infected (- - -) NFS/N mice stained with Fl-R283. The mean fluorescence values for control and infected thymocytes were 77 and 106, respectively. Mean fluorescence values for uninfected and infected spleen cells were 126 and 808, respectively.*

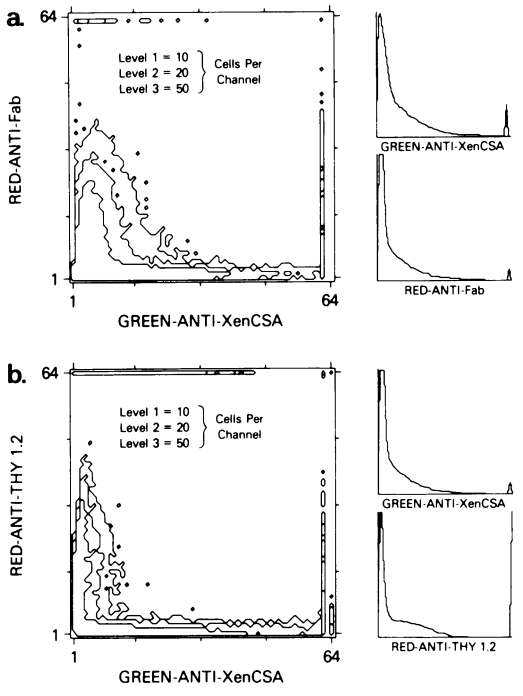

FIG. 4. *Contour maps of spleen cells from Cas-Br-M MuLV-infected mice stained for XenCSA and cell surface immunoglobulin (a) or XenCSA and Thy-1.2 (b). Cells were stained in order with Fl-R283, biotin-labeled anti-Fab or anti-Thy-1.2, and X-RITC-labeled avidin. Single-parameter profiles of stained cells are shown to the right of the contour maps.*

Fig. 4 demonstrated that the cells with the highest levels of XenCSA staining (greater than channel 16) did not stain intensely with either anti-Fab (Fig. 4a) or anti-Thy-1.2 (Fig. 4b).

Expression of MCF gp70 in cells of Cas-Br-M MuLV-infected mice. To determine whether increased XenCSA expression on spleen cells but not on thymocytes of Cas-Br-M MuLV-infected mice was related to de novo expression of MCF or xenotropic MuLV genomes, we tested extracts of spleen cells and thymocytes for the presence of MCF gp70-related antigens by radioimmune competition assays (Fig. 5). Thymocyte extracts from infected mice did not compete at anytime after inoculation (data not shown), but spleen extracts from infected mice showed increasing levels of competing antigen from 4 to 7 weeks of age. MCF MuLV gp70-related antigens were not detected in brain extracts from these mice (data not shown).

The competing antigen detected by radioimmunoassay was characterized further by metabolic labeling and immune precipitation. Labeled extracts from Cas-Br-M MuLV-infected SC-1 cells or SC-1 and NIH 3T3 cells infected with the spleen homogenate obtained from Cas-Br-M MuLV-infected NFS mice inoculated 2 weeks previously were immune precipitated with a broadly cross-reactive goat anti-Rauscher MuLV gp70, goat MCF MuLV-specific antibodies, or normal goat serum, and the precipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 6). All three cell extracts contained antigen reactive with anti-Rauscher MuLV gp70, but only SC-1 and NIH 3T3 cells infected with virus from Cas-Br-M MuLV-inoculated NFS/N mice expressed MCF MuLV-related gp70 determinants. Evidence that true MCF MuLV-related gp70 determinants are being detected in Cas-Br-M MuLV-infected mouse spleens includes the findings that SC-1 but not NIH 3T3 cells can be exogenously infected with xenotropic MuLV, and no immune precipitate was seen when SC-1 cells were reacted with goat anti-Moloney MCF gp70 serum (Fig. 6, lane 2). In a separate experiment, spleen cells from a 7-week-old Cas-Br-M MuLV-infected mouse were cocultivated with mink lung cells to determine whether infectious MCF virus could be detected. Foci characteristic of MCF viruses were detected 10 days later.

DISCUSSION

NFS/N mice injected at birth with Cas-Br-M MuLV develop hind limb paralysis and nonthymic lymphomas. The mechanisms involved in the pathogenesis of either of these disorders are poorly understood. The data presented in this report demonstrate that MCF MuLV were produced in spleens of infected animals before

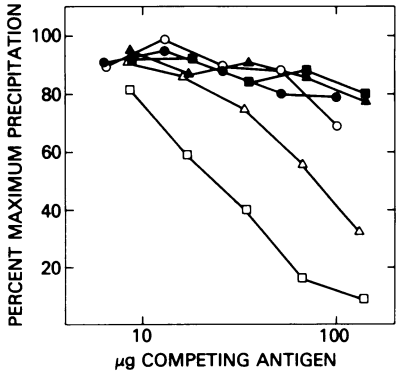

FIG. 5. *Detection of MCF gp70-related antigens in spleens of mice infected with Cas-Br-M MuLV. Competition radioimmunoassays were carried out with 50,000 rpm of ^{125}I-labeled Moloney gp70, a 1:500 dilution of rat anti-SFFV-NRK serum, and competing-antigen extracts of spleens from uninfected NFS mice (■) and Cas-Br-M MuLV-infected NFS mice at 2 (▲), 3 (●), 4 (○), 6 (△), and 7 (□) weeks after infection.*

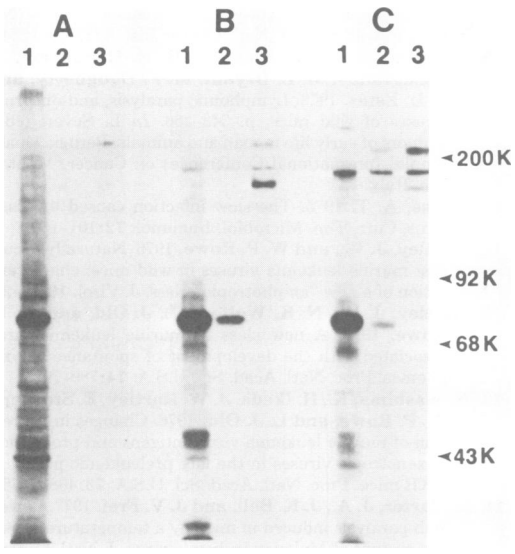

FIG. 6. *Polyacrylamide gel electrophoresis of labeled-cell extracts precipitated with MCF-specific gp70 antiserum. Cells were labeled for 30 min with [^{35}S]methionine and then immune precipitated with goat anti-R-MuLV gp70 serum (lanes 1); goat anti-Moloney MCF gp70 serum absorbed with ecotropic M-MuLV (lanes 2); or normal goat serum (lanes 3). Labeled cells extracts included (A) SC-1 cells infected with Cas-Br-M MuLV; (B) SC-1 cells infected with a spleen homogenate from an NFS mouse infected 7 weeks earlier with Cas-Br-M MuLV; or (C) NIH 3T3 cells infected with the same spleen homogenate as in (B). Molecular weight markers are as indicated.*

the onset of neurological signs or lymphoma, raising the possibility that recombinant viruses may play a role in the development of one or both of these disease manifestations.

The appearance of MCF MuLV in spleen cells was preceded by the development of high titers of infectious ecotropic MuLV in thymus, spleen, and brain, and the expression of markedly increased levels of MuLV-related antigens (XenCSA) on the surface of spleen and lymph node cells, but not thymocytes. The exact nature of XenCSA remains to be determined, but the fact that it is not expressed on spleen cells of 1-week-old infected mice and thymocytes of older mice, both of which produce high titers of ecotropic virus, strongly suggests that these antigens are unrelated to ecotropic MuLV. These results suggested that cell surface antigens coded for by xenotropic or MCF MuLV (or shared by these MuLV classes) were responsible for the striking elevation of XenCSA detected on spleen cells of Cas-Br-M MuLV-infected mice.

The possibility that nonecotropic MuLV might be responsible for enhanced expression of MuLV antigens on spleen cells was confirmed in three different systems. First, competition radioimmunoassay demonstrated increasing levels of MCF gp70-related antigens detectable in spleen cells but not thymocytes of infected mice between 4 and 7 weeks of age. Second, metabolic labeling of cells infected with MuLV obtained from spleen cells of 7-week-old Cas-Br-M MuLV-inoculated mice and precipitated with a goat anti-Moloney MCF gp70 antiserum demonstrated the presence of a protein immunologically cross-reactive with the gp70 of MCF MuLV but not the gp70 of Cas-Br-M MuLV. Finally, in preliminary experiments, the spleen cells of 7-week-old Cas-Br-M MuLV-infected mice produced infectious MCF MuLV.

In Cas-Br-M MuLV-associated nonthymic lymphomas, a pathogenic role of MCF MuLV is supported by several observations. First, Cas-Br-M MuLV-infected mice developed tumors involving the spleen and lymph nodes but not the thymus, and MCF gp70 expression was found in the spleen but not the thymus (lymph nodes were not studied). Second, spleen cells expressing the highest levels of MuLV-related antigens had a very low expression of cell surface Thy-1 or immunoglobulin, and analysis of three tumors obtained from Cas-Br-M MuLV-infected mice indicated that they were negative for expression of Thy-1, Lyt-1, Lyt-2, and cell surface or cytoplasmic immunoglobulin (A. R. Yetter, H. C. Morse III, T. Fredrickson, and P. M. Hoffman, unpublished data). It would thus appear that the MCF viruses are preferentially expressed in the organ and cell type which eventually undergo neoplastic transformation. Finally, we have recently shown that lymphomatous spleen extracts containing MCF virus are capable of accelerating the appearance of lymphoma in NFS/N mice (P. M. Hoffman and H. C. Morse III, manuscript in preparation). Studies to better define the cell type involved in MCF virus production and transformation and to evaluate the leukemogenic potential of Cas-Br-M MuLV MCF strains are in progress.

The role of MCF viruses in paralysis induction is more difficult to evaluate. MCF gp70 could not be identified by radioimmunoassay of brain extracts of mice either before the onset of hind limb weakness or in paralyzed mice (P. M. Hoffman, S. K. Ruscetti, and H. C. Morse III, J. Neuroimmunol., in press). Low levels of MCF virus production or highly localized lesions could explain our failure to detect MCF gp70 in whole-brain extracts. Current immunohistochemical studies of brain and spinal cords from Cas-Br-M MuLV-infected mice may elucidate this point. Furthermore, MuLV can recombine in genes other than *env*, and the possibility remains open that other recombinant viruses with neither se-

rological nor biological properties of MCF could be implicated in the development of the neurological disease. It is interesting that paralytic disease has been reported in association with reticulum cell sarcoma in BALB/c mice (20), with lymphoma induced by a temperature-sensitive mutant of Moloney MuLV (14), and in the high-lymphoma BXH-2 recombinant inbred strain (H. Bedigian, B. Taylor, and H. Meier, personal communication). The viral or genetic factors that determine why paralysis associated with lymphoma occurs in these instances but not in the spontaneous lymphomas of AKR or C58 mice or after Cas-Br-M MuLV inoculation in these strains (P. M. Hoffman and H. C. Morse III, manuscript in preparation) are worthy of further study.

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