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Accelerated Appearance of Multiple B Cell Lymphoma Types in NFS/N Mice Congenic for Ecotropic Murine Leukemia Viruses

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SUMMARY: Spontaneous lymphomas occur at high frequency in NFS.V$^+$ mice, strains congenic for ecotropic murine leukemia virus (MuLV) proviral genes and expressing virus at high titer. In the present study, a total of 703 NFS.V$^+$ lymphomas were studied by histopathology, immunophenotypic analysis, immunoglobulin heavy chain or T cell receptor β chain rearrangements, and somatic ecotropic MuLV integrations; 90% of the lymphomas tested were of B cell lineage. Low-grade tumors included small lymphocytic, follicular, and splenic marginal zone lymphomas, while high-grade tumors comprised diffuse large-cell (centroblastic and immunoblastic types), splenic marginal zone, and lymphoblastic lymphomas. Comparison of mice of similar genetic background except for presence (NFS.V$^+$) or absence (NFS.V$^-$) of functional ecotropic MuLV genomes showed that NFS.V$^-$ clonal lymphomas developed at about one-half the rate of those occurring in NFS.V$^+$ mice, and most were low-grade B cell lymphomas with extended latent periods. In NFS.V$^+$ mice, clonal outgrowth, defined by Ig gene rearrangements, was associated with acquisition of somatic ecotropic proviral integrations, suggesting that, although generation of B cell clones can be virus independent, ecotropic virus may act to increase the rate of generation of clones and speed their evolution to lymphoma. The mechanism remains undefined, because only rare rearrangements were detected in several cellular loci previously associated with MuLV insertional mutagenesis. (*Lab Invest 2000, 80:159–169*).

One of the earliest mammalian animal models in cancer research was the AKR mouse strain developed by Jacob Furth about 70 years ago. Selective breeding yielded mice with nearly 100% mortality due to thymic lymphoma in 7 to 14 months (Cole and Furth, 1941). AKR and the limited number of other T cell lymphoma-prone mouse strains (C58, HRS, and some AKXD recombinant inbred (RI) lines) (Gilbert et al, 1993; Green et al, 1980; MacDowell and Richter, 1935; Meier et al, 1969; Mucenski et al, 1986, 1988) have been intensively studied, yielding a partial molecular understanding of the role of ecotropic and recombinant mink cell focus-inducing (MCF) murine leukemia viruses (MuLV) in pathogenesis of this early-onset lymphoma (Cloyd et al, 1980; Hartley et al, 1977; Herr and Gilbert, 1983; Holland et al, 1985; M^{c}Grath and Weissman, 1979; Rowe and Hartley, 1983; Stoye et al, 1991; Thomas et al, 1984). In contrast to those of T cell lineage, spontaneous B cell lymphomas are most commonly seen in aged mice of strains in which T cell lymphoma does not predominate. For example, the incidence of B cell lymphoma of various types may be 30% or more in unmanipulated, aged BALB/c, C57BL (Frith and Wiley, 1981), AKR.*Fv-1*^{b} (Haran-Ghera et al, 1993), CWD (Angel and Bedigian, 1984; Mucenski et al, 1988; Thomas et al, 1989), or certain AKXD RI lines (Gilbert et al, 1993; Mucenski et al, 1986) held for up to 3 years, as well as in thymectomized AKR (Peled and Haran-Ghera, 1985) and *Eμ-myc* transgenic mice (Adams et al, 1985). In SL/Kh (Shimada et al, 1993; Yamada et al, 1994) and SJL/J (Haran-Ghera et al, 1967) mice, a high incidence of pre-B and lymphoplasmacytic B cell lymphomas, respectively, occurs earlier in life.

Previous studies of NFS.V congenic mice (NFS carrying the loci from AKR/N or C58/Lw that encode infectious ecotropic MuLV [Chattopadhyay et al, 1975; Rowe, 1978; Rowe and Kozak, 1980]) established that lymphomas occurred frequently (Fredrickson et al, 1984) and that the majority were nonthymic and of B cell origin, as determined by analysis of expression of Ig and lineage-defining cell surface antigens (Fredrickson et al, 1985). Lymphomas developed within 200–600 days, and high incidence was associated with high levels of ecotropic MuLV expressed early in life. NFS.V$^+$ mice, represented by five strains descended

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from NFS.*Akv1*, NFS.*Akv2*, and NFS.*C58v1*, express high levels of ecotropic MuLV (NFS.V$^+$). NFS.V$^-$ strains are genetically similar but lack ecotropic viral coding genes. The primary aims of studying this mouse population were (a) to examine the morphologic diversity of spontaneous B cell lymphomas, and (b) to study in detail the enhancing effect on B cell lymphomagenesis of expression of ecotropic MuLV, including examination for association with new somatic ecotropic proviral integrations, as has been reported for some myeloid leukemias in AKXD mice (Mucenski et al, 1987a, 1988) and for a few cases of B cell lymphoma, notably in CWD and SEA and certain AKXD mice (Gilbert et al, 1993; Justice et al, 1994; Mucenski et al, 1987a, 1988).

Over 700 hematopoietic neoplasms, 95% of which were lymphomas, occurring over a 5-year period in NFS.V$^+$ and NFS.V$^-$strains were identified morphologically. Many were analyzed by Southern blot hybridization for identification of T or B lineage, clonal patterns, and new ecotropic proviral integrations. Lymphomas of B cell origin were 10-fold more frequent than T-lineage tumors, and all lymphomas tested comprised monoclonal or oligoclonal populations. New somatic integrations of ecotropic MuLV were found in 88% of NFS.V$^+$ lymphomas tested, consistent with the reported close association of high titer of infectious ecotropic virus early in life with high lymphoma incidence (Fredrickson et al, 1984). Also, however, long-latency, low-grade lymphoma was detected in about 40% of mice without inducible ecotropic MuLV integrations, demonstrating that ecotropic MuLV expression is not an absolute requirement for initiation of lymphomagenesis but rather is important in accelerating progression.

Results

Occurrence of Lymphomas in NFS.V$^+$ Mice

Mice were chosen for necropsy when they had developed enlarged lymph nodes and/or spleen, dyspnea due to enlargement of thymus or hilar nodes, or hind limb paralysis due either to compression of the spinal cord or to meningeal infiltration. In addition, some mice were selected for necropsy when spleens were determined by palpation to be about three times their normal size. Illness other than lymphoma was rare, but occasional mice succumbed to other hematopoietic neoplasms, epithelial tumors of the ovary, lung, mammary or adrenal gland, or *Citrobacter freundii*-induced colitis. Histopathologic diagnosis was based on published criteria (Fredrickson et al, 1995; 1999) conforming to those used for the Kiel Classification of human lymphomas (Lennert and Feller, 1992). Because REAL (Harris et al, 1994) and, more recently, the proposed WHO classifications (Jaffe et al, 1999) of human lymphomas are supplanting the Kiel system, we have proposed a "NIAID/NCI" classification of mouse lymphomas that parallels the WHO system in many respects (Morse et al, 1999; Taddesse-Heath and Morse, in press). The relations of this formulation to the Kiel-based system for the mouse (Fredrickson et al, 1995; Fredrickson and Harris, 1999) are presented in Table 1 as they pertain to diagnoses of lymphoma types occurring at greater than 1% frequency in our series.

The following considerations are important in understanding the new classification scheme. First, mouse B cell lineage lymphomas with lymphoblastic morphology identical cytologically to human precursor lymphoblastic lymphoma (LL) include precursor B cell as well as surface immunoglobulin (sIg$^+$) lymphomas. Almost all cases of B lineage LL tested for Ig expression by immunocytochemistry or flow cytometry were positive, indicating that pre-BLL were not common in this series. At least some of the sIg$^+$ LL comprise homologs of Burkitt-like lymphoma, but for the majority this is not yet established. Until the nature of the sIg$^+$ "non-Burkitt" type is better understood, we will use the term B cell lymphoblastic lymphoma (B-LL) for all the sIg$^+$ lymphomas with this morphology. Second, it has not been shown that mouse T cell lymphomas with lymphoblastic morphology are of the precursor type. We will refer to all T cell lymphomas with this morphology as T cell lymphoblastic lymphomas (T-LL). Third, splenic marginal zone lymphomas (MZL)

Table 1. Comparison of Classification Systems for Human and Mouse Lymphomas

	Proposed WHO (human)	Proposed NIAID/NCI (mouse)	Kiel-based (mouse)
Lymphomas of mature B cells			
Low grade	Small lymphocytic (SLL)	SLL	SLL
	Follicular (FL)	FL	Centroblastic-Centrocytic (CBCCL)
	Marginal zone, splenic (MZL)	MZL	MZL
Intermediate grade	Not defined	MZL+	MZL+
High grade	Not defined	MZL++	MZL++
	Diffuse large cell (DLCL)	DLCL	
	Centroblastic type (CB)	DLCL (CB)	Centroblastic follicular (CBLfol)
			Centroblastic diffuse (CBLdiff)
	Immunoblastic type (IB)	DLCL (IB)	Immunoblastic (IBL)
	Burkitt-like (BL)	DLCL (BL)	Lymphoblastic (B-LL)
Lymphomas of T cells	Precursor T cell LL (pre-TLL)	Pre-TLL	T-LL

have been shown to exhibit progression in grade (Fredrickson et al, 1999), and we indicate low-, intermediate-, and high-grade tumors of this type as MZL, MZL+, and MZL++. Finally, the Kiel definitions of lymphomas falling under the category of diffuse large cell (DLCL) in the WHO and NIAID/NCI systems have been termed immunoblastic (IBL), centroblastic follicular (CBLfol) to indicate probable origin from follicular cells and centroblastic diffuse (CBLdiff) to indicate tumors where the origin in the marginal zone or follicle could not be identified.

With these considerations in mind, the following provides a brief description of the histogenesis and morphology of lymphomas seen in NFS.V$^+$ mice. Among the low-grade tumors, small lymphocytic lymphoma (SLL) initially is manifested as an increase in the small lymphocyte population of the splenic white pulp (Fig. 1A) with eventual spread into the red pulp and dissemination to lymph nodes, liver, lungs, and kidneys. Mitotic figures and large cells are rare, but leukemia is often seen. Follicular lymphomas (FL) form grossly discernible nodular growths that are seen mainly in the spleen and less frequently in lymph nodes. In these enlarged follicles, the normal population of small lymphocytes is displaced peripherally by one composed of centroblasts and centrocytes (Fig. 1B). Low-grade MZL presents as an increase in the normally sparse population of marginal zone cells (Fig. 1C) to form distinctive perifollicular rings (Fredrickson et al, 1999). These rings increase in size and coalesce, filling the red pulp and fragmenting the follicles. Such progression to high grade is further manifested by accumulation of centroblasts and immunoblasts (Fig. 1D), often without changes in clonal populations (Fig. 1E).

High-grade lymphomas designated as DLCL(CB) (Fig. 1F) or DLCL(IB) (Fig. 1G) are of splenic origin but often spread to lymph nodes and extranodal sites, particularly the liver (Fig. 1G). Primary sites of LL involve lymph nodes for those of B cell lineage or thymus in the case of T cell LL, with variable degrees of extension to the spleen. These are aggressive lymphomas with a characteristic growth pattern in which normal nodal structures are replaced with sheets of lymphoma cells that extend into surrounding adipose tissue, and there is frequent marked infiltration of liver, lungs, and kidneys. Cytologically, cells are generally of medium size, interspersed with numerous mitotic figures and variable numbers of tingible body macrophages engulfing apoptotic bodies (Fig. 1H).

Among 708 cases of hematopoietic neoplasms in NFS.*Akv1*, NFS.*Akv2*, and NFS.*C58v1* mice, there were 677 lymphomas. Of these, 636 represented morphologic lymphoma types occurring at a frequency greater than 1%, as shown in Table 2 for the five NFS.V$^+$ families combined. The relative frequency of lymphoma types was very similar for each family.

Lymphomas of the splenic marginal zone (MZL) were the most frequent, representing 38% (240/636) when all morphologic grades (Fredrickson et al, 1999) were combined. MZL was largely limited to the spleen except for relatively minor involvement of splenic and mesenteric lymph nodes. The frequency of very large spleens (0.8 g or more) increased with progression in grade, being 5% for low-grade and 56% for high-grade MZL. Lymphoblastic lymphoma (LL) was next most frequently seen, at 27%, and was diagnosed on average 3 months earlier than other lymphoma types. Early detection was facilitated by easily observed peripheral lymphadenopathy that was often more marked than splenomegaly even in B-LL. The remaining lymphoma types were about equally distributed, and the most consistent gross finding was splenomegaly. The frequency of 0.8 g or greater spleen weight was 65% for follicular lymphoma and ranged from 71% to 88% for DLCL of different morphologic subtypes. In low-grade SLL, only 39% of spleens reached this size. Overall, most mice not succumbing to LL developed other types of lymphoma within 350 to 450 days; few mice survived beyond 600 days.

Although an accurate measure of overall incidence of lymphoma cannot be determined from these data because of periodic discards and early sacrifice, in an earlier study 52 NFS.V$^+$ mice were observed for diseases throughout their lifespan. Of these, 44 (85%) developed terminal hematopoietic neoplasms, including lymphoma (36 cases), myeloid leukemia (4 cases), or histiocytic sarcoma. The frequency of lymphoma types was closely similar to that presented in Table 1 (data not shown).

Molecular and Phenotypic Characterization of Lymphomas

DNAs prepared from lymphoma tissue of 483 cases were analyzed by Southern blotting for rearrangements within immunoglobulin heavy chain (IgH) and TCRβ genes in order to establish B or T cell lineages and to identify clonal populations (Table 3). In addition, 105 cases were examined by flow cytometry for cell surface antigen expression. In all DNAs, we detected clearly defined hybridization bands of random size and variable density, typically lighter in those samples with a high proportion of normal cells as judged by flow cytometry and/or histology. These bands were used to define clonal lymphoma populations as monoclonal, represented by one band or two of equal intensity, interpreted as indicating a rearrangement of one or both alleles; monoclonal/oligoclonal, represented by several bands of variable density but with a clearly predominant monoclonal population; and oligoclonal, typically three to five bands.

Ninety-two percent of all lymphomas tested, including 75% of the LL, demonstrated rearrangements of IgH but not TCRβ and were classed as B cell lymphomas. In 39 other DNAs, including 37 LL, clear clonal rearrangements, usually monoclonal, of the TCRβ locus established a T cell lineage. As shown in Table 3, in the majority of cases of each lymphoma type the rearrangements of IgH and TCRβ were of monoclonal pattern (333/444 B cell and 36/39 T cell). Correlation of lineage determinations made by FACS analysis with those made by Southern blotting for rearrangements in immunoglobulin or T cell receptor genes was high.

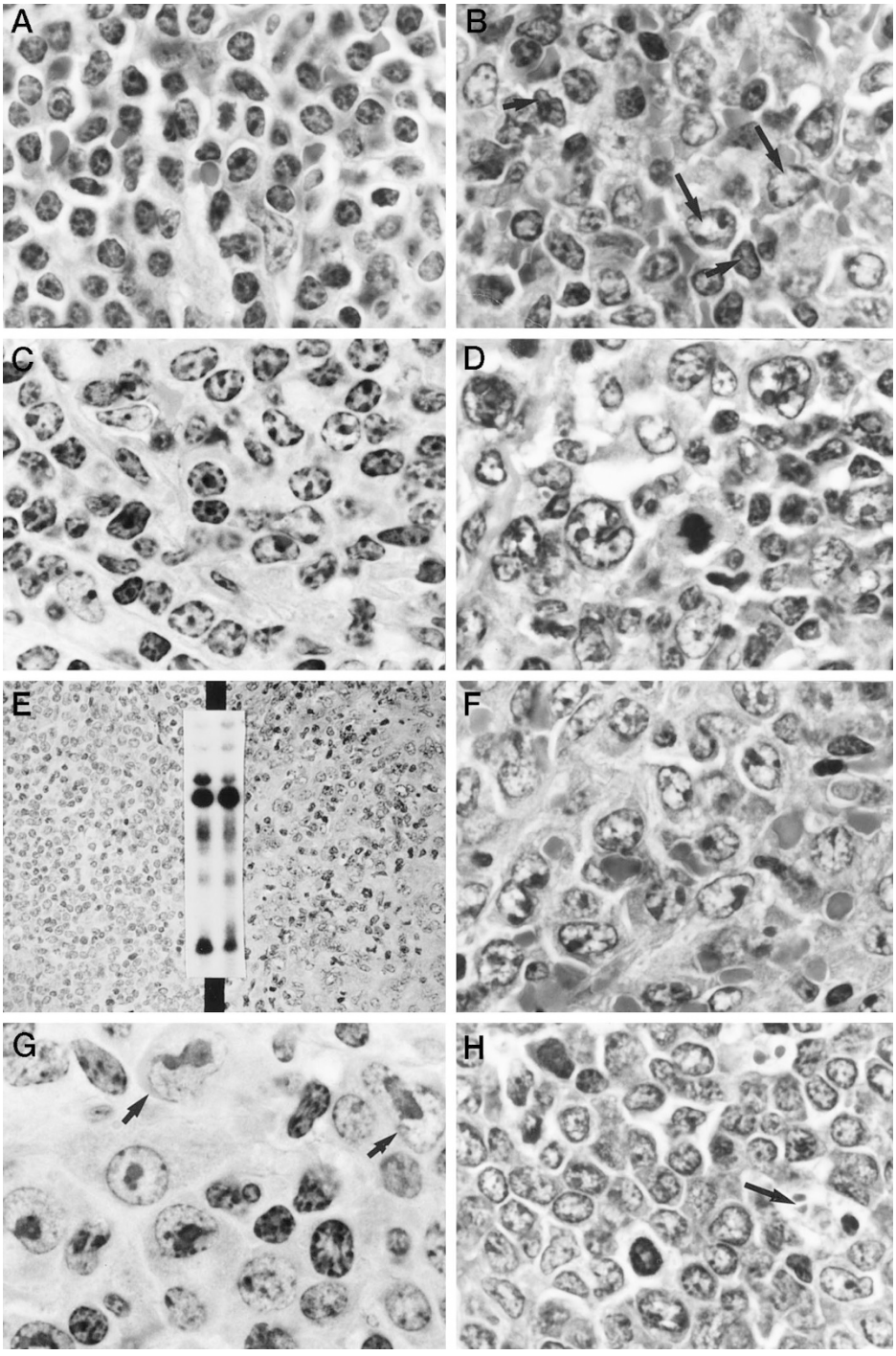

Figure 1.

H&E ×300 except as noted. *A*, SLL, showing mainly round nuclei containing one to several nucleoli; a rare mitotic figure is shown at upper center. *B*, FL, containing both centroblasts (*arrows*) and centrocytes with smaller darker nuclei (*arrowheads*). *C*, Low-grade MZL. Typical MZL cells form an expanded marginal zone and have round to ovoid nuclei separated by plentiful lightly staining cytoplasm. *D*, High-grade MZL++ composed of a heterogeneous population dominated by large cells and featuring numerous mitotic figures (E) MZL as detected in spleen biopsy (*left*) and necropsy (*right*) samples, showing progression from low grade to high grade, respectively, with retention of the same clonal J_H rearrangements (*center panel*). The interval from biopsy to necropsy was 40 days, with spleen weight increasing from approximately 300 to 600 mg (×50). *F*, DLCL(CB) composed of large cells with round, vesicular nuclei containing two distinct nucleoli adhered to the nuclear membrane. (G) DLCL(IB), containing a high proportion of typical immunoblasts (*arrows*). *H*, LL, composed of fairly uniform, closely packed cells with ovoid nuclei, containing one to several nucleoli, interspersed with macrophages (*arrow*) containing apoptotic bodies.

Of cases that were analyzed by FACS and later found to have mono- to oligoclonal populations, 98 of 99 with IgH alterations were identified as B lineage lymphomas, while 6 of 6 with TCRβ rearrangements alone were T cell lymphomas. In about 70% of T cell lymphomas, rearrangements within the IgH locus were also detected, as observed previously (Fredrickson et al, 1993; Herr et al, 1983); the T cell lineage in a

Table 2. Frequency of Different Morphologic Lymphoma Types in Lymphomas Occurring in NFS.V$^+$ Mice

Lymphoma type	No. of cases	% of lymphomas (n = 636)	Age at diagnosis[a]
Low grade			
SLL	57	9	408 ± 101
FL	37	6	436 ± 111
MZL	97	15	393 ± 85
Subtotal	191	30	
Intermediate grade			
MZL+	54	8	425 ± 88
High grade			
MZL++	89	14	407 ± 84
DLCL			
CBLfol	24	4	394 ± 109
IBL	38	6	396 ± 126
CBLdiff	58	9	394 ± 89
LL	182	27	282 ± 112
Subtotal	391	61	

* Average age at diagnosis in days ± standard deviation.

representative sample of these cases was confirmed by flow cytometric or immunocytochemical analysis.

Ecotropic Viral Gene Analyses in Relation to B Cell Lymphomagenesis in NFS.V Congenic Mice

Comparison of NFS.V$^+$ and NFS.V$^-$ strains. It was reported earlier (Fredrickson et al, 1984) that among NFS mice segregating for ecotropic MuLV sequences, lymphomas developed preferentially in those individuals expressing high titers of infectious ecotropic MuLV early in life, as compared with those with low titers or virus negative. In the course of the present study, it was noted that lymphomas occasionally were seen in a strain of similar genetic background but in which the ecotropic MuLV integration had been lost: NFS.*c58v2*eco-negative. Lymphoma was frequently detected only by histopathology, there being little or no splenomegaly. Overall, among 29 lymphomas seen during the 5-year period, there were 14 low-grade, 5 intermediate-grade, and 10 high-grade, with average latencies of 642 ± 143 days, 632 ± 92 days, and 364 ± 195 days.

Further evaluation of lymphomagenesis in virus-negative mice employed mice generated in the course of a recent re-derivation of one of the high-virus strains, NFS.*Akv2* (4–2K). This strain presently carries three ecotropic MuLV integrations detectable in *Eco*RI digests: 25 kilobase (kb), representing *Emv12*, the originally described *Akv2* locus (Kozak and Rowe, 1980), and two additional bands of 22 kb and 15.3 kb, respectively. 4–2K mice were bred to the NFS.V$^-$ stock and F2, backcross, and inbred progeny typed for presence of these bands and expression of ecotropic MuLV. The mice used in these matings were from inbred lines that had been backcrossed to NFS/N for 13 generations and could thus be considered as congenics. Groups were set aside for lifetime monitoring for lymphoma. Assays for infectious ecotropic virus (data not shown) revealed that the 25 kb band was consistently associated with expression of virus, detected by XC plaque assays of tail extract, splenic infectious centers or, in a minority of cases, by IUdR induction of tail cultures. Mice segregating for only the 22 kilobase (kb) or 15.3 kb bands, as well as mice lacking a reactive band, were completely negative for infectious virus even upon IUdR treatment. Table 4

Table 3. Clonal Analysis of NFS.V$^+$ Lymphomas: Southern Blot Hybridization to Detect Rearrangements of Immunoglobulin Heavy-Chain (IgH) and T Cell Receptor β Chain (TCRβ) Genes

	Clonal Patterns†							
	IgH (No. with clonal pattern)				TCRβ (No. with clonal pattern)			
Lymphoma type*	No. cases	Mono	Mono/Oligo	Oligo	No. cases	Mono	Mono/Oligo	Oligo
Low grade								
SLL	53	36 (68)	12 (23)	5 (9)	2	2 (100)	0	0
FL	28	21 (75)	3 (11)	4 (14)	0			
MZL	47	26 (55)	9 (19)	12 (26)	0			
Intermediate grade								
MZL+	37	25 (68)	10 (27)	2 (5)	0			
High grade								
MZL++	80	57 (71)	18 (23)	5 (6)	0			
DLCL								
CBLfol	17	14 (82)	0 (0)	3 (18)	0			
IBL	33	22 (67)	6 (18)	5 (15)	0			
CBLdiff	37	34 (92)	3 (8)	0 (0)	0			
LL	112	98 (87)	12 (11)	2 (2)	37	34 (92)	3 (8)	0
Total cases	444	333 (75)	73 (16)	38 (9)	39	36 (92)	3 (8)	0

* Abbreviations of diagnoses as for Table 1. Data for MZLs from Fredrickson et al, 1999.

† Number of cases and number and (percent of total) with clonal rearrangement in each diagnostic category. DNAs were prepared from spleen, lymph node, or thymus, digested with EcoRI, and hybridized with J_H probe or digested with HpaI and hybridized with TCRβ probe to detect IgH and TCRβ rearrangements, respectively. Mono, monoclonal; Mono/Oligo, monoclonal/oligoclonal; Oligo, oligoclonal.

Table 4. Lymphoma Incidence and Frequency of Morphologic Types in NFS.*Akv2* (4-2K) Populations Segregating for Germline Ecotropic MuLV Sequences

	Ecotropic MuLV expression*							
	Positive				Negative			
Lymphoma type†	No.	% of lymphomas	Age‡	Fraction clonal§	No.	% of lymphomas	Age	Fraction clonal
Low grade								
SLL	8	12	570 ± 106	3/3	3	12	706 ± 19	
FL	11	16	570 ± 74	5/5	1	4	658	1/1
MZL	24	36	526 ± 118	12/12	16	64	612 ± 104	4/4
Composite¶	3	4	451 ± 97	1/1	0			
Subtotal	46	69			20	80		
Intermediate grade								
MZL+	0				2	8	675	
High grade								
MZL++	2	3	553	1/1	2	8	675	
IBL	3	4	499 ± 83	1/1	0			
CBLdiff	3	4	551 ± 14	2/2	0			
LL	10	13	373 ± 198	6/6	1	4	744	1/1
Composite#	3	4	474 ± 110	2/2	0			
Subtotal	21	31			3	12		
Total lymphomas/no. of mice	67/83	81	511 ± 132	33/33	25/61	41	640 ± 97	6/6

* Ecotropic virus-positive mice carry the 25 kb germline EcoRI fragment hybridizing in Southern blotting with the EcoSp probe and associated with spontaneous and/or induced expression of infectious ecotropic MuLV. Mice in the virus-negative group carry EcoRI EcoSp-reactive fragments of 22 kb, 15.3 kb, or no reactive sequences.

† Abbreviations and definitions as for Table 1. Composite tumors are defined as two distinct lymphoma types occurring in the same spleen.

‡ Age at diagnosis in days ± standard deviation.

§ Fraction clonal = no. of cases with monoclonal or oligoclonal IgH or TCRβ rearrangements divided by no. tested.

¶ Includes FL/MZL (3 cases).

Includes CBL/MZL (2), IBL/SLL (1).

presents the frequency of lymphoma types and the incidence after at least 18 months of observation. In virus-expressing mice carrying the 25 kb band alone or in combination with the 22 kb or 15.3 kb band, the incidence was 67 of 83 (81%) with an average age at diagnosis of 511 ± 132 days, while for virus-negative segregants (pooled results of 22 kb, 15.3 kb, and no band) the incidence was 25 of 61 (41%) with a latency of 640 ± 97 days (chi square = 13.9; $p = 0.0002$). All lymphomas tested, whether in virus-positive or -negative mice, were monoclonal or oligoclonal.

Somatic provirus integration. As a basis for evaluating insertional mutagenesis by somatically acquired ecotropic MuLV proviral genomes, 181 NFS.V$^+$ B cell lymphomas representing all morphologic types were examined by Southern blot hybridization for new provirus integrations as determined by comparison with matching tail DNAs. Somatic integrations were detected in 162 lymphomas (90%), and frequency was comparable in all morphologic types. The number of new integrations varied from one to six but was usually one or two. Bands were of variable hybridization intensity, many being quite faint. Examples of somatic and germline integrations are illustrated in Figure 2 for three lymphomas and one reactive spleen as compared with the respective tail samples. Three germline bands are seen in all samples; for 32472 and 32800, there are one or two additional bands, indicative of reintegrations of proviruses in the germline. For the splenic lymphomas, but not the reactive spleen, there are additional bands of variable intensity representing new somatic integrations.

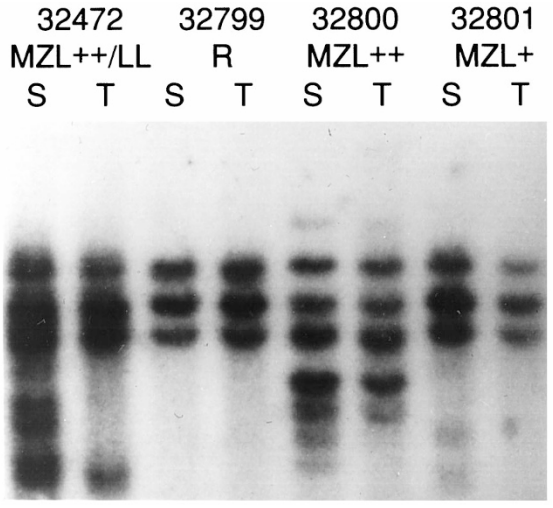

Figure 2.
Southern blot analyses for identification of somatic MuLV integrations in lymphomas of NFS. V$^+$ mice. DNA samples of spleens (S) and corresponding tail biopsies (T) from NFS.Akv2 mice were digested with *Eco*RI and hybridized with the ecotropic-specific probe EcoSp. Germline integrations are those also present in tail DNA. One to two somatic integrations are seen in the lymphoma DNAs, whereas the spleen with reactive (R) changes had none.

Rearrangement of cellular genes. Probes representing several cellular genes identified in other lymphoma

systems as activated or dysregulated by somatic MuLV integrations were used to screen DNA samples from B cell lymphomas for disruption of germline configurations of each oncogene. Forty to 80 examples of LL, SLL, MZL, FL, and each DLCL type were tested. Only rare rearrangements were detected in *Myc* (2/266) and *Pim1* (4/100). All the rearrangements were found in LLs, except for *Myc* disruption in one DLCL(CB). It has not been determined whether these changes were occasioned by proviral insertion. No alterations were detected in *Evi5*, *Fis1*, or *Ccnd1* (S. K. Chattopadhyay, X. Liao, and C.–F. Qi, unpublished data). Thus, these oncogenes are not frequent targets for insertional mutagenesis in NFS.V$^+$ mice.

Pathogenicity of NFS.V$^+$ Viruses. MuLV isolates from three B cell lineage NFS.V$^+$ lymphomas, including one LL, one SLL, and one high-grade MZL, were inoculated into newborn NIH Swiss or NFS/N mice. Only isolates from case 32458, the MZL, induced lymphoma; this mouse also had an osteosarcoma, but the significance of this is not known. Ten litters were inoculated with harvests of tissue culture passaged material containing both ecotropic and MCF viruses. Each litter yielded mice with lymphoma, the overall incidence being 30 of 61 (49%) with an average latent period to disease of 347 $\pm$ 125 days. In the range of lymphoma types seen, LL predominated with 16 cases, 10 B cell and 6 T cell. Also found were six FL, five DLCL (two CBL and three IBL), two MZL, and one SLL. All of the 25 lymphoma DNAs tested contained one or more clonal integrations of ecotropic MuLV sequences. In contrast, 10 litters inoculated with MCF virus isolates alone yielded only five mice with lymphoma (5/60, 8%; average latency 412 $\pm$ 66 days); there were four LL (one B cell and three T cell) and one FL. As would be expected, none of these lymphoma DNAs displayed ecotropic MuLV integrations.

Because pathogenic MCF viruses often require the presence of ecotropic virus for in vivo infection (Cloyd et al, 1981), five litters of NFS.V$^+$ mice were inoculated with selected MCF-only harvests and observed for 7 months to test for acceleration of lymphoma development. Eight of 34 mice developed lymphoma (24%), four LL (one B cell, three T cell), two MZL, and two SLL, with an average latency of 188 $\pm$ 27 days. Four of seven DNAs contained new somatic ecotropic virus integration. Although the average latency to tumor development differed markedly in the two groups of mice infected with MCF alone, the difference in incidence is not statistically significant (chi square = 2.7; $p = 0.1$). In the single litter inoculated with ecotropic MuLV alone, one tumor developed, an unusual splenic composite of myelogenous leukemia and histiocytic sarcoma. These data indicate that although lymphomas did develop in exogenously infected low-lymphoma mice, the complex representing pathogenic virus was not specific for a single lymphoma phenotype.

More efficient lymphoma induction observed with mixed ecotropic and MCF MuLV inocula could also suggest the presence of a replication-defective, pathogenic component. To examine this possibility, Hirt DNA was prepared from *M. dunni* cells infected with early *M. dunni* passage harvests of 32458 and examined by Southern blotting to determine the size of MuLV-reactive genomes. The DNA was digested with various restriction enzymes and hybridized with the EcoSp and MCF-xenoSp probes. Only full-size genomes were detected. Although the ecotropic virus seems to be a pure population, there appear to be two or more components to the MCF virus population, one predominating (data not shown). Further analysis will be required to determine whether one or more have pathogenic potential.

Discussion

NFS.V$^+$ mice, congenic for loci derived from the AKR and C58 strains that code for infectious ecotropic MuLV, develop a wide spectrum of B cell-lineage lymphomas, including several with striking similarities to human neoplasms. Lymphomas occur at high incidence, within about 8 to 18 months, and, in contrast to the T cell lymphomas of AKR and C58, the majority are of B cell lineage. These include lymphoblastic, lymphocytic, and follicular types as well as MZL, only recently recognized as an important mouse lymphoma (Fredrickson et al, 1999; Ward et al, 1999; Yumoto et al, 1980). Although there is some variation in latency and frequency of individual lymphoma types, in three studies of hematopoietic neoplasms in NFS.V$^+$ mice carried out at intervals over the past 18 years, the lymphoma incidence in high-virus mice was 73% (Fredrickson et al, 1984), and 82% and 81% (this study) for mice observed at least 18 months.

The majority of lymphomas early in life were LL, 85% of cases occurring before 1 year of age, whereas MZL, SLL, FL, and DLCL lymphomas predominated in the second year of life. All of 483 NFS.V$^+$ lymphomas tested contained clonal populations defined by IgH or TCRβ rearrangements, the majority being monoclonal.

Of the NFS.V$^+$ clonal B cell lymphomas tested, 90% contained novel somatic ecotropic integrations, a frequency consistent with MuLV insertional mutagenesis as an important lymphomagenetic mechanism. In marked contrast to T cell lymphomas, targeting of cellular oncogenes (Mucenski et al, 1987b) or involvement of MCF MuLVs has been reported only infrequently for B lineage tumors (Armstrong et al, 1980; Gilbert et al, 1993; Mucenski et al, 1987b, 1988; Pals et al, 1986; Thomas et al, 1990; Zijlstra et al, 1983, 1986), but association of somatic ecotropic virus integrations in spontaneous pre-B or B cell lymphoma has been reported in certain AKXD RI strains (Gilbert et al, 1993; Justice et al, 1994; Mucenski et al, 1987a); CWD (Mucenski et al, 1988); SEA (Mucenski et al, 1988), C57BL (Zijlstra et al, 1986), SL/Kh (Yamada et al, 1994), and in graft-versus-host reaction in CAF_1 mice (Pals et al, 1986).

The present study shows clearly, however, that mice of comparable genetic background to NFS.V$^+$ but lacking sequences expressing infectious virus (NFS.V$^-$ mice) are not free of lymphoma. Such mice do develop clonal lymphomas, but at about one-half the frequency of those segregating for a virus

induction-competent locus. Also, NFS.V$^-$ lymphomas were predominantly low grade and occurred with longer latency than NFS.V$^+$ tumors (Table 4). T and B cell lymphomas also occur in p53-deficient mice, independent of any evidence of somatic integration of ecotropic proviruses (Ward et al, 1999) or expression of infectious virus (J. W. Hartley, unpublished data). The majority of B cell lesions involve premalignant hyperplasia of the marginal zone or relatively early stage MZL (Ward et al, 1999; L. Taddesse-Heath, unpublished data). We suggest, therefore, that a major influence of ecotropic MuLV on B cell splenic lymphomagenesis may be in hastening and intensifying an ongoing, presumably multistep process rather than its initiation. In virus-positive mouse strains, virus integration and spread also may play an important initiation role by stimulating the first emergence of clones at higher frequency. Mechanisms could include insertional mutagenesis and/or a direct or indirect effect of ecotropic or MCF MuLV-derived viral proteins. A mechanistically distinct role for MuLV in B cell lymphomagenesis could be through chronic low-level stimulation of the B cell receptor, a possibility supported by the observations that NFS.V$^+$ lymphomas transplant to virus-positive but not to virus-negative mice (Fredrickson et al, 1985). Essentially all NFS.V$^+$ B cell lymphomas express CD5 (Fredrickson et al, 1999), and CD5 has been shown to interact with Ig V_H framework sequences (Pospisil et al, 1996) as well as CD72 (Luo et al, 1992). Thus, signals provided by MuLV and CD5 could combine to promote B cell survival or proliferation, thus functioning as promoting factors in the evolution of the transformed state.

The demonstration of development of a variety of lymphomas following exogenous infection of very low lymphoma-incidence mice with uncloned mixtures of ecotropic and MCF MuLVs isolated from several B cell tumors is of potential importance. Although MCF viruses have been readily isolated from most NFS.V$^+$ lymphomas tested, those characterized molecularly were of class II structure (Chattopadhyay et al, 1982; Lung et al, 1983) and were not oncogenic in AKR or NFS.V$^+$ mice (Cloyd et al, 1980). It is noteworthy that studies using the Swiss mouse strain NMRI (Lovmand et al, 1998; Speth et al, 1995) uncovered a B lymphomagenic potential for Akv, the AKR-derived prototypic ecotropic MuLV strain, long considered nononcogenic, based on assays in other mouse strains. Other instances of B cell lymphoma induction by exogenous infection with either ecotropic or MCF MuLVs do not appear to be highly efficient (Thomas et al, 1990; Zijlstra et al, 1983; 1986). B cell lymphomas, mostly pre B-LL, can be induced by retroviruses that contain oncogenes such as *v-abl* (Abelson and Rabstein, 1970), *v-cbl* (Langdon et al, 1989), *v-myc* (Morse et al, 1986), or constructs of *v-myc* and *v-raf* (Kurie et al, 1990) or by infection of *Eμ-myc* transgenic mice with Moloney MuLV (Adams et al, 1985). In the case of pristane-induced plasmacytomas in BALB/c mice, the great majority of tumors carry translocations between *c-Myc* and the IgH locus (Potter and Wiener, 1992), but neither ecotropic nor MCF MuLV plays a role (Potter et al, 1984). In spontaneous mouse lymphoma, association of any morphologic types with specific chromosomal translocations that result in structural changes in oncogenes has not been seen with any frequency or consistency. Recent studies, however, suggest a functional role for p53 in pathogenesis of MZL (Ward et al, 1999), for BCL6 in high-grade mouse B cell lymphomas (C.F. Qi, et al. unpublished data, 2000), and possibly for cyclin D1 in low-grade lymphomas (Qi et al, 1998).

Materials and Methods

Mice

NFS.V congenic mice were bred and housed under conventional conditions at MA BioSystems, Inc. (Rockville, Maryland); both male and female mice were studied. These strains were established beginning in 1970 by Wallace Rowe, who developed several families of NFS/N mice bearing genes for independently segregating ecotropic proviruses of the high virus-expressing, high thymic lymphoma strains AKR/N and C58/Lw. Strain NFS/N lacks genetic information for ecotropic MuLV (Chattopadhyay et al, 1974) and has a very low incidence of lymphomas and other tumors (Fredrickson et al, 1984). Strains derived from AKR/N were initially bred to C57BR, then mated to NIH Swiss, backcrossed, and finally crossed to inbred NFS/N (Chattopadhyay et al, 1975; Rowe, 1978; Rowe and Kozak, 1980). C58/Lw was mated and backcrossed to NIH Swiss, then to NFS (Chattopadhyay et al, 1975; Fredrickson et al, 1985). Selection was by biological assay for high ecotropic virus expression, and segregation patterns in progeny tests were the basis for establishing families carrying single genes representing the parental provirus complement. Inbreeding of virus-positive segregants was begun at the N8 to N20 backcross to NFS. Since establishment of homozygosity, several inbred families have been maintained by brother-sister mating: five high-virus NFS.V$^+$ families, including two NFS.*Akv1* families carrying ecotropic MuLV locus *Emv11* (Rowe et al, 1972); two families of NFS.*Akv2*, carrying *Emv12* (Kozak and Rowe, 1980); and NFS.*C58v1*, carrying *Emv26* (Kozak and Rowe, 1982). In addition to the originally described loci, ecotropic viral gene reinsertions have occurred (Rowe and Kozak, 1980; Buckler et al, 1982; S. K. Chattopadhyay, T. N. Fredrickson, H. C. Morse, III, and J. W. Hartley, unpublished data), and thus the number of germline integrations in each family is now generally increased and may vary among litter mates. Three sublines, each carrying a single provirus, were derived recently from one NFS.*Akv2* family, 4–2K, which now carries three proviral integrations. These are designated 4–2K(25 kb), 4–2K(22 kb), and 4–2K(15.3 kb) and are described further in Results. NFS.V$^-$ mice derive from NFS.*C58v2* (Fredrickson et al, 1984), a strain in which the original proviral integration was lost and that therefore carries no sequences hybridizing to the ecotropic MuLV-specific probe.

Tissue Sampling

Spleen and occasionally lymph node and infiltrated nonlymphoid tissues, as indicated by gross findings, were routinely sampled for histopathology and frozen for later DNA extraction. In many cases, samples for flow cytometry and immunocytochemistry were also obtained.

Histology

Tissue samples were fixed in 10% buffered formalin for sectioning and staining with hematoxylin and eosin.

Molecular Studies

High molecular weight DNAs were prepared from lymphoid tissues or tail samples (Gross-Bellard et al, 1973) and analyzed by Southern blotting as previously described (Fredrickson et al, 1999). For immunoglobulin heavy chain (IgH) rearrangements, DNAs were digested with *Eco*RI and hybridized with the J11 J_H probe (Lang et al, 1982). To confirm similarity of hybridizing band size in paired DNAs from spleen biopsy and necropsy samples, *Sac*I and *Xba*I were used as required. For T cell receptor β chain (TCRβ) rearrangements, digestion was with *Hpa*I and the probe was CTβ (Hedrick et al, 1985). To screen for somatic integrations of ecotropic MuLV, DNAs were digested with *Eco*RI and occasionally *Pvu*II and probed with the ecotropic-specific probe EcoSp, a 400-bp *Sma*I fragment (Chattopadhyay et al, 1980). For analysis of MCF MuLV genomes, an MCF-xenoSp probe (referred to as B-E in Chattopadhyay et al, 1982), which detects MCF and xenotropic MuLV sequences, was used.

Flow Cytometry and Immunocytochemistry

Single-cell suspensions prepared from splenic or lymph node lymphomas were stained with a panel of antibodies for two-color analyses using a FACScan (Becton Dickinson, San José, California) by established techniques using previously described antibodies (Fredrickson et al, 1999). Immunocytochemistry was performed on cryostat-sectioned frozen samples, as described previously (Fredrickson et al, 1999).

Virus Assays and Mouse Inoculation

Tests for expression of ecotropic MuLV used XC cell (ATCC CCL 165) plaque assays (Rowe et al, 1970) on SC-1 cells (ATCC CRL 1404) of tail extracts from 6- to 10-week-old mice or mitomycin C-treated spleen cell infectious centers (Cloyd et al, 1981). In some cases, virus expression was determined by induction of cultured tail cells with 5-iododeoxyuridine (Kozak and Rowe, 1982). Selected cell-free harvests of cocultivations of mitomycin C-treated lymphoma cells with SC-1 or *M. dunni* (Lander and Chattopadhyay, 1984) (ATCC CRL 2017) cells, tested for ecotropic virus by XC plaque assay or MCF MuLV by immunofluorescence with MCF-reactive monoclonal antibody 514 (ATCC CRL 1914), were inoculated (0.04 ml, divided intraperitoneally and in the region of the thymus) into 1- to 3-day-old NFS/N or NIH Swiss mice obtained from the colonies of the National Institutes of Health.

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