Neurodegenerative Disease Induced by the Wild Mouse Ecotropic Retrovirus Is Markedly Accelerated by Long Terminal Repeat and gag-pol Sequences from Nondefective Friend Murine Leukemia Virus

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The wild mouse ecotropic retrovirus (WM-E) induces a spongiform neurodegenerative disease in mice after a variable incubation period of 2 months to as long as 1 year. We isolated a molecular clone of WM-E (15-1) which was weakly neurovirulent (incidence, 8%) but was highly leukemogenic (incidence, 45%). Both lymphoid and granulocytic leukemias were observed, and these leukemias were often neuroinvasive. A chimeric virus was constructed containing the env and 3' pol sequences of 15-1 and long terminal repeat (LTR), gag, and 5' pol sequences from a clone of Friend mouse leukemia virus (FB29). FB29 has been shown previously to replicate to high levels in the central nervous system (CNS) but is not itself neurovirulent. This finding was confirmed at the DNA level in the current study. Surprisingly, intraperitoneal inoculation of neonatal IRW mice with the chimeric virus (FrCasE15) caused an accelerated neurodegenerative disease with an incubation period of only 16 days and was uniformly fatal by 23 days postinoculation. Introduction of the LTR of 15-1 into the FrCasE8 genome yielded a virus (FrCasE15-8) with a degree of neurovirulence intermediate between those of 15-1 and FrCasE8. No differences were found in the levels of viremia or the relative levels of viral DNA in the spleens of mice inoculated with 15-1, FrCasE8, or FrCasE15-8. However, the levels of viral DNA in the CNS correlated with the relative degrees of neurovirulence of the respective viruses (FrCasE8 > FrCasE15-8 > 15-1). Thus, the env and 3' pol sequences of WM-E (15-1) were required for neurovirulence, but elements within the LTR and gag-pol regions of FB29 had a profound influence on the level of CNS infection and the rate of development of neurodegeneration.

The wild mouse ecotropic retrovirus (WM-E) originally isolated by Gardner and co-workers (15) causes a chronic neurodegenerative disease associated with spongiform degeneration of the grey matter and tremulous paralysis (1). In addition, this virus also causes leukemia of both lymphoid and granulocytic cell types (14, 16). The neurodegenerative disease is generally slow in onset, with incubation periods ranging from 2 to 12 months, and the incidence of disease can be unpredictable, even when molecularly cloned virus and inbred strains of mice are used. These features have made systematic study of the pathogenesis of the neurologic disease cumbersome.

In the original description of the neurologic disease, the overall incidence in wild mice over a 14-month observation period was only 15% (15). The heterogeneity in susceptibility appeared to be due in part to segregation of the Akv-r1 (Fv-4) resistance gene (17). More subtle effects have been observed in inbred laboratory mice in which host resistance genes affect both the incidence and the tempo of the neurologic disease (20). These latter host genes have not been characterized.

In addition to host genetic resistance, the tempo of the disease is affected by stage of development of the mouse at which inoculation of the virus occurs. The most rapid disease has been observed in mice inoculated in utero during midgestation (35). In contrast, mice generally are completely resistant when the virus is inoculated after postnatal day 6 (22). It is thought that at least a component of this age dependence is mediated by the immune response (21).

The nature of the virus inoculum also influences the tempo of the disease. Brooks et al. (3) found that inoculation of concentrated virus shortened the incubation period and increased the severity of the disease. This could have been due to either the increased initial viral load per se or perhaps the existence of a minor population of highly virulent virus variants in the original virus stock. DesGroseillers et al. (10, 12) have used molecular approaches to define the viral sequences which influence neurovirulence. These studies revealed that the viral env gene of WM-E is an important determinant of neurovirulence (10, 28) but that viral long terminal repeat (LTR) sequences appeared to influence the tempo of the disease (12). They replaced the LTR of their infectious clone of WM-E (NE-8) with that of the thymotropic virus Moloney murine leukemia virus (Mo-MuLV). Interestingly, intrathymic inoculation of the chimeric virus induced accelerated neurologic disease with an incubation period of 40 to 100 days (compared with 100 to 200 days for NE-8).

Using similar approaches, we constructed a chimeric virus containing envelope sequences derived from an infectious molecular clone of WM-E and nonenvelope sequences derived from a strain of nondefective Friend murine leukemia virus (F-MuLV). This chimeric virus induced markedly accelerated neurodegenerative disease (incubation period, only 16 days) which affected 100% of the inoculated mice and was invariably fatal. Sequences within the viral LTR, as well as the gag-pol region of F-MuLV, were found to be

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responsible for this remarkable disease acceleration, and these sequences specifically enhanced the level of infection of the central nervous system (CNS).

**MATERIALS AND METHODS**

**Mice, viruses, and inoculations.** IRW mice are an inbred strain of the Fv-1™ genotype derived, bred, and raised at the Rocky Mountain Laboratories. These mice are highly susceptible to leukemia induced by neonatal inoculation of both F-MuLV (36) and Mo-MuLV (M. Sibton, H. Ellerbrok, F. Pozo, J. Nishio, S. F. Hayes, L. H. Evans, and B. Chesebro, J. Virol., in press) helper viruses and are also susceptible to CNS disease induced by neonatal inoculation of WM-E (26). Mice (24 to 48 h old) were inoculated intraperitoneally and, in one experiment, intracerebrally with 30 µl of virus stocks. Mice were weaned at 21 days, except for mice inoculated with the virus FrCasE, which were not weaned. Virus stocks were prepared as described previously (32). The titers of virus stocks were as follows (in focus-forming units per milliliter): 15-1, 1.3 × 10³; FrCasE, 5.4 × 10³; FrCasF, 3.0 × 10³; CasFrE, 1.0 × 10³; FB29, 4.0 × 10⁵. Clinical evaluations involved examination of the movements of mice in the cage and lifting each mouse by the tail to evaluate the normal reflex abduction of the hind limbs (I). Starting at 11 to 12 days postinoculation, mice were examined daily until 30 days of age. Mice were examined weekly thereafter for evidence of neurologic disease and after 4 months, when the first cases of leukemia were noted; the examinations included palpation for splenomegaly and lymphadenopathy under ether anesthesia.

**Virus quantified by focal immunofluorescence assay using type-specific anti-gp70 monoclonal antibodies 48, for viruses containing the F-MuLV env gene (8), and 667, for viruses containing the WM-E env gene (26). Virus titers are expressed as in vitro fluorescent focus-forming units per milliliter. Fv-tropism was analyzed by “fitness” curves using NIH 3T3 and Balb 3T3 cells (29).**

**Pathologic studies.** Clinically affected mice were killed by exsanguination after ether anesthesia and examined for gross pathologic changes. In some cases, the brain and a representative section of the cervical spinal cord were fixed in 5% formaldehyde in phosphate-buffered saline (pH 7.4) for 24 h and paraffin-embedded blocks were sectioned and stained with hematoxylin and eosin. Diagnoses of leukemia types were made on the basis of the sites and character of organomegaly, as well as examination of imprints stained with Giemsa. Thymic lymphomas were characterized by gross thymic enlargement in addition to splenomegaly and lymphadenopathy. Nonthymic lymphomas were characterized by splenomegaly and lymphadenopathy but no thymic enlargement. Chloroleukemia was diagnosed when there was splenomegaly and lymphadenopathy, the lymph nodes having a characteristic green tinge. These latter leukemias were unaccompanied by thymic enlargement. Leukemic infiltration of the CNS was diagnosed either by imprint preparations of the meninges over the neurocortex or by routine histologic examination of embedded material.

**Molecular cloning of viral DNA and isolation of a WM-E-specific probe.** NIH 3T3 cells were infected at a high multiplicity of infection with the Cas-Br-M strain of WM-E (originally obtained from Janet Hartley, National Institute of Allergy and Infectious Diseases), and to assure confluent infection, the cells were passaged three times at a 1:10 dilution. These cells were used to infect new NIH 3T3 cells by cocultivation at a ratio of four uninfected cells to one infected cell. After 21 h, the DNA was extracted and subjected to high-salt precipitation as described by Hirt (19). The unintegrated closed circular viral DNA in the supernatant was digested with SalI, which cuts once in the viral genome within the polymerase gene (7, 24), and cloned into plasmid pUC19, which was also cut with SalI and subjected to alkaline phosphatase treatment. After transformation into Escherichia coli JM109, bacterial colonies were blotted onto nylon filters (Nytran; Schleicher & Schuell, Inc., Keene, N.H.). Of 2,500 recombinant plasmids, 12 hybridized to a WM-E-specific probe, WM13 (described below), and 8 of these contained inserts of ~8.2 kilobases (kb). Plasmid DNAs from these eight clones were partially purified by the alkaline lysis method (25) and cut with SalI. DNA was transfected into NIH 3T3 cells as previously described (36), and virus was recovered from one of the clones (15-1) after four passages of the cells at a 1/5 dilution. This virus was used to infect Fisher rat embryo cells, from which virus stocks were prepared.

The 1504E strain of WM-E (4), obtained from M. Gardner (University of California, Davis, Dixon), was cloned by similar techniques with some modifications. In this case, the Hirt supernatant was subjected to further fractionation by ion-exchange chromatography to purify supercoiled DNA (18) and this DNA was digested with HindIII, which cuts the viral DNA 3′ of the SalI site within the polymerase gene (7). After ligation into pUC19 and transformation, colonies were screened with a full-length viral DNA probe derived from FB29 (see below). We recovered 14 clones of ≥8.2 kb, one of which, clone 12-4, was used to produce a WM-E-specific probe essentially as described by Rassart et al. (33). As predicted on the basis of restriction endonuclease analysis of Hirt supernatant preparations of this virus (7), WM1504E contains an XbaI site 263 bases 3′ of the env start codon and a BamHI site 760 bases 3′ of the env start. This 0.5-kb XbaI-BamHI fragment was ligated into pUC19 cut with the same enzymes. The insert (WM1504E) was purified by electrophoresis, and its specificity was tested by Southern blot analysis using cloned viral DNAs from WM-E clones 15-1 and 12-4; ecotropic viruses Mo-MuLV, F-MuLV, and Akv; xenotropic virus NZB-IU6; and polytropic virus 13MCF (the latter two of which viruses were kindly supplied by A. Khan, National Institute of Allergy and Infectious Diseases). At high stringency (washing at 65°C in 0.1× SSPE [1× SSPE is 150 mM NaCl, 10 mM NaH₂PO₄, 1 mM EDTA [pH 7.4]]-0.1% sodium dodecyl sulfate for 30 min), significant hybridization was detected only to the wild mouse viral DNAs (data not shown).

**Construction of chimeric viral genomes.** The two parental viruses used in construction of chimeric viruses were 15-1 (the infectious WM-E clone) in pUC19 and FB29, a clone of F-MuLV strain 1-3 (36) cloned at the HindIII site in the 3′ end of the pol gene into a modified pUC19 plasmid from which the multiple cloning site between SpHl and EcoRI had been excised. The latter plasmid was kindly supplied by M. Sibton (Laboratoire d’Immunologie et Virologie des Tumeurs, Hopital Cochin, Paris, France). DNA fragments were purified by electrophoresis from agarose gels (DNA Grade UltraPure Agarose; Bio-Rad Laboratories, Richmond, Calif.). Enzymes were from New England BioLabs, and reactions were performed under conditions recommended by the manufacturer.

The chimeric viral genomes are illustrated in nonpermuted form in Fig. 1. For construction of FrCasE, the FB29 plasmid was cut with SpHl and Clal and the 8.6-kb fragment containing 5.7R gag and 5′ pol sequences, along with plasmid pUC19, was ligated to a 2.6-kb SpHl-Clal fragment contain-
FIG. 1. Schematic representation of the DNA genomes of parental viruses 15-1 and FB29 and chimeric viruses CasFrE, FrCasE, and FrCasEL. The gene boundaries are depicted above. The restriction sites used in the constructions and for confirmation of the respective structures were ClaI (C), EcoRI (E), HindIII (H), KpnI (K), PvuI (P), SalI (Sa), and SphI (Sp). Asterisks indicate the sites at which the viral DNA was cloned into the vector. Note that the FB29 and FrCasE plasmids contain one LTR, whereas the 15-1, CasFrE, and FrCasEL plasmids contain 2 LTRs. After transfection and subsequent infection, the proviruses contain both 5' and 3' LTRs. Fv-1 tropism as a marker of the p30 gene was determined by hybridization analysis on NIH 3T3 and Balb 3T3 cells. Anti-gp70 monoclonal antibody reactivity was analyzed on live NIH 3T3 cells infected with the respective viruses. The viremia titers represent the levels of viremia of IRW mice infected as neonates and bled at 21 to 26 days postinoculation. Titers are represented as mean fluorescent focusing units per milliliter of serum. The numbers in parentheses represent the numbers of mice analyzed. n.t., Not tested.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Tropism</th>
<th>gp70 Reactivity</th>
<th>Viremia</th>
</tr>
</thead>
<tbody>
<tr>
<td>15-1</td>
<td>N</td>
<td>4.0 X 10^4</td>
<td>(6)</td>
</tr>
<tr>
<td>FB29</td>
<td>+</td>
<td>6.8 X 10^4</td>
<td>(9)</td>
</tr>
<tr>
<td>CasFrE</td>
<td>+</td>
<td>2.6 X 10^4</td>
<td>(6)</td>
</tr>
<tr>
<td>FrCasE</td>
<td>+</td>
<td>5.6 X 10^4</td>
<td>(10)</td>
</tr>
<tr>
<td>FrCasEL</td>
<td>+</td>
<td>1.5 X 10^4</td>
<td>(6)</td>
</tr>
</tbody>
</table>

Southern blot analysis. For Southern blot analysis of genomic DNA, tissue was homogenized in 10 mM Tris–1 mM EDTA with a Dounce homogenizer. Sodium dodecyl sulfate and proteinase K were added to final concentrations of 1% and 0.36 mg/ml, respectively, and the homogenate was incubated for 4 h at 50°C. After phenol–chloroform extraction, samples were treated with RNase (70 units) at 37°C for 60 min. After phenol–chloroform extraction, the DNA was ethanol precipitated in 2 M ammonium acetate and washed twice with 95% ethanol and the pellet was dissolved in 10 mM Tris–1 mM EDTA (pH 7.4). This technique consistently yielded high-quality DNA with 260/280 ratios of 1.7 to 1.8.

For Southern blot analysis, 10 μg of tissue DNA was cut with BamHI, yielding an internal 3.4-kb fragment from 15-1 and the chimeric viruses which hybridized to probe WM6. BamHI was also used to cut DNAs from tissue of FB29-inoculated mice, yielding a 0.8-kb internal fragment which hybridized to F-MuLV-specific probe E57BS (27), kindly provided by Serge Fischelson (Laboratoire d’Immunologie et Virologie des Tumeurs). The DNA was fractionated in 1% agarose, gels and blotted onto nitrocellulose paper. Preincubation was in 0.05 mg of sheared salmon sperm DNA per ml×5 Denhardt solution–3× SSPE at 63°C for 4 h (25). The probes were labeled with [32P]dCTP using the random-primer technique (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) to a specific activity of 1 × 10^8 to 2 × 10^8 dpm/μg. Hybridizations were performed with 200 to 300 ng of labeled probe in a 5-ml volume at 63°C for approximately 24 h. Blots were washed for 30 min at the same temperature with 2× SSPE–0.1% sodium dodecyl sulfate and subsequently in 0.1% SSPE–0.1% sodium dodecyl sulfate at 63°C. The blots were exposed to Kodak X-Omat AR film with intensifying screens at −70°C, and the autoradiograms were scanned with a laser densitometer (LKB Ultrascan XL; LKB Produkter AB, Bromma, Sweden).

RESULTS

Neurovirulence and leukemogenesis of WM-E molecular clone 15-1. The virus derived by transfection of clone 15-1 was inoculated intraperitoneally or intracerebrally into 74 neonatal IRW mice (9 × 10^3 FFU per mouse), and the mice were observed for evidence of clinical disease (Fig. 2). Mice which exhibited clinical signs of disease were killed and autopsied. By 43 weeks of observation, 97% of the mice had either been killed because of clinical disease (62%) or died between the weekly observation periods without pathologic diagnosis (35%). An unexpectedly high incidence of leukemia was observed (45% of mice inoculated). Most of the leukemias were lymphocytic, but a significant number of granulocytic leukemias (choloreukemias) were also observed (Table 1).

The incidence of neurological disease was relatively low and could be divided clinically into two types. Tremulous hindlimb paralysis associated with muscle atrophy, characteristic of WM-E disease, was seen in only six (8%) of the mice and had a relatively long incubation period of more than 25 weeks. The autopsies of these mice were essentially unremarkable, with no evidence of leukemia or gross CNS abnormalities. These mice, unfortunately, were not examined histologically. A second type of neurologic disease seen in 11 mice (15%) consisted of acute flaccid paralysis of the hindlimbs without tremor. These are signs suggestive of cord transection, perhaps due to an acutely developing space-
occupying lesion. Autopsies of these mice revealed evidence of leukemia in 6 of 11 mice, with leukemic infiltrates in the meninges (Fig. 3A). Of the remaining five mice with flaccid paralysis, three had no evidence of leukemia, but gross and microscopic examination of the CNS revealed massive intraparenchymal hemorrhages involving the hindbrain and rostral spinal cord (Fig. 3B). The symmetry of the lesions suggested that the vascular defect occurred in one of the larger feeder arteries. Of the 11 mice with flaccid paralysis, 2 were not autopsied. This form of paralysis was rapidly fatal, and mice rarely survived for more than 24 h after the clinical diagnosis was made. As mentioned above, in addition to leukemia and neurologic disease, a significant proportion of the inoculated mice (35% overall) died between the weekly examinations. These mice exhibited neither signs of chronic neurologic disease (tremor or reflex abnormalities) nor evidence of significant splenomegaly or lymphadenopathy by palpation. Although the cause of death is not certain, it is possible that these mice developed acute flaccid paralysis due to either hemorrhage or undetected leukemia. Cumulatively, the results of this study indicated that molecularly cloned virus 15-1 was highly leukemogenic but only weakly neurovirulent. Furthermore, among the 17 mice which ultimately developed neurologic disease, only 6 exhibited clinical evidence of the chronic degenerative disease caused by WM-E.

Rationale for construction of chimeric viruses. Previous studies using an infectious center assay indicated that a strain of F-MuLV (FB29) replicated to high levels in the

TABLE 1. Types of leukemia induced by 15-1

<table>
<thead>
<tr>
<th>Leukemia type*</th>
<th>No. of mice</th>
<th>%</th>
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</thead>
<tbody>
<tr>
<td>Thymic lymphoma</td>
<td>9</td>
<td>27</td>
</tr>
<tr>
<td>Nonthymic lymphoma</td>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>Chloroleukemia</td>
<td>12</td>
<td>37</td>
</tr>
<tr>
<td>Thymic lymphoma and</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>chloroleukemia</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Diagnoses were based on gross examination at autopsy and, in some cases, imprint preparations and histopathologic examinations. The criteria used are elaborated in Materials and Methods.

CNS but did not cause neurologic disease (31). A comparison of the levels of viral DNA in the CNS relative to those in the spleens of mice inoculated as neonates with either 15-1 or FB29 revealed a striking difference (Fig. 4). Whereas 15-1 and FB29 viral DNAs were detected in the spleens of mice 14 days postinfection, detectable levels of viral DNA were observed in the CNS only in mice inoculated with FB29. These results suggested that the weak neurovirulence of 15-1 was due in part to its limited ability to replicate in the CNS, a function which might be complemented by sequences from FB29. We therefore constructed chimeric viruses with 15-1 and FB29 (Fig. 1).

Pathogenicity of recombinant viruses. Since the neurovirulence of WM-E has previously been mapped to the env gene (10, 28), we introduced the env and 3' pol sequences of 15-1 into FB29 (FrCasE; Fig. 1). Neonatal IRW mice inoculated with this chimeric virus exhibited a severe neurologic disease characterized by tremor and paralysis of both hind- and forelimbs beginning on day 16 postinoculation. This was
mice were first noted at 27 days postinoculation, and the disease incidence increased relatively slowly thereafter. Thus, the incidence, tempo, and severity of the neurologic disease induced by FrCasEL was intermediate between those of FrCasE and 15-1. These results indicated that although the FB29 LTR in FrCasE contributed to its markedly accelerated neurovirulence, this effect could also be influenced by elements within the FB29 gag-pol region. Whether the accelerated neurovirulence of FrCasE was a consequence of additive effects of these two regions of the viral genome is not known.

Both LTR and gag-pol sequences from FB29 specifically increase the level of CNS infection. Differences in the tempo of the neurodegenerative diseases induced by FrCasE, FrCasEL, and 15-1 could be due to differences in the initial doses of virus inocula or possibly to differences in the relative levels of virus replication in the mice. However, the titers of the initial inocula of the viruses were comparable (3 × 10^5 to 5 × 10^5/ml; see Materials and Methods). In addition, the levels of viremia measured at 21 to 26 days postinoculation were also comparable for these viruses (Fig. 1, viremia). To examine the levels of virus replication specifically within the CNS, the relative levels of viral DNAs in spleens and CNS tissue were examined (Fig. 8). Whereas the levels of viral DNAs in the spleens of mice inoculated with 15-1, FrCasE, and FrCasEL were comparable, the levels of viral DNAs in the CNS were different (FrCasE > FrCasEL > 15-1). These data are summarized in Table 2, which also shows that although viral DNA was not detected in the CNS of 15-1-inoculated mice 14 days postinoculation, low but detectable levels were seen in two of three mice at 60 days postinoculation. Taken together, the results indicate that both LTR and gag-pol sequences from FB29 had a profound influence on the level of CNS infection. However, the CNS/spleen ratio of viral DNA in FrCasE-inoculated mice was similar to that of mice inoculated with FB29 (Table 2).
indicating that the level of CNS infection correlated with relative neurovirulence only for those viruses containing 15-1 pol-env sequences.

**LTR gag-pol sequences of 15-1 contain the determinants of leukemogenesis.** Although the pol-env sequences of 15-1 contain determinants of neurovirulence, this phenotype was only weakly expressed in combination with 15-1 LTR and gag-pol sequences. 15-1 itself was highly leukemogenic (Fig. 2). Chimeric virus CasFrE, which consisted of FB29 pol-env sequences on a 15-1 background (Fig. 1) was also leukemogenic and induced leukemias of the same cell types and tempo as those induced by the parent virus, 15-1. Of 39 IRW neonates inoculated intraperitoneally with this chimeric virus, 13 (33%) had developed leukemia during 31 weeks of observation. This incidence is comparable to that induced by the donor of the non-env sequences (15-1) at this time after inoculation (34%) (Fig. 2). In addition, the incubation period of the leukemias induced by CasFrE was similar to that of those induced by 15-1 (18 weeks) (Fig. 2). Of the 13 leukemias induced by CasFrE during this period, there were 7 thymic lymphomas, 2 nonthymic lymphomas, and 4 chloroleukemias. This mixture of leukemia types is reminiscent of the leukemias induced by 15-1 (Table 1). Unlike the virus from which the pol-env sequences were derived (FB29), no erythroleukemias were seen. This result was expected, since previous studies by DesGroseillers and Jolicoeur (11) and Chatis et al. (6) indicated that strong determinants of leukemogenesis are harbored by the viral LTR. Interestingly, many of the leukemias induced by CasFrE were neuroinvasive, like those induced by 15-1. Meningeal infiltrates were observed in four of seven thymomas, two of two nonthymic lymphomas, and three of four chloroleukemias. Only two of these mice, however, exhibited signs of neurological disease (flaccid paralysis of the hindlimbs). We have never detected CNS hemorrhage in CasFrE-inoculated mice. These results indicated that introduction of FB29 pol-env sequences into the 15-1 genome had little influence on the leukemogenesis determined by the 15-1 LTR gag-pol sequences.

**DISCUSSION**

An infectious molecular clone of WM-E (15-1) was isolated which, after neonatal inoculation, was found to be leukemogenic but exhibited only weak neurovirulence. IRW mice inoculated with this virus developed leukemias of both
study, we observed no measurable differences in leukemogenicity between 15-1 and CasFrE, in terms of either the incubation period or the spectrum of leukemia cell types. This suggests that the proposed WM-E pol-env determinant of leukemogenicity (23) is probably shared by FB29 and 15-1.

Two types of neurologic disease were induced by 15-1, chronic tremulous paralysis, which was seen in only 8% of the inoculated mice, and acute flaccid paralysis, which was observed in 15% of the mice. The latter syndrome was caused by either leukemic infiltration of the CNS or acute intraparenchymal hemorrhage. The cell type of the leukemia did not appear to affect its capacity to infiltrate the meninges, since this occurred in both lymphomas and chloroleukemias. It is not clear whether this observation is unique to leukemias induced by 15-1 or whether it is a more general phenomenon. Unless neurologic signs occur, one is not inclined to look at the CNS for evidence of leukemic infiltrates. It was apparent from the studies of mice inoculated with CasFrE that leukemic infiltration of the meninges often occurred in mice which exhibited no clinical neurologic signs, and further damage caused by necrosis and hemorrhage is probably required to precipitate clinical disease. The acute CNS hemorrhage seen in 4% of the inoculated mice was curious but unexplained. WM-E is thought to replicate in endothelial cells (30), and it is thus conceivable that infection of these cells could lead to vascular fragility. Although we are not aware of studies with WM-E, infection with F-MuLV (9) and Rauscher leukemia virus (2) is associated with thrombocytopenia which could secondarily lead to CNS hemorrhage. On the basis of these observations, it is suggested that care should be taken in evaluating the etiology of paralysis that appears late in the course of infection with leukemogenic retroviruses.

Despite the rather unimpressive primary neurovirulence of 15-1, a chimeric virus (FrCasE) containing the env gene and 3' pol sequences from 15-1 and LTR, gag, and 5' pol sequences from F-MuLV strain FB29 was highly neurovirulent. FrCasE induced paralytic disease with an incubation period of only 16 days and an incidence of 100% and was fatal by 23 days postinoculation. Examination of the CNS revealed typical spongiform degeneration of the grey matter with evidence of inflammatory cells. Thus, this inflammatory cells. Thus, this inflammatory cell response appeared to recapitulate the disease induced by WM-E (1) but with a remarkably accelerated tempo and enhanced severity. This result was unexpected, since FB29 itself induces erythroblastosis in IRW mice (36) but has no known potential for neurovirulence. It is unlikely that the accelerated neurovirulence of FrCasE was due to a cloning artifact, since three independently derived clones yielded viruses with the same phenotype.

Although FB29 does not induce neurologic disease, both infectious-center assays (31) and Southern blot analysis (Fig. 4; Table 2) indicated that FB29 replicated to high levels in the CNS. In contrast, 15-1 replicated in the spleen but viral DNA was not detected in the CNS until late postinoculation (Table 2). Introduction of pol-env sequences from 15-1 into the genome of FB29 resulted in a virus which replicated to high levels in the CNS and also caused neurologic disease. These results confirmed the importance of the env gene in the neurovirulence of WM-E (10, 28) but also indicated that non-env sequences strongly influenced the level of virus replication in the CNS and consequently the tempo and severity of the neurologic disease. We showed through construction of chimeric virus FrCasEL that both the LTR and gag-pol regions of FB29 increased the level of virus

FIG. 8. Southern blot of DNAs extracted from spleen (S) and spinal cord-cerebellum (C) tissues 14 days postinoculation of neonatal mice with 15-1, FrCasE, or FrCasEL. Samples (10 µg) of DNA were treated as described in the legend to Fig. 4. The relative levels of viral DNA were comparable for spleens but differed significantly for CNS tissue, in which they correlated with the relative neurovirulence of the respective virus.

lymphoid and granulocytic types after approximately an 18-week incubation period. This variety of cell types was similar in terms of both frequency and tempo to those induced by biologically cloned Cas-Br-M (14), from which 15-1 was molecularly cloned. Chimeric viruses constructed between WM-E and amphotropic viruses (23) indicated that determinants of leukemogenicity exist within the LTR, gag-pol, and pol-env regions of the WM-E genome. In the current

<table>
<thead>
<tr>
<th>Virus</th>
<th>Days post-inoculation</th>
<th>Mean (range) densitometric unitsa</th>
<th>CNS/Spleen ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>FB29</td>
<td>14</td>
<td>6.0 (5.8-6.3)</td>
<td>7.5 (6.8-8.8)</td>
</tr>
<tr>
<td>15-1</td>
<td>14</td>
<td>&lt;0.1b</td>
<td>7.5 (7.3-7.7)</td>
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<td>15-1</td>
<td>60</td>
<td>≤0.2b(≤0.1-0.25)</td>
<td>6.2 (4.7-8.0)</td>
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<tr>
<td>FrCasE</td>
<td>14</td>
<td>7.7 (6.9-9.1)</td>
<td>8.7 (8.0-9.0)</td>
</tr>
<tr>
<td>FrCasEL</td>
<td>14</td>
<td>2.2 (1.0-2.9)</td>
<td>8.4 (7.2-9.6)</td>
</tr>
</tbody>
</table>

a DNA samples (10 µg) from three mice per group were digested with BamHI, and Southern blots were probed with the F-MuLV-specific probe for FB29-inoculated mice and the WM-E-specific probe for mice inoculated with 15-1 and the chimeric viruses. Digestion was performed after 4 days of exposure of the autoradiogram for FB29 and 12 days of exposure for 15-1 and the chimeric viruses.

b No signal was detected for 15-1-inoculated mice at 14 days postinoculation.

c A weak but detectable signal was seen in two of three mice analyzed at 60 days postinoculation with 15-1.
infection of the CNS and contributed to the acceleration of disease. The influence of the LTR on CNS replication was not unexpected. RetroviralLTRs contain transcriptional enhancer elements which bind trans-acting cellular proteins (37) and can function in a cell type-specific fashion (13, 38). DesGrosellers et al. (12) have previously shown that introduction of the Mo-MuLV LTR into WM-E resulted in a chimeric virus which induced accelerated neurologic disease after intrathymic but not intraperitoneal infection. Since the Mo-MuLV LTR appears to contain thymotopic enhancer elements, it was suggested that high-level virus replication in the thymus might secondarily increase the level of CNS infection. In the current study, however, the level of splenic infection (Fig. 4; Table 2) and the level of viremia were independent of the source of the viral LTR. Thus, the CNS should have been exposed to similar levels of FrCasEL and FrCasE. However, FrCasE infected the CNS at higher levels than did FrCasEL. This suggests that the enhanced CNS replication exhibited by FrCasE was CNS specific. The influence of gag-pol sequences from FB29 on the level of CNS infection was unexpected. This effect also appeared to be CNS specific, since 15-1 and FrCasEL infected spleens at comparable levels (Fig. 4; Table 2) and produced similar levels of viremia (Fig. 1). Robinson and co-workers (34) have found that the capacity of avian leukemia viruses to induce osteopetrosis mapped to a region including the 5′ half of the gag gene and immediately adjoining 5′ noncoding sequences. Interestingly, a sequence has been identified in Rous sarcoma virus within the 5′ half of the gag gene which functions as a transcriptional enhancer in vitro (5). Whether this element is the determinant for the osteopetrotic potential of avian leukemia virus is not clear. Such an enhancer has not been identified in murine retroviruses. Finer-mapping studies of the FB29 gag-pol sequence will be required to identify the sequence responsible for increasing CNS replication. For viruses 15-1, FrCasEL, and FrCasE, there was a direct correlation between the level of CNS infection and both the titer and severity of the neurologic disease. However, CNS infection, although necessary, was not sufficient for induction of neurologic disease. The levels of viral DNA in the CNS relative to the spleens of mice inoculated with FB29 and FrCasE were comparable (Table 2). However, only the latter virus was neurovirulent. Two possible mechanisms appear to be consistent with these observations. FB29 and FrCasE might infect distinct populations of cells in the CNS, and this tropism is determined by the respective envelope glycoproteins. This hypothesis implies that unique receptors exist for the WM-E glycoprotein in the CNS. Alternatively, FB29 and FrCasE could infect the same cells in the CNS, but the presence of WM-E pol-env sequences in the CNS is cytotoxic. Immunohistochemical studies (to be reported elsewhere) tend to support the former hypothesis, since FB29 has been detected exclusively within CNS vascular cells (endothelial cells and pericytes), whereas FrCasE was found both within vascular and extravascular elements of the CNS. In the current study, we found that major determinants of CNS tropism map within the viral LTR as well as the gag-pol region but that induction of neuropathologic changes depends on the pol-env region of the viral genome. Combining the LTR gag-pol region of the FB29 strain of F-MuLV with the pol-env sequences of WM-E clone 15-1 yielded a chimeric virus with markedly enhanced neurovirulence compared with any other isolate of WM-E so far described. By virtue of the highly reproducible disease induced by FrCasE, this virus should provide a useful model for more systematic study of the pathogenesis of retrovirus-induced neurodegenerative disease. LITERATURE CITED


