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Expression of Murine Leukemia Viruses in B-Cell Lymphomas of CWD/Agl Mice

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The inbred mouse strain CWD/Agl has a high incidence of spontaneous B-cell lymphomas characterized by gross splenomegaly and lymph node enlargement. The endogenous ecotropic retrovirus of CWD/Agl mice is expressed in the spleen within the first 2 weeks of age and in the thymus by 1 month of age. Endogenous xenotropic virus is expressed in the spleen and bone marrow of the earliest age group examined (4 months). Restriction enzyme analysis of DNA extracted from tumorous tissues suggests that mink cell focus-forming viruses are not required for B-cell lymphomagenesis in CWD/Agl mice. CWD/Agl mice provide an important new experimental model for the study of B-cell lymphoma.

Whereas B-cell lymphocytic tumors make up a significant proportion of human lymphoid tumors, few suitable mouse models exist for the study of this type of tumor. The majority of spontaneous leukemias and lymphomas described in mice have been of T-cell origin. Retroviruses have long been implicated as an etiological factor in spontaneous T-cell lymphomagenesis of high-tumor-incidence strains of mice, such as AKR and C58, and recent evidence suggests that mink cell focus-forming (MCF) viruses play an important role in T-cell lymphomagenesis in these mice (3, 12). The development of mouse strains with a high incidence of spontaneous B-cell lymphocytic tumors would provide a useful model for the study of retroviral involvement in B-cell lymphomas. We characterized retrovirus expression in a inbred mouse strain, CWD/Agl, which has a high incidence of spontaneous B-cell lymphomas.

CWD mice have a recessive gene, referred to as curly whisker, (*cw*), which arose as a mutation in a subline of the CBA/Cbi inbred strain at the Chester Beatty Research Institute. Mice carrying the *cw* mutation were imported to this laboratory, and the mutation was maintained on the (C57BL/6J × C3H/HeJ)F_1 background, where it presumably acquired the endogenous N-ecotropic provirus of C3H/HeJ mice and the nonagouti (*a/a*) locus. Offspring from this cross were mated to strain DBA/2J, where they acquired the *Emv-3* provirus. Mice from this cross were brother-sister mated and are now in their F37 generation. These mice are homozygous for nonagouti (*a*) and dilute (*d*) loci (22) and are referred to as the inbred mouse strain CWD/Agl. The *cw* mutation is mapped to chromosome 9 (2 centimorgans from the centromere and 34 map units from the *d* locus). Homozygotes (*cw/cw*) have strongly curled whiskers, easily recognizable soon after birth (10).

CWD/Agl mice have a high incidence of lymphomas (85%, with a mean survival time of ca. 500 days), histologically characterized as being primarily B-lymphocyte-derived follicular germinal center-cell lymphomas. The disease is characterized by gross splenomegaly and lymph node enlargement. The thymus is enlarged in less than 20% of the cases. Immunofluorescence assays demonstrated that these tumors did not bear thymus cell antigens (Thy 1), but nearly 100% of the cells were positive for surface immunoglobulin. A leukemic blood picture is not usually associated with the lymphoma.

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Examination of 27 male and female mice showed that lymphoma incidence between heterozygotes (+/*cw*) and homozygotes (*cw/cw*) was similar (85 and 86%, respectively). Also, no significant difference in tumor incidence was observed between males and females (93 and 83%, respectively) (J. Roths and E. Murphy, personal communication).

Embryos of CWD/Agl mice of different ages were examined for ecotropic virus expression by the UV-XC assay previously described (1). Embryos were removed and immersed for several seconds in diethyl ether to inactivate any maternal virus that may have contaminated the embryos during their removal. Cell cultures established from these embryos rarely expressed any XC-positive virus. To quantitate ecotropic viral expression in tissues of mice of various ages, single-cell suspensions were prepared separately from the spleen and thymus, and virus expression was determined by the infectious center assay as previously described (17). Ecotropic virus was expressed in the spleen of CWD mice as early as 2 weeks of age; however, virus was not detected in the thymus of this age group. The results of the infectious-center assay for 1- and 3-month-old mice are presented in Table 1. Virus was detected in spleen cells of 58% of the 1-month-old mice. This increased to 93% of the mice by 3 months of age. The viral titer ranged from 0 to >100 PFU per 10^7 cells at both ages. The number of mice with a virus-positive thymus increased from 42% at 1 month of age to 64% by 3 months of age. The results suggest that ecotropic virus expression occurs in the spleen sometime within the first 2 weeks of age and that the thymus is either infected with this virus or that the virus is expressed within the thymic environment at a later time. No significant difference in virus expression was observed between heterozygotes and homozygotes (data not shown).

Lymphoid cells also were tested for the production of xenotropic virus as previously described (16). Mink lung cells were infected with 10^7 lymphoid cells. After the mink lung cells were subcultured three times, the presence of virus was determined by the RNA-dependent DNA polymerase (RT) assay (6) and by the indirect immunofluorescence (IF) assay. In the IF assay, cells were grown on coverslips, fixed for 10 min in cold acetone, and incubated with a 1:40 dilution of a broad-reacting goat anti-murine leukemia virus (a gift of John Elder, Research Institute of Scripps Clinic, La Jolla, Calif.) for 1 h at 37°C. This was followed by incubation with a second antibody (fluorescene-conjugated rabbit anti-goat immunoglobulin G [Cappel Laboratories, Dowingtown,

TABLE 1. Ecotropic virus expression in CWD/Agl mice

Age of mice	Incidence of expression[a] in:	
	Spleen	Thymus
1 mo	25/43 (58%)	18/43 (42%)
3 mo	55/59 (93%)	38/59 (64%)

[a] Number positive/number of mice.

Pa.]) for 1 h at 37°C. The coverslips were examined by fluorescent microscopy with a Zeiss fluorescent microscope. Xenotropic virus was expressed in the spleen and bone marrow of 40% of the mice in the youngest age group examined (4 months of age). Xenotropic virus was not detected in the thymus of the mice in this age group. Xenotropic virus was detected in the spleen and bone marrow of 100% of the mice by 7 months of age. Xenotropic virus was also detected in the thymus of 40% of the mice 7 months or older. Further studies are being conducted to determine the age at which xenotropic virus can first be detected in CWD/Agl mice.

To analyze the possible involvement of MCF-like viruses in B-cell lymphomagenesis, single-cell suspensions obtained from tumors of CWD/Agl mice were treated with mitomycin C (25 μg for 30 min at 37°C) and overlaid on mink lung cells. The cultures were passaged three times and analyzed for virus expression by the RT and IF assays as described above. To distinguish MCF-like viruses from xenotropic viruses, supernatant fluids from positive mink lung cultures were transferred onto SC-1 cells and examined by the IF and RT assays for virus expression. Supernatant fluids from IF- and RT-positive cultures were transferred back to mink lung cultures in a limiting dilution. Any virus that replicated on both SC-1 and mink lung cells was considered to be an MCF-like virus. Of 10 tumors examined, no MCF-like virus were isolated.

Recently, a gene designated *Rmcf* which governs the spread of AKR-MCF viruses has been identified (13). The restrictive allele of the gene, $Rmcf^r$, is dominant or semi-dominant to the susceptible allele, $Rmcf^s$ · $Rmcf^r$ has been identified in the strains DBA/1, DBA/2, CBA/N, and CBA/Ca. In addition to this gene, at least one additional gene has been identified which affects MCF virus expression and thymocyte amplification (20). This second gene has been identified in several C57 strains and SJL mice; all these strains have been identified as carrying the $Rmcf^s$ allele. Since the progenitors of the CWD mutant stock carry both the resistant and susceptible alleles of *Rmcf*, the susceptibility of CWD mice to MCF viruses is not known. Studies are being conducted to determine which allele CWD/Agl mice carry.

CWD/Agl mice have two endogenous ecotropic loci, *Emv-1* and *Emv-3* (15). Fingerprint analysis of the virus produced by CWD/Agl mice showed the presence of oligonucleotide spots that are characteristic of both *Emv-1*- and *Emv-3*-encoded viruses, suggesting that both proviruses are expressed. The *Emv-3* provirus, however, has been found to be defective, and recent evidence suggests that the *Emv-1* provirus may also be defective as determined by the XC assay (5, 23; K. Hutchison, personal communication), suggesting that in CWD/Agl mice *Emv-3* and *Emv-1* sequences may interact in vivo to produce infectious virus. Other mapping studies will be required to determine whether spontaneous virus expression in CWD/Agl mice is a result of recombination between defective viral genomes or between ecotropic and xenotropic virus.

To determine whether amplification of ecotropic viral DNA sequences occurs in lymphomatous CWD/Agl mice, we characterized the viral DNA content of these tissues. High-molecular-weight cellular DNAs were extracted from frozen mouse spleens as previously described (15). DNAs (15 μg per lane) were digested to completion (60 U of enzyme, 2 h, 37°C) under reaction conditions described by the manufacturer (Bethesda Research Laboratories, Inc., Rockville, Md.), electrophoresed through 0.8% agarose gels, and transferred to nitrocellulose filters as previously described (7). Hybridization, washing, and autoradiography of the filter was performed as previously described (15). The probe, a 167-base-pair fragment cloned from the amino-terminal end of the p15E region of the ecotropic *env* gene (N-p15E, kindly provided by Winship Herr, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) was labeled with ^{32}P by the T4 polymerase system. This probe detects both ecotropic and certain oncogenic MCF-like proviruses. Furthermore, N-p15E has very little cross-reactivity with endogenous xenotropic murine leukemia virus sequences (14).

The results of *Pvu*II digestion of brain DNA and the DNA extracted from three tumorous spleens is shown in Fig. 1 (lanes 1 through 4). *Pvu*II cleaves once within the viral genome, generating viral-cellular junction fragments (18). As expected, all tumors contained the *Pvu*II viral DNA fragments of the endogenous *Emv-1* (4.3 kilobases [kb]) and *Emv-3* (5.4 kb) N-ecotropic genomes acquired during the generation of this stock. In addition, the tumors contained additional proviral sequences ranging in size from ca. 2 to 10 kb, whereas these new fragments were not detected in brain DNA. Analysis of 16 independent spontaneous CWD/Agl tumors revealed that the DNA of all tumors contained one to six somatically acquired tumor-specific fragments. The proviruses appeared to be integrated in many sites in DNAs of different CWD/Agl mice, but within each individual, the tumors appeared to be clonal with respect to proviral insertions. Furthermore, DNAs prepared from different tumor tissues of individual lymphomatous CWD/Agl mice showed the same pattern of tumor-specific proviral insertions, again demonstrating that the tumors are of monoclonal origin (manuscript in preparation). The results of *Pst*I-*Eco*RI digestion of these DNAs is shown in Fig. 1 (lanes 5 through 7). *Pst*I cleaves only within the long terminal repeat sequences of ecotropic proviruses and yields a single detectable viral DNA fragment of 8.2 kb (18). *Eco*RI does not cleave within the ecotropic murine leukemia virus genome, but it does cleave within the genome of many MCF viruses (2). Double digest (*Pst*I-*Eco*RI) of DNAs extracted from tumorous spleens yielded only a fragment of 8.2 kb, suggesting that the tumor-specific proviral sequences detected in CWD/Agl tumors neither are derived from MCF viruses nor are defective N-ecotropic murine leukemia virus genomes. Whereas N-p15E detects only class I MCF viruses, analysis of tumor DNAs with other restriction enzymes and probes also suggests that tumor-specific proviral sequences in these tumors are not derived from MCF viruses (manuscript in preparation). It is possible that these proviral sequences could be derived from recombinational events between other defective viruses, as has been suggested in some instances of spontaneous avian leukosis virus expression in chickens (4).

Rowe recently reported marked abnormalities, including erratically curved whiskers, of mice infected at birth with replication-competent murine leukemia viruses (19). Mice with $Rmcf^{r/r}$ had reduced frequencies of the anomalies, whereas mice with $Rmcf^{s/s}$ alleles had more extensive alterations than did $Rmcf^{r/s}$ mice. The curly-whisker mutation

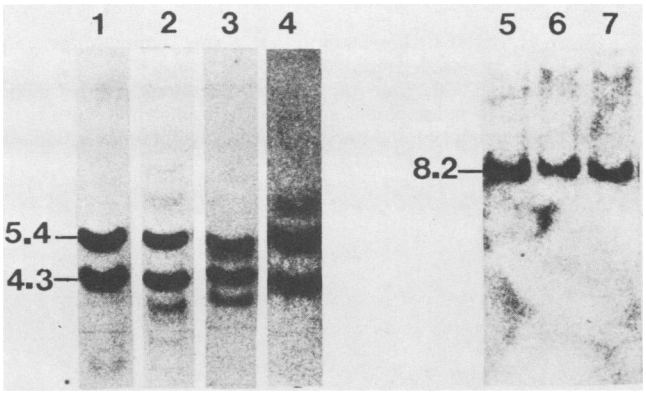

FIG. 1. DNAs extracted from the brain (lane 1) or tumorous spleens T-1 (lanes 2 and 5), T-2 (lanes 3 and 6), and T-3 (lanes 4 and 7) were digested with *Pvu*II (lanes 1 through 4) or *Pst*I and *Eco*RI (lanes 5 through 7). The digested DNAs were electrophoresed on 0.8% agarose gels and transferred to nitrocellulose paper by the method of Southern (21). The paper was incubated with a hybridization mixture containing ^{32}P-labeled N-p15E and autoradiographed.

appears not to influence either ecotropic virus expression or lymphoma incidence. It is not known, however, whether the mutation has any effect on xenotropic virus expression.

The inbred strain CWD/Agl provides an important and useful new model for the study of the role of retroviruses in B-cell lymphomas. Like other mouse strains with a high incidence of lymphocytic tumors, the endogenous ecotropic virus is expressed early in the life of CWD/Agl mice. The role of the endogenous ecotropic viruses is not clear, however. Two other inbred mouse strains, HRS/J and SEA/GnJ, contain the *Emv-1* and *Emv-3* loci (15). Like CWD/Agl mice, HRS/J mice express high levels of ecotropic virus early in life. HRS/J mice, however, have a high incidence of spontaneous T-cell leukemia associated with MCF viruses (11). SEA/GnJ mice, on the other hand, express low levels of ecotropic virus and have a low incidence of lymphomas (unpublished data). These observations suggest that other factors are involved in lymphomagenesis in these strains. To date, no MCF-like viruses have been isolated from CWD/Agl tumors. This, along with the molecular analysis of tumor DNAs, suggest that MCF viruses are not required for lymphomagenesis in CWD/Agl mice. MCF-like viruses have been isolated from tumorous spleens of spontaneous B-cell lymphomas of SJL/J(v^+) (8, 9); however, the oncogenicity of these MCF viruses has not been established.

From one to six tumor-specific *Pvu*II fragments have been observed in all tumor DNAs examined. Although the tumor-specific sequences appear to be integrated in many sites in DNAs of different CWD/Agl mice, it is possible that one of these proviruses is integrating at a site close to a cellular oncogene. This could result in an increase in the transcription of the proto-oncogene and initiate leukemogenesis. The observation of only a single tumor-specific fragment in the DNA of one tumor suggests that only one integration would be required for the transformation event. Research on whether there are chromosomal rearrangements involving specific proto-oncogenes or increased expression of these oncogenes in spontaneous B-cell lymphomas of CWD/Agl mice is ongoing.

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