Host Genes Conferring Resistance to a Central Nervous System Disease Induced by a Polytropic Recombinant Friend Murine Retrovirus

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Infection of certain strains of mice with the ecotropic Friend murine leukemia virus results in the generation of recombinant polyclonal mink cell focus-inducing viruses and the development of erythroleukemia. We isolated a Friend mink cell focus-inducing virus (F-MCF-98D) from a Friend murine leukemia virus-infected BALB/c mouse which caused primarily a neurological disease as well as a low incidence of leukemia in susceptible IRW mice. Through genetic studies with the resistant C57BL/10 strain, we identified two genes which correlated with restricted viral replication and resistance to the development of disease caused by F-MCF-98D. One gene correlated with the expression of an endogenous gp70 linked to the Rmcf gene and might act by viral interference. The mechanism of action of the second gene was less clear, but it appeared to be associated with development of an antiviral antibody response.

The study of host resistance genes has been important in the elucidation of the pathogenesis of retroviral diseases. Strains of laboratory mice have been found to possess a variety of resistance mechanisms effective against retroviruses. For the most part, these resistance mechanisms fall into two broad categories, immunological and nonimmunological. Pertaining to the former group, it has been found that the ability to mount an effective humoral or cellular antiviral immune response may be important for the control of viral replication and abrogation of pathology in some strains of mice (8, 12, 15, 20, 37). A variety of nonimmunological resistance mechanisms are also known. The Fv-1 locus (26) blocks a postpenetration step in the viral replication cycle and has been found to segregate with resistance to retroviral disease in several systems (22, 25). The Fv-2 gene causes resistance by influencing cell division cycling in a critical target cell population (34). Expression of an endogenous polyclonal env gene linked to the Rmcf locus (13) of DBA/2 mice has been shown to specifically restrict the replication of polyclonal viruses in vivo (4). Similarly, the Fv-4 gene, which was discovered in several populations of wild mice (33), encodes an endogenous ecotropic env protein which restricts replication of ecotropic virus, most likely by viral interference (17). This gene might be of importance in resistance to central nervous system (CNS) disease induced by ecotropic murine leukemia virus (MuLV). Additionally, other resistance genes for CNS disease caused by ecotropic murine retroviruses are known to exist in laboratory strains of mice; however, mechanisms of action of these genes have not been characterized (14).

In the present report, we describe a novel pathogenic Friend mink cell focus-inducing (F-MCF) virus, F-MCF-98D, isolated from a BALB/c mouse infected with Friend murine leukemia virus (F-MuLV). This biologically cloned virus was found to cause primarily CNS disease as well as leukemia in the susceptible IRW strain. Genetic backcross studies with resistant C57BL/10 (B10) mice revealed that two genes were involved both in the ability to restrict viral replication in vivo and in the resistance to disease. One of these genes segregated with the expression of an endogenous xenotropic retroviral envelope glycoprotein (gp70) which might act by decreasing virus spread in vivo owing to viral interference, while the other gene appeared to correlate with the development of an antiviral antibody response. Thus, resistance appeared to involve cooperation between immunological and nonimmunological resistance factors.

MATERIALS AND METHODS

Mice. Mice used in this study were bred and housed in the animal care facility of the Rocky Mountain Laboratories, Hamilton, Mont. C57BL/10SnJ mice were purchased from the Jackson Laboratory, Bar Harbor, Maine. Inbred Rocky Mountain White (IRW) mice (7) were obtained from a colony derived and maintained at the Rocky Mountain Laboratories.

Virus, viral assay, and cells. The F-MCF virus 98D was isolated from an F-MuLV-infected BALB/c mouse and biologically cloned by limiting dilution as described previously (9). F-MCF-98D, F-MCF-1 (35), and the molecular clone of F-MuLV, FB29 (32), were propagated on Mus dunni cells (18). C57BL/6 3T3 cells were derived in this laboratory by the procedure of Aaronson and Todaro (2) and express a gp70 reactive with monoclonal antibody (MAb) 18-6 (27). BALB 3T3 cells (2) used in this study express no detectable gp70. Dunni and 3T3 cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (RPMI-FCS), sodium bicarbonate (2.0 g/liter), and penicillin (200 U/ml of RPMI-FCS). Tail skin cultures were maintained in similar medium with the addition of gentamicin (5 μg/ml) and amphotericin B (2.5 μg/ml). All mice in this study were infected intraperitoneally when less than 24 h old with 30 to 50 μl of a stock with a titer of 1 × 10⁸ to 5 × 10⁸ focus-forming units (FFU)/ml. No effect of dosage was noted within this range. Mice were considered to have died of leukemia if they had enlarged lymph nodes or spleens detected by palpation while under anesthesia at one or more examinations prior to death. Mice were considered to have died of CNS disease if they had previously exhibited neuro-

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logical symptoms such as hind limb weakness or paralysis and had no evidence of enlarged lymph nodes or spleen. To assay virus-producing spleen cells (infectious centers) or viremia, dunni cells were overlaid with different dilutions of spleen cells or plasma from infected mice. Foci of infection were enumerated through the use of a focal immunofluorescence assay (FIA) (31) employing the MCF-specific MAb 514 (6).

**Typing of mice for expression of 18-6-reactive endogenous gp70.** The technique employed here has been described previously (4). Briefly, within 24 h of birth, the tails of neonatal mice were clipped and used to start tail skin cultures according to the method of Lander et al. (19). After sufficient growth, the cells were analyzed by flow microfluorometric analysis for expression of cell surface 18-6-reactive gp70 as described previously (3). In brief, tail skin culture cells were removed and dispersed by trypsin, filtered through nylon mesh, and added to the wells (5 × 10^5 cells per well) of a 96-well round-bottom plastic tray. Cells were pelleted by centrifugation, suspended in 0.1 ml of MAb 18-6, and incubated for 30 min on ice. After being washed, the cells were suspended in 0.1 ml of a 1/200 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Organon Teknika, Malvern, Pa.) and incubated for an additional 30 min on ice. After washing, 10,000 cells were analyzed with a FACS analyzer (Becton Dickinson and Co., Mountain View, Calif.). The analyzer was operated in log gain. Controls consisted of cells reacted with an irrelevant MAb of the same isotype (immunoglobulin G3) as MAB 18-6, followed by the conjugate.

**Antiviral antibody assay.** To quantitate antibody to F-MCF-98D, we used an enzyme-linked immunosorbent assay. To perform this assay, a 96-well flat-bottom tissue culture plate (Flow Laboratories, McLean, Va.) was first seeded with 5 × 10^4 dunni cells or F-MCF-98D-infected dunni cells per well in 150 μl of RPMI-FCS. The cells were incubated overnight at 37°C and then washed once with RPMI-FCS. Fivefold dilutions of sera from infected mice in 50 μl were then added to each well and allowed to incubate for 1 h at room temperature. After four washes as above, 50 μl of a 1/200 dilution of F(ab')2 fragments of sheep antimouse immunoglobulin conjugated to β-galactosidase (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was added per well followed by a 2-h incubation at room temperature with shaking. Wells were washed four times, after which 50 μl of substrate (1 mg of p-nitrophenyl-β-D-galactoside [Bethesda Research Laboratories] per ml) was added. After a 1-h incubation at room temperature, 50 μl of 0.5 M sodium carbonate was added per well and the plate was read at 405 nm on an automated enzyme-linked immunosorbent assay reader. A sample was considered positive for antibody if at least two dilutions gave readings which were at least twice the control reading obtained from uninfected cells.

**RESULTS**

Results of initial experiments indicated that the biologically cloned F-MCF-98D virus was capable of inducing both neurological disease and leukemia in susceptible mice. The neurological disease consisted of dyscoordination followed by hind limb paralysis and atrophy of skeletal muscle. The disease differed somewhat in clinical appearance from that induced by the wild mouse ecotropic virus Cas-Br-E in that tremor was not a prominent feature. IRW mice were highly susceptible to both leukemia and neurological disease induced by F-MCF-98D. The predominant form of disease was neurological, with the incidence of leukemia gradually rising at later times. By 8 months of age, 90% of IRW mice had died of either CNS disease or leukemia. In contrast, B10 mice were resistant (data not shown), and this resistance was dominant in (IRW × B10)F₁ (F₁) mice (Fig. 1). The maximal combined frequency of both diseases in F₁ mice was quite low (24%). and disease of either type did not occur until 10 months of age, well after the majority of IRW mice had succumbed. F₁ mice were strikingly resistant to the CNS disease induced by F-MCF-98D, exhibiting a longer incubation period as well as a significantly lower frequency of neurological disease, 9% versus 75% for IRW mice. IRW and F₁ mice infected with F-MCF-98D had similar frequencies of leukemia, 15% in the F₁ group versus 16% among IRW mice, although the incubation period was significantly longer in the F₁ group. Therefore, the resistance to disease induction seen in F₁ mice was evidenced by a pronounced resistance to neurological disease and a prolonged incubation period for leukemia.
Replication of F-MCF-98D in susceptible and resistant mice. To study the genetic resistance to disease induced by F-MCF-98D, we examined viral replication in susceptible and resistant mice. Figure 2 displays viral replication, as measured by splenic infectious centers (IC), in IRW, B10, and F1 mice. At 8 to 14 days postinfection, IRW mice replicated F-MCF-98D to high levels, whereas both the B10 and F1 groups had significantly lower splenic IC titers. At 30 to 50 days, IRW mice had high splenic IC titers that were virtually the same as those of the group assayed at 8 to 14 days. In contrast, at this later time the majority of resistant B10 and (IRW × B10)F1 mice had splenic IC titers which were below the level of detection ($10^6$ IC per $5 \times 10^6$ splenocytes), demonstrating recovery from infection in most B10 and F1 mice.

Because F1 mice were found to act like the B10 parent with respect to the pattern of viral replication, the resistance gene(s) appeared to be dominant. Therefore, to determine the number of genes controlling viral replication, we also tested backcross mice resulting from the cross F1 × IRW for viral replication at 30 to 50 days. Of the backcross population, 66 of 95 (70%) had a high viral titer phenotype ($>10^9$ IC per $5 \times 10^6$ splenocytes), while 29 of 95 (30%) had a low viral titer phenotype ($<10^3$ IC per $5 \times 10^6$ splenocytes) (Fig. 2). This finding was consistent with two genes controlling viral replication.

Similar results were also observed when infected mice were assayed for viremia 3 to 4 months postinfection with F-MCF-98D (Fig. 3). IRW mice clearly displayed high levels of viremia, whereas the majority of F1 mice had levels of viremia which were below the level of detection ($<10^2$ FFU/ml). F1 × IRW backcross mice segregated into two groups based on level of viremia at 4 months, with 20 of 33 (61%) having a high level of viremia ($>10^3$ FFU/ml) and 13 of 33 (39%) having low levels ($<10^3$ FFU/ml). Thus, control of viral replication, as measured by both splenic IC and viremia, was consistent with a two-gene model.

Genes influencing viral replication also influence clinical disease. Data presented in Fig. 1, 2, and 3 indicated that resistance to disease induced by F-MCF-98D was associated with recovery from virus expression in B10 and F1 mice. To determine whether these events were controlled by the same genetic mechanisms, F1 × IRW backcross mice were analyzed for viremia at 4 months post-inoculation and subsequently observed for development of clinical disease. The results indicated that the presence of viremia of $>10^3$ FFU/ml at 4 months was associated with subsequent death due to disease in 20 of 20 mice (Table 1). Furthermore, of 13 mice who had low plasma virus levels at 4 months, 10 were still alive at 13 months. Thus, level of viremia at 4 months was a very good predictor of clinical outcome, and the genetic mechanisms of resistance to this disease appeared to act by influencing viral replication. Because of the demonstrated role of the immune response in resistance to certain retroviral diseases, we examined the antiviral antibody response to F-MCF-98D as a potential mechanism controlling the level of viral replication. An initial examination of B10, F1, and IRW mice indicated that the presence of antiviral antibody correlated with resistance to disease in the B10 and F1 groups, while the susceptible IRW mice had no detectable antiviral antibody response (data not shown). We therefore examined the relationship between viremia and the antiviral antibody response in F1 × IRW backcross mice at 4 months (Table 1). These data indicated a direct correlation between the presence of antiviral antibody and low levels of viremia. Thus, the ability to restrict viral replication correlated with resistance to disease and indicated that one of the factors restricting viral replication might have been the antiviral immune response.

### Table 1. Correlation of viremia, antiviral antibody response, and long-term survival in backcross mice infected with F-MCF-98D

<table>
<thead>
<tr>
<th>Mouse status</th>
<th>No. with viremia</th>
<th>$&lt;10^3$ FFU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dead</td>
<td>20</td>
<td>3</td>
</tr>
<tr>
<td>Alive</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Antibody positive</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>Antibody negative</td>
<td>20</td>
<td>0</td>
</tr>
</tbody>
</table>

*Infected (IRW × B10)F1 × IRW backcross mice were inoculated neonatally with F-MCF-98D and were observed for survival to 13 months of age. Plasma viremia was assayed by FIA at 4 months of age. Presence of antiviral antibody in the same sample assayed for viremia was determined by an enzyme-linked immunosorbent assay method as described in Materials and Methods.*

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**Fig. 2.** Replication of F-MCF-98D in IRW, B10, (IRW × B10)F1, and (IRW × IRW)F1 × IRW backcross mice as measured by splenic IC. Spleen cells from neonatally inoculated mice 8 to 14 days postinfection and 30 to 50 days postinfection were assayed on M. dunnii cells. Foci of infection were counted by FIA with the MCF-specific MAb 514. Each point represents the titer from a single mouse. Dotted line at $10^3$ IC per $5 \times 10^6$ spleen cells discriminated IRW mice from B10 and F1 mice at 30 to 50 days; therefore, this was used to compare groups of backcross mice.

**Fig. 3.** Replication of F-MCF-98D in IRW, (IRW × B10)F1, and (IRW × IRW)F1 × IRW backcross mice as measured by viremia. Dilutions of plasma from infected mice were added to M. dunnii cells, and foci of infection were counted by FIA with MAb 514. Each point represents the titer from a single infected mouse. The lower limit of detection in this assay was $10^2$ FFU/ml.
Expression of endogenous retroviral envelope gene segregates with restricted viral replication. Another mechanism by which some strains of mice restrict the replication of retroviruses is interference mediated by expression of endogenous retroviral gp70. Previous work from this laboratory demonstrated the existence of two apparently allelic endogenous retroviral env genes which are linked to the Rmcf locus (3). The gp70 molecules encoded by these genes can be distinguished by MAb 514, reactive with most MCF viruses (6), and MAb 18-6, reactive with a subgroup of xenotropic viruses (27). Both 514- and 18-6-reactive gp70 are expressed by immature hematopoietic cells (5) of some strains of mice, and expression of the 514-reactive gp70 was previously shown to correlate with restriction of MCF virus replication and resistance to F-MuLV-induced erythroleukemia in DBA/2 mice (4). Since B10 mice express the 18-6-reactive gp70 (5), it seemed possible that this endogenous gp70 was involved in the resistance of B10 mice to disease caused by F-MCF-98D. To examine this hypothesis, F1 × IRW backcross mice were typed for expression of 18-6-reactive gp70, inoculated with F-MCF-98D, and then tested for the level of viral replication at 5 weeks. Previous data suggested that two genes were needed for restriction of viral replication (Fig. 2). The data in Fig. 4 show that the 18-6-reactive env gene was likely one of these two genes as 39 of 39 18-6-negative backcross mice had high viral titers. In the 18-6-positive mice, the additional genetic effects on virus titer could be observed. In this group, 22 mice had high viral levels and 13 had low levels of virus, a finding consistent with one or two additional genes influencing viral replication (Fig. 4).

Previous studies from this laboratory demonstrated that the expression of MAb 514-reactive MCF gp70 restricts the replication of MCF viruses in vitro (3). Since xenotropic MuLV gp70 interferes with infection by MCF viruses but not by ecotropic viruses (9), we attempted to determine whether MAb 18-6-reactive xenotropic gp70 expressed on an in vitro cell line might act similarly to restrict replication of F-MCF-98D. Therefore, F-MCF-98D, F-MCF-1, and ecotropic F-MuLV titers were determined on BALB 3T3 cells, which express on endogenous gp70, and B6 3T3 cells, which express the 18-6-reactive xenotropic gp70. The results indicated that the titer of the ecotropic virus was reduced to the same extent in B6 3T3 cells as that of the MCF viruses (Table 2). Thus, we were unable to demonstrate MCF virus-specific interference in these B6 3T3 cells.

**TABLE 2. Comparative titration of viruses on cells which differ in expression of endogenous gp70 reactive with MAb 18-6a**

<table>
<thead>
<tr>
<th>Virus</th>
<th>No. of foci of infection (FFU/ml)</th>
<th>Ratio (B6/BALB)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-MCF-98D</td>
<td>2.4 x 10^3</td>
<td>3.1 x 10^4</td>
</tr>
<tr>
<td>F-MuLV</td>
<td>2.8 x 10^4</td>
<td>4.3 x 10^4</td>
</tr>
<tr>
<td>F-MCF-1</td>
<td>6.0 x 10^5</td>
<td>1.2 x 10^5</td>
</tr>
</tbody>
</table>

* B6 3T3 cells were derived from a C57BL/6 mouse embryo. These cells express gp70 reactive with MAb 18-6. BALB 3T3 cells do not express endogenous gp70 and were included as controls. Dilutions of high-titer viral stocks were added to cells, and foci of infection were counted by an FLA. Standard error of replicate titers was <10% of values shown.

**DISCUSSION**

The results of the present study can be summarized as follows. First, we presented the initial report of a novel pathogenic F-MCF virus capable of causing both neurological disease and leukemia after neonatal inoculation of susceptible mice. Second, through backcross studies utilizing susceptible and resistant strains of mice, we identified two genes which appear to be involved with restriction of viral replication in vivo and resistance to disease.

The isolation of MCF viruses from strains of F-MuLV-infected mice which develop erythroleukemia has been widely recognized (16, 28). Reports of F-MCF isolates cloned from F-MuLV that are capable of causing disease have been less frequent. Ishimoto et al. (16) reported that pathogenic F-MCF viruses arose quite rapidly after neonatal infection of certain mouse strains with F-MuLV. However, these isolates were reported to be XC positive, thus raising the possibility of contaminating ecotropic F-MuLV (16). Another report detailed isolating an F-MCF virus which was pathogenic but had reduced virulence when compared with F-MuLV (10). Therefore, the F-MCF-98D virus is of interest for two reasons. First, it was clearly pathogenic in vivo in the absence of ecotropic F-MuLV, a fact confirmed by our recent production of a pathogenic molecular clone of F-MCF-98D (data not shown), and second, it produces CNS disease as well as leukemia. A comparison of the genome of F-MCF-98D with that of other F-MCF viruses which do not induce CNS disease may permit mapping of sequences responsible for the neurological disease.

C57BL mice possess genes which restrict the generation of MCF viruses after infection with F-MuLV (30). Direct inoculation of the pathogenic F-MCF-98D bypassed the recombinational events responsible for the generation of pathogenic MCF viruses and allowed the study of genes which function at later steps in the disease process. It was evident that one of the factors that correlated with lack of disease in B10 mice was the ability of these mice to restrict the replication of the virus. The pattern of viral replication seen in F1 and F1 × IRW backcross mice indicated that restriction was dominant and under the control of at least two genes. When viral replication was examined in B10 and F1 mice at early and late time points, the results suggested that restriction was not absolute but rather that some factor(s) was suppressing the high-level viral replication seen in the IRW mice. Thus, at 1 to 2 weeks postinfection, although viral titers were low compared with those in IRW mice, the majority of B10 and F1 mice had detectable levels of virus. However, at later times, most of the mice in both groups expressed viral titers below the level of detection, suggesting
that the low-level infection was established early and then cleared. An examination of backcross mice for the presence of antiviral antibodies and viremia showed a strong correlation between the presence of antibody and restricted viral replication, indicating that one of the genes controlling viral replication might be acting by facilitating this antiviral antibody response.

The role of antiviral antibody in resistance to murine retroviral disease is varied. Some strains of mice are unable to mount detectable antibody responses against certain retroviruses encountered during the neonatal period owing to either the immaturity of the immune system (15, 29) or the presence of an immune defect (21). Conversely, it is well documented that numerous strains are able to produce antibody in response to either endogenous or exogenous retroviruses, even when exposed neonatally (1, 11, 23, 24). In some cases, it appears that genes of the H-2 complex control the ability to produce antiretroviral antibody (36–38). It is interesting in relation to our study that H-2d strains such as B10 have been reported to be good responders to retroviral antigens in other systems (36–38). However, the presence of an antiretroviral antibody response does not always segregate with resistance to disease in genetic studies (11, 36). The fact that the presence of antibody correlated with restricted viral replication and resistance to F-MCF-98D-induced disease in B10, F1, and backcross mice does not prove that antibody played a protective role because antibodies could appear secondary to elimination or reduction of virus infection by another unknown mechanism.

Our finding that the expression of an endogenous xenotropic MuLV envelope protein appeared to be the second resistance gene was unexpected. Previously, we demonstrated that this endogenous retroviral gene is linked to the Rmcf locus and that its expression segregates with the Rmcf-sensitive allele (3). These previous results showed that expression of the MAb 18-6-reactive gp70 by primary embryonic cell cultures did not specifically restrict MCF virus replication in vitro, a result we confirmed in the present study using the B6 3T3 cell line. Although we observed a significantly lower level of infection for F-MCF-98D on cells expressing 18-6-reactive gp70, a control ecotropic virus was also restricted on these cells. Thus, the data did not demonstrate MCF-specific interference in B6 3T3 cells. However, the fact that we were unable to demonstrate specific interference in vitro does not mean that interference could not have occurred in vivo. Earlier work from this laboratory demonstrated that, in addition to embryonic cell cultures, 18-6-reactive gp70 is expressed by hematopoietic progenitor cells of several strains of mice, including B10, but not IRW (5). It is possible that interference occurred in vivo in cells expressing this protein within the hematopoietic or CNS compartment or both. In support of this hypothesis, it has been shown that retroviral interference patterns can depend on the cell type (9). The resistance allele of the Rmcf gene itself provides only a modest level of restriction (ca. 30- to 100-fold) (13); thus, it is possible that even quite low-level viral interference mediated by 18-6-reactive gp70 in vivo slows replication of the virus to a point where the antibody response could then clear the infection. Such a mechanism might explain our observation that two genes were necessary for the resistance phenotype. Alternatively, the expression of 18-6-reactive gp70 might be closely linked to some other unknown resistance gene capable of influencing virus replication.

LITERATURE CITED


