Multiparameter Analyses of Spontaneous Nonthymic Lymphomas Occurring in NFS/N Mice Congenic for Ecotropic Murine Leukemia Viruses

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Mouse strains congenic for ecotropic retrovirus genes have a much higher frequency of spontaneous lymphomas than the background NFS/N strain. In this study, most of these lymphomas have been identified as B-cell in origin by morphologic features, identification of immunoglobulin class, and cell-surface antigens. The classification suggested by Pattengale and Taylor proved to be applicable to the lymphomas studied. Most were of large follicular center cells and are considered typical of the type formerly designated as "reticulum cell sarcoma, type B." Many lymphomas contained a large proportion of nonneoplastic cells which partially obscured their neoplastic component. The role of ecotropic murine leukemia viruses as etiologic agents for B-cell lymphomas remains equivocal. However, because the only difference between the NFS/N and congenic mice is the expression of viruses in the latter, it appears that these viruses are somehow involved in induction of B-cell lymphomas. (Am J Pathol 1985, 121:349–360)

MURINE LYMPHOMAS are derived from either T or B lymphocytes. T-cell lymphomas are more fully understood than B-cell neoplasms in terms of their histopathology and pathogenesis because of their ready availability—they can be induced at high frequency with short latent periods by inoculation of newborn mice with replication-competent murine leukemia viruses (MuLV), and they are the predominant spontaneous neoplasms of AKR and C58 mice less than 1 year of age.[1] In these strains, endogenous ecotropic proviral sequences are expressed at high levels from early in life.[2] After spreading through the animals, these viruses recombine with endogenous nonecotropic viral sequences to produce a new class of MuLV, termed mink cell focus-inducing (MCF) viruses.[3] Some of these recombinants are capable of infecting thymocytes and causing their clonal transformation.[4]

The role of MuLV in the pathogenesis of B-cell lymphomas is less clear because it has only recently been appreciated that these lymphomas are the predominant spontaneous neoplasms of aged mice in several strains. Until recently the designation most applied to these neoplasms was that proposed in 1954 by Dunn, namely, the reticulum cell sarcoma, type B (RCS-B). This has been a useful classification, but it is now apparent that it includes several different kinds of lymphomas. Thus immunomorphologic studies of "RCS-B" neoplasms in aged BALB/c mice and other strains suggested a new classification system that takes advantage of the distinguishing features of these lymphomas to define them as lymphocytic, lymphoblastic, follicular center cell, and immunoblastic lymphomas of B lymphocytes.[6,7] With this classification system most of the BALB/c lymphomas were found to be follicular center cell lymphomas of B-cell origin.[7] The development of a system for

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clearly defining B-cell lymphomas thus makes it possible to approach the question of the possible role for MuLV in B-cell lymphomagenesis.

One system particularly well suited to analyses of this problem is lymphomas of NFS/N mice congenic for ecotropic MuLV induction loci from AKR or C58 mice (V-congenics[8-10]). Normal NSF/N mice do not express ecotropic MuLV because they lack the genetic information for this class of virus[11]; these mice have a low frequency (7%) of spontaneous nonthymic lymphomas.[9] By comparison, NFS V-congenic mice contain one or more copies of ecotropic provirus loci, express varying levels of infectious ecotropic MuLV, and have a high frequency (25%) of nonthymic lymphomas and a lower frequency (9%) of thymic lymphomas. In earlier studies, it was shown that these lymphomas appeared predominantly in mice that expressed high systemic levels of ecotropic virus early in life.[9]

In the present study, nonthymic lymphomas of NFS V-congenic mice were studied for determination of whether they were of B-cell origin. Histologic diagnoses of the lymphomas were made with the use of the Pattengale-Taylor classification system.[6] Lymphomas were also analyzed by flow microfluorometry (FMF) for a series of cell-surface markers characteristic of T cells and B cells. Finally, immunohistochemical staining for cytoplasmic immunoglobulin (cIg) was used for determination of the clonality of the lymphomas. The results demonstrate that most of the nonthymic lymphomas of NFS V-congenic mice were B-cell lymphomas, predominantly of the follicular center cell type. The combined approaches to diagnosis applied in this study were essential to clearly establishing the nature of these lymphomas.

Materials and Methods

Mice

Normal NFS/N mice were obtained from the colonies of the National Institutes of Health. NFS/N mice congenic for ecotropic MuLV induction loci from AKR/N and C58/Lw (ie, V-congenics) were bred as previously described.[8,9] They included two sublines congenic for *Akv-1*, designed NFS.*Akv-1* (1-4) and NFS-*Akv-1* (1-2); one strain congenic for *Akv-2*, designated NFS.*Akv-2* (4-2); and another congenic for *C58v-1* designated NFS.*C58v-1* (298, 3-4). Congenic mice of both sexes with lymphomas, detected by splenic and lymph node palpation, were sacrificed along with age-matched V-congenic mice without splenic or nodal enlargement. Sixteen young, noncongenic NFS/N mice were also used as controls for assurance of reagent efficacy in the detection of surface markers on the basis of previous experience with these mice. Thus, there were three sets of mice studied for the presence of cell surface markers: V-congenics, ranging in age from 8 to 24 months (mean, 18 months) with lymphoid cell neoplasms; V-congenics in the same age range (8–24 months with a mean of 20) without lymphoid neoplasms; and healthy young NFS/N mice 1–3 months of age (mean, 2).

Flow Microfluorometry (FMF)

Single-cell suspensions from spleens of normal and lymphomatous mice were prepared and stained for FMF analyses as described.[12] Cells ranging in number from 2×10^4 to 5×10^4 were analyzed for fluorescence intensity of viable cells (determined by light-scatter gating) on a fluorescence-activated cell sorter (FACS II, Becton Dickinson FACS Systems, Mountain View, Calif) with the use of established techniques.[12] Neoplastic cells were usually readily distinguishable from normal or reactive lymphocytes, as judged by narrow forward-angle light scatter, which correlates with cell size. Antibodies employed in these assays included 1) fluorescein-labeled $F(ab')_2$ fragments of goat anti-mouse Fab (reactive with all cell surface immunoglobulins; provided by Dr. R. Asofsky and Dr. B. J. Fowlkes, National Institute of Allergy and Infectious Diseases, National Institutes of Health); 2) mouse monoclonal anti-Lyb-2.1 (reactive with all B cells and pre-B cells; purchased from New England Nuclear Corp., Boston, Mass); 3) rat monoclonal anti-ThB (reactive with all B cells and some pre-B cells; a gift from Dr. Jeffrey Ledbetter, Stanford University); 4) mouse monoclonal anti-Ia^k (cross-reactive with Ia^s of NFS/N and reactive with all B cells; purchased from Becton Dickinson Monoclonal Antibodies Division, Sunnyvale, Calif); 5) fluorescein-labeled mouse monoclonal anti-Thy-1.2 (reactive with all T cells; purchased from New England Nuclear Corp., Boston, Mass); and 6) fluorescein-labeled $F(ab')_2$ fragments of rabbit antibodies to XenCSA (xenotropic and MCF MuLV-related cell-surface gp70 antigens; prepared as described by Morse et al[12]). Cells treated with unlabeled anti-Lyb-2 or anti-ThB were reacted with fluorescein-labeled goat anti-mouse IgG_2 or rabbit anti-rat Ig, respectively, as a second-step reagent. The number of cells fluorescing above background levels is given as a percentage of the total population tested. Detection of XenCSA was measured as the degree of fluorescence per cell of the sample examined, a value always less than 100 for young NFS/N controls.

Electron Microscopy

Pieces of spleen about 2 cu mm were placed in ice-cold 2.5% glutaraldehyde maintained at pH 7.3 by a

Table 1—Morphologic and Phenotypic Characteristics of Splenic Cells From NFS/N V-Congenic Mice With Spontaneous Lymphomas

Morphologic diagnosis	Age (mos)	Spleen weight (g)	Cell surface antigens						Cytoplasmic Ig	Family‡
			sIg*	Lyb-2*	ThB*	Ia*	Thy-1*	XenCSA†		
Small lymphocyte lymphoma										
1	18	0.67	84	89	57	NT	3	159	Negative	1–2
2	19	1.81	75	64	34	81	10	174	Negative	1–2
3	20	1.08	88	91	89	NT	7	337	Negative	1–2
4	14	2.60	82	79	73	NT	11	2992	Negative	4–2
5	13	2.00	94	83	NT	NT	4	992	Negative	1–4
Small FCC lymphoma										
6	12	2.08	87	68	84	NT	30	452	Kappa	4–2
7	21	2.54	73	NT	NT	83	24	857	NT	4–2
8	22	4.84	64	NT	96	48	7	664	IgA, kappa	4–2
Mixed small and large FCC lymphoma										
9	22	0.79	65	73	68	63	31	201	IgM, lambda	1–2
10	12	1.88	62	20	18	NT	30	225	IgG_3, kappa	1–4
11	24	NT	35	NT	32	20	22	1010	NT	1–2
12	17	1.50	41	75	27	NT	52	289	Negative	3–4
13	15	1.30	60	51	63	NT	41	348	IgM, kappa	4–2
Large FCC lymphoma										
14	24	0.78	33	58	35	43	20	101	NT	4–2
15	24	3.74	53	82	NT	68	24	192	IgG_3, kappa	1–4
16	14	0.96	64	63	52	60	25	252	IgM, kappa	1–2
17	22	3.48	63	81	66	64	15	1470	Kappa	4–2
18	15	2.49	84	87	77	67	12	1002	IgM, kappa	4–2
19	16	5.09	77	68	69	68	11	347	IgA, lambda	1–4
20	16	2.48	20	26	NT	19	74	97	Negative	1–4
21	13	4.87	75	90	74	52	9	233	NT	1–4
22	19	1.28	39	35	49	NT	50	1472	NT	1–4
23	8	2.72	56	33	49	69	24	1816	Kappa	3–4
24	23	0.79	46	44	NT	NT	56	233	Kappa	3–4
25	12	NT	NT	53	45	55	43	1271	NT	1–4
26	18	2.30	37	63	37	NT	30	452	Negative	4–2
27	16	2.10	58	60	59	NT	23	2408	IgA, kappa	4–2
28	22	0.48	51	NT	49	60	37	406	IgA, lambda	301
29	13	2.17	57	NT	30	25	39	925	IgG_3, kappa	4–2
30	16	2.30	53	54	21	55	21	1544	IgM, lambda	298
31	15	NT	61	NT	69	70	18	1593	NT	298
32	13	3.20	66	58	53	NT	9	NT	Kappa	1–4
33	13	NT	43	NT	36	41	34	629	Negative	298
34	18	1.42	88	96	75	94	6	183	IgG_{2b}, kappa	4–2
35	18	1.72	41	63	59	NT	17	991	NT	1–2
Immunoblastic lymphoma										
36	16	3.06	57	73	30	69	38	526	Negative	1–2
37	23	1.05	35	71	NT	NT	4	754	Kappa	1–4
38	12	2.48	33	NT	NT	21	27	1410	IgM, kappa	298
Lymphoblastic lymphoma										
39	16	2.59	42	NT	NT	44	NT	NT	NT	4–2
40	17	1.53	17	18	5	11	10	231	Negative	1–4
41	10	5.00	96	86	NT	NT	35	2992	NT	1–4
42	16	2.59	42	NT	NT	44	58	1028	NT	3–4

* Numbers indicate percent positive cells.
† Numbers indicate mean fluorescence.
‡ Families 1–2 and 1–4 are congenic for *Akv-1*, Family 4–2 for *Akv-2*, Family 298 for *C58v-1*, and Family 301 for *C58v-2*.
§ NT, not tested.

Table 2—Comparison of Lymphomatous Mice With Age-Matched Nonlymphomatous Congenic Mice and With Young Adult, Noncongenic NFS/N Mice

Number	Diagnosis	Age (mos)	Spleen weight (mg)	Percentage cells positive ± SD*					
				sIg	Ia^k	Lyb-2	ThB	Thy1.2	XenCSA
5	Small lymphocyte lymphoma	17 ± 3	1632 ± 342	85 ± 3	81	81 ± 5	63 ± 12	7 ± 1	931 ± 537
3	Small FCC	12 ± 6	3153 ± 854	75 ± 7	66	68	65	20	658 ± 117
5	Mixed FCC	19 ± 3	1368 ± 227	50 ± 8	43	52 ± 12	42 ± 10	31 ± 3	426 ± 149
22	Large FCC	17 ± 4	2335 ± 300	55 ± 4	57 ± 5	62 ± 5	53 ± 4	27 ± 4	757 ± 146
3	Immunoblast	17 ± 3	2197 ± 597	42 ± 8	45	72	28	23	897 ± 265
3	Lymphoblast	14 ± 2	3040	52 ± 23	28	52	5	34	1417 ± 820
13	Young NFS/N noncongenics	2 ± 1	150 ± 80	60 ± 6	NT	66 ± 7	60 ± 6	30 ± 7	89 ± 19
15	Age-matched NFS/N congenics nonlymphomatous	20 ± 4	270 ± 110	60 ± 9	59 ± 10	64 ± 12	55 ± 11	35 ± 14	90 ± 16

* Standard deviation not given when only 1 or 2 were tested for that marker in that particular group. XenCSA values are mean fluorescence ± 1 SD.
NT, not tested.

0.1 M phosphate buffer. The tissue was fast fixed in 1% OsO_4, washed in buffer, dehydrated, and mounted in an Epon-Araldite mixture. Specimens were photographed with a Philips electron microscope, Model EM 300.

Light Microscopy

Splenic and lymph node tissue was either placed in B5 or Tellyeznickie's fixative, embedded in paraffin, and sectioned at 5 μ. Sections were routinely stained with hematoxylin and eosin (H&E). Unstained sections were used for the immunoperoxidase procedure (see below). All other tissues examined were fixed in Tellyeznickie's fixative.

Immunoperoxidase

For immunoperoxidase staining, rabbit antimouse heavy ($A,M,G_1,G_{2a},G_{2b},G_3$) and light (kappa and lambda) chains were purchasd from Litton Bionectics Inc. (Kensington, Md).

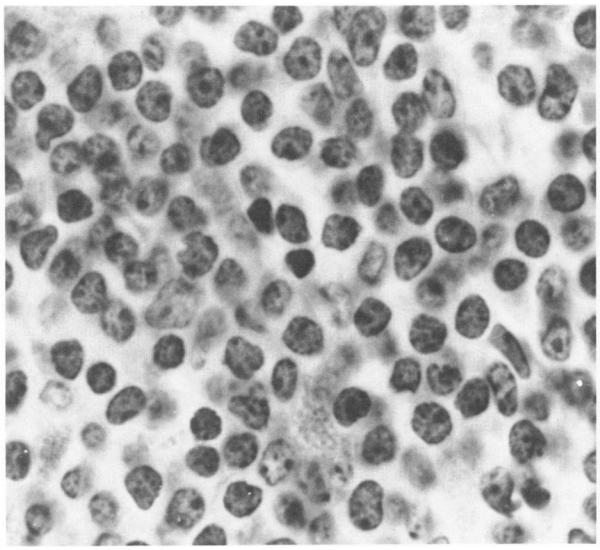
Immunoperoxidase techniques were performed by means of the peroxidase-antiperoxidase method as described previously.[7] In brief, rabbit anti-mouse sera at concentrations of varying titer (IgA, 1/200; IgG_1, 1/200; IgG_{2a}, 1/100; IgG_{2b}, 1/200; IgG_3, 1/50; IgM, 1/200; kappa, 1/400; and lambda, 1/400), swine anti-rabbit IgG (1/30), and rabbit peroxidase-antiperoxidase (1/200) were used sequentially, with washes between each stage. Diaminobenzidine was used as the chromogen, giving a permanent brown reaction product that contrasted well with the hematoxylin counterstain used. Normal rabbit and swine sera were used as controls. Antigens were titered for maximum positivity and specificity on both monoclonal derived, mineral oil-induced plasmacytomas of BALB/c mice and nonneoplastic reactive spleens and lymph nodes. Positivity was defined as the presence of either cytoplasmic kappa or lambda light chain (ie, alone or associated with one or more heavy chains) within 25% or more of the "critical" neoplastic lymphoid cells, as ascertained morphologically.

Results

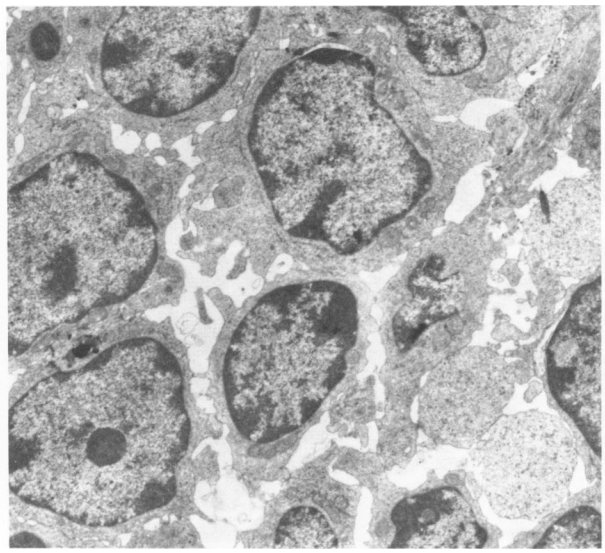
Population analysis for splenic cell-surface markers of 42 mice with nonthymic lymphomas is given in Table 1 and compared with spleen cells from age-matched and young controls in Table 2. By FMF criteria, all the lymphomas but 3 were of B-cell origin; Case 42 was diagnosed as a T-cell lymphoma, and Cases 39 and 40 as null-cell lymphomas. The diagnoses presented in these Tables are grouped according to the classification proposed by Pattengale and Taylor,[6] and the majority (30/42, or 71%) were follicular center cell (FCC) lymphomas, mostly of large cell type, with the remaining cases consisting of small lymphocytic (5/42, or 12%), lymphoblastic (3/42, or 7%), or immunoblastic (3/42, or 7%) lymphomas. The following gross and histologic

All photographs are of splenic tissue fixed in B5. Light micrographs are at a magnification of × 1000, and electron micrographs are at × 4100 for Figures 1–6. Figures 8–11 are at a magnification of ×400. **Figure 1**—Small lymphocyte lymphoma. **A**—Light micrograph showing a uniform population of small, round lymphocytes (Mouse 2). **B**—Electron micrograph showing that the cytoplasm, almost absent in light microscopy, contains only occasional mitochondria and ribosomes (Mouse 3). **Figure 2**—Small follicular center cell lymphoma. **A**—Light micrograph showing irregularly shaped, small nuclei (Mouse 6). **B**—Electron micrograph showing margination of chromatin, indistinct nucleoli, and moderate amounts of cytoplasm containing mitochondria and ribosomes (Mouse 6). **Figure 3**—Large follicular center cell lymphoma. **A**—Light micrograph shows irregularly shaped nuclei with moderate amounts of ill-defined cytoplasm (Mouse 19). **B**—Electron micrograph showing nucleoli attached to nuclear membrane and cytoplasm containing numerous mitochondria and some lamellas of rough endoplasmic reticulum (Mouse 19).

1A



1B



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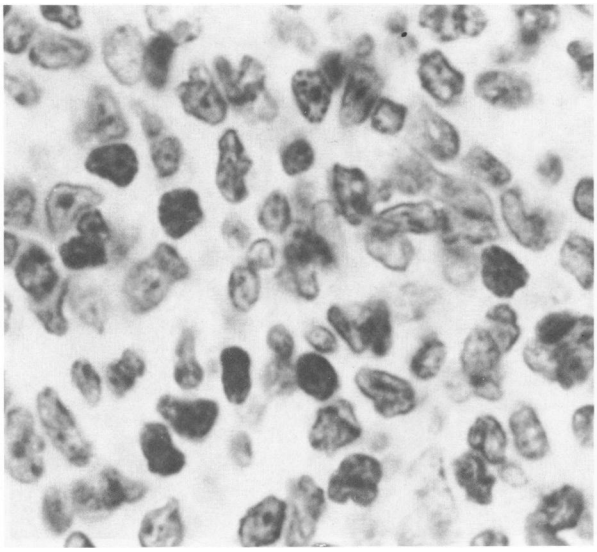

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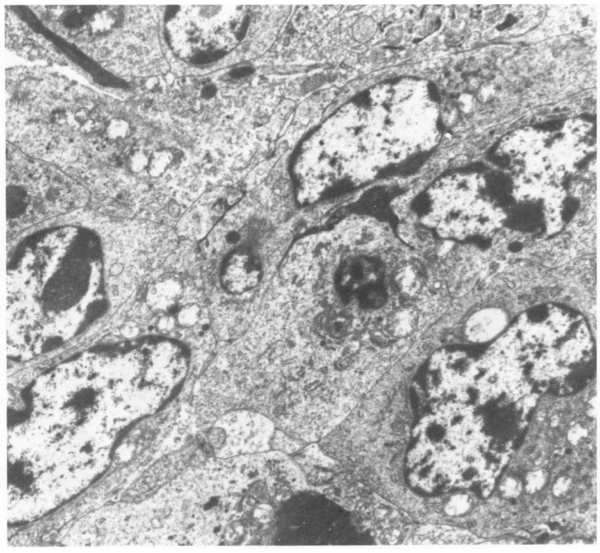

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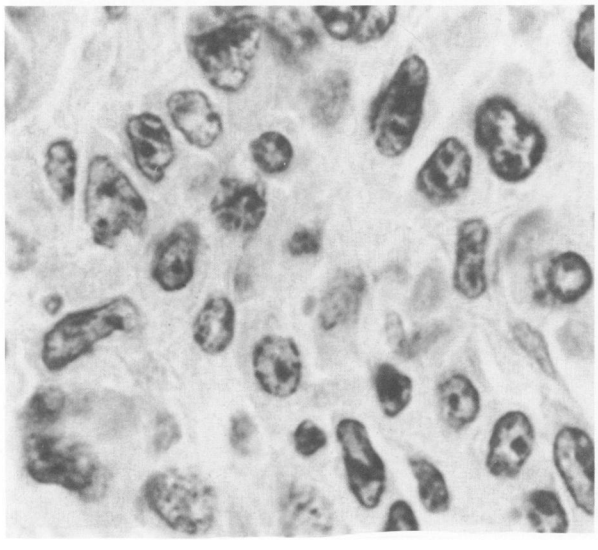

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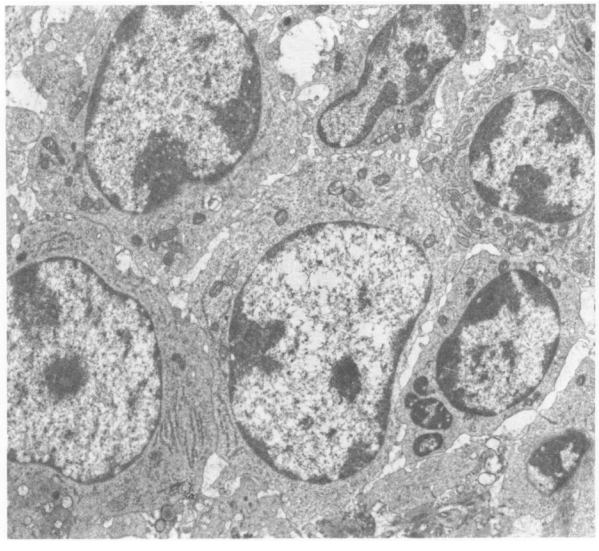

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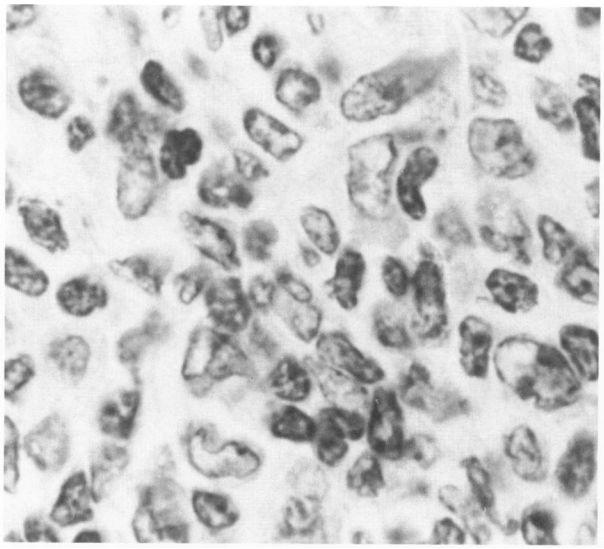
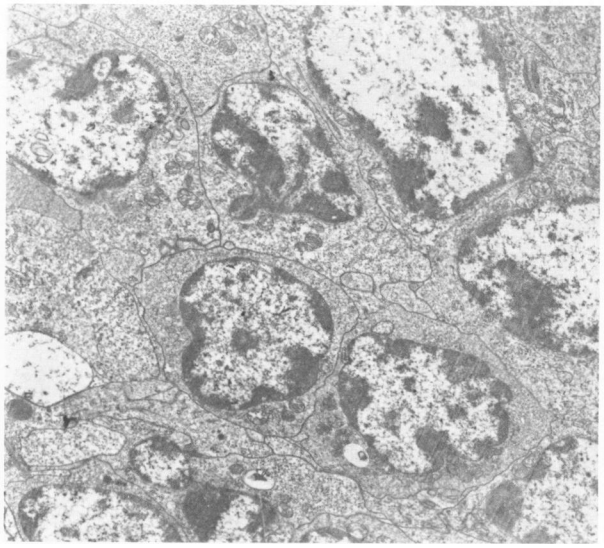
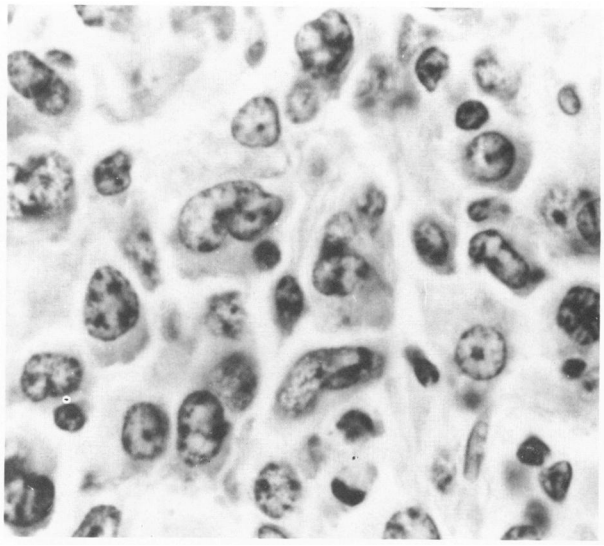
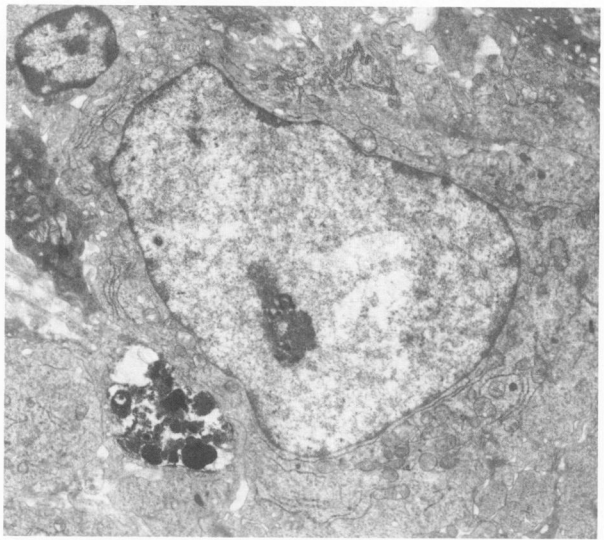




4B

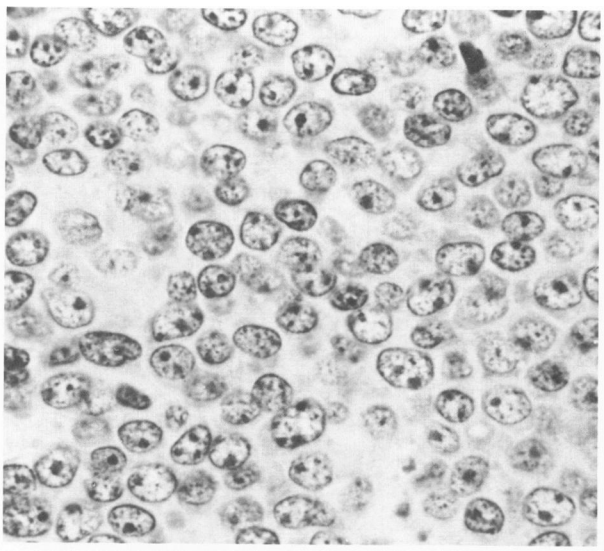
5A

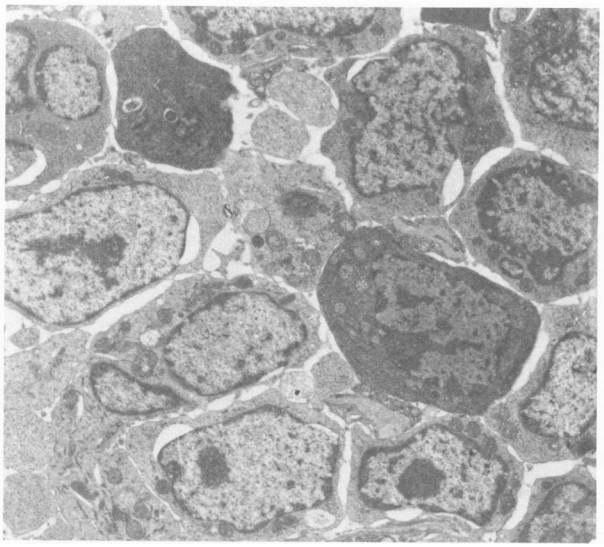




5B

6A





6B

Table 3—FMF Analyses of Primary and Transplanted Lymphomas of V-Congenic Mice

Lymphoma*	Status†	Cell surface antigens				
		sIg‡	ThB‡	Ia‡	Thy-1‡	XenCSA§
11674	G0	20	15	19	60	97
	G1	58	54	56	8	449
12080	G0	39	35	47	23	1593
	G1	80	73	91	11	3084
12083	G0	61	69	70	18	1593
	G1	78	83	84	5	2949
12570	G0	33		21	27	1410
	G1	66		70	26	2151

* Lymphoma 11674 corresponds to Lymphoma 19 and 12083 to Lymphoma 30 in Table 1.
† G0, primary tumor; G1, first generation transplant.
‡ Percent positive cells.
§ Mean fluorescence.

characteristics of each type of lymphoma were used as the criteria for diagnosis.

Small Lymphocytic Lymphomas

This was the most uniform type of lymphoma in the series according to cell-surface markers, morphologic features and gross appearance. Affected mice had large, very dark, evenly colored red spleens without white mottling of the cut surface. The liver was enlarged and reddish-gray, but lymph nodes generally showed only moderate enlargement. As for all B-cell lymphomas in this series, the thymus was atrophic, although thymic lymphomas occur in V-congenic mice.[9] Generally, small lymphocytic lymphomas were associated with fairly severe leukemia, but unequivocal marrow involvement was observed in only two cases. A moderate anemia accompanied the leukemic phase. Histologically, the lymphoma was composed of diffuse sheets of small lymphocytes replacing most of the normal splenic structures. Within these sheets of small lymphocytes, plasma cells or immunoblasts were seen rarely, but the general picture was one of a monomorphic cell type with very few mitotic figures or size variation (Figure 1). In the liver there was extensive stasis of small lymphocytes throughout the sinusoids, in contrast to the periportal localization of other lymphomas of both T- and B-cell origin. The neoplastic cells had typical ultrastructural features of small lymphocytes. A high percentage of cells were positive for surface immunoglobulin (sIg) and the other B-cell markers Lyb-2, Ia, and ThB. The number of splenic T lymphocytes was reduced to about one-third of normal controls. Values for XenCSA were variable, and all cells were negative for cIg, in contrast to sIg (Tables 1, and 2).

Follicular Center Cell (FCC) Lymphoma

FCC lymphomas were generally seen grossly as large, nodular mases throughout the spleen. Nodal involvement was also usually evidenced by symmetric enlargement, particularly of abnormal and mediastinal nodes, although most lymphomas appeared to originate in the spleen and then spread to nodes. In advanced cases, the lungs and kidneys, and particularly the liver, were sites of nodular tumors similar to those seen in the spleen. A leukemic phase was rarely observed, anemia was nonexistent or mild, and marrow involvement was limited to rare circumscribed foci of tumor cells. In contrast to the uniformly high frequency of sIg^+ cells detected among spleen cells of mice with small lymphocytic lymphomas, analyses of spleens from mice with FCC lymphomas revealed wide variability (20–88%) in the frequencies of Ig-bearing cells (Table 1). For individual lymphomas, there was generally close agreement among the frequencies of cells expressing the B-cell markers sIg, ThB, and Ia, but there were numerous discrepancies in the number of $Lyb\text{-}2^+$ cells. The overall frequency of B cells (sIg^+) in normal spleens of 60% was increased in only 3 cases of this type of lymphoma. However, abnormal sIg^+, Ia^+, ThB^+, and $Lyb\text{-}2^+$ cells could often be distinguished from normal B cells by virtue of their increased size, as judged by narrow forward-angle light scatter and/or more uniform reactivity with antibodies to cell-surface antigens. An example of a FCC lymphoma stained with antibody to

Figure 4—Mixed follicular center cell lymphoma. **A**—Light micrographs showing both large and small irregularly shaped nuclei (Mouse 9). **B**—Electron micrographs showing a mitotic figure, possibly of a large follicular center cell (Mouse 9). **Figure 5**—Immunoblastic lymphoma. **A**—Light micrograph showing vesicular nuclei and some large amounts of clearly defined cytoplasm (Mouse 35). **B**—Electron micrograph showing central nucleolus and heavy membrane, cytoplasm containing numerous mitochondria, and rough lamellas (Mouse 35). **Figure 6**—Lymphoblastic lymphoma. **A**—Light micrograph showing uniform population of cells with round, vesicular nuclei and indistinct cytoplasm (Mouse 40). **B**—Electron micrograph showing diffuse chromatin, prominent nucleoli, thick nuclear membranes, and well-demarcated cytoplasm containing mainly ribosomes (Mouse 40).

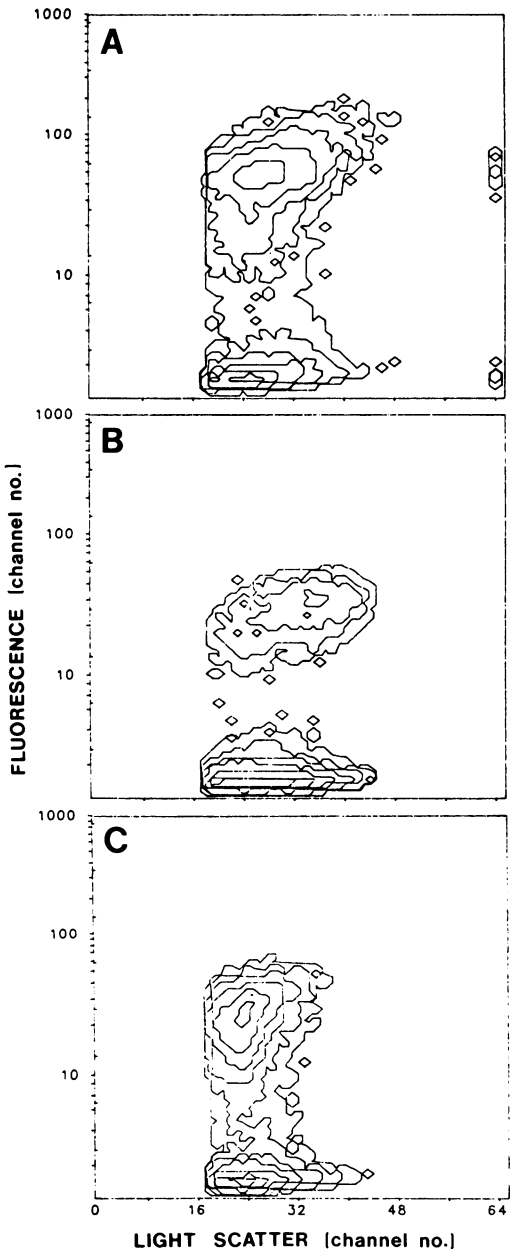


Figure 7—Characterization of B-cell lymphomas by two-parameter (fluorescence versus light scatter) FMF studies of spleen cells. **A**—Large FCC lymphoma with 53% sIg⁺ cells (Table 1, Number 15). Most sIg⁺ cells are slightly larger and express considerably higher levels of sIg than normal splenic B cells (**C**; 60% sIg⁺). Cytoplasmic staining showed this lymphoma to be uniformly IgG3⁺, kappa⁺. **B**—Immunoblastic lymphoma with 33% sIg⁺ cells (Table 1, Number 38). Neoplastic cells are readily identified on the basis of increased size (72% of sIg⁺ cells larger than most normal sIg⁺ cells) and the uniformity of staining with anti-Ig. Cytoplasmic staining showed this lymphoma to be uniformly IgM⁺, kappa⁺.

sIg and examined by two-parameter FMF is shown in Figure 7A.

The frequency of unidentified (sIg⁻, Thy-1⁻) cells in spleens of mice with FCC lymphomas was doubled over normal from 5% to 10% in 14/29 cases (Table 1). This finding paralleled histopathologic observations, because macrophages, and, less frequently, myeloid cells and erythroid cells, can sometimes be a prominent feature.

Mitotic figures were generally numerous in mixed and large cell FCC lymphomas. The ultrastructural features of the lymphoid cells constituting the neoplastic component shown in Figures 2–4 are typical for large and small FCC, although it should be noted that nuclear indentations were not a constant feature. As would be expected, the presence of cIg was detected in most of these cases. The predominant light chain was kappa but there were 2 cases, 29 and 19, in which lambda was the monoclonal type. In some cases it was not possible to identify the heavy chain component (Cases 6, 17, 22, 23, and 32), but in others it was clearly IgG_3 (Case 10), IgM (Case 29), or IgA (Case 19). The XenCSA values varied considerably, but with two exceptions (Cases 14 and 20), they were all clearly above control values.

Immunoblastic Lymphomas

Grossly, these neoplasms were indistinguishable from FCC lymphomas. Histologically they were composed of a fairly uniform population of large cells characterized by a single, large central nucleolus with a heavy margin of chromatin adjacent to the nuclear membrane (Figure 5). The amphophilic cytoplasm was plentiful and often clearly outlined. Generally, these lymphomas were characterized by more numerous mitotic figures than in the other types. Infiltration of spleen and nodes was usually extensive, with complete obliteration of normal structures. Medullary areas of the nodes often contained cells with plasmacytoid features, as well as immunoblasts. The ultrastructural features of the immunoblasts were numerous elongated mitochondria, numerous polyribosomes, and moderate amounts of endoplasmic lamellas.

As for some FCC lymphomas, immunoblasts sometimes were represented at only a low frequency among spleen cells but could be distinguished from normal B cells by FMF on the basis of abnormal size and staining characteristics. An example of FMF studies of an immunoblastic lymphoma comprising only 24% of spleen cells is shown in Figure 7B. The remaining cells in the spleen included a heterogeneous mixture of histiocytes, erythroid, myeloid, and lymphoid elements.

Lymphoblastic Lymphomas

The 4 tumors of this type were fairly similar histologically with uniform sheets of intermediate size lymphoid cells with round, vesicular nuclei with usually a single nucleolus (Figure 6). As found previously,[7,13] such cells may be of T or B lineage or composed of cells without antigenic markers. Infiltration of lymphoma cells

in the spleen, lymph nodes, liver, and kidneys was diffuse, rather than nodular, and extensive. Mitotic figures were moderately numerous. As with lymphomas of small lymphocytes, and in contrast to those of FCC origin, there was a minimal number of macrophages or other reactive cells in these lymphomas. This monomorphism is indicated by the histologic appearance.

Transplantation of Lymphomas

As noted above, FCC and immunoblastic lymphoma cells often represented only a minor component of the total spleen cell population. For determination of whether cells were truly neoplastic, single-cell suspensions (10^7 cells) prepared from spleens of animals with FCC lymphomas were inoculated intraperitoneally into adult V-congenic mice treated with Pristane (0.5 ml) 4 days prior to inoculation. Most recipient animals developed lymphadenopathy and splenomegaly within 3 months of inoculation. Histologic studies of their tissues in relation to those of the donor mice revealed a significantly more morphologically homogeneous population of neoplastic cells (Figures 8–11) which correlated with an increased frequency of B cells as determined by FMF (Table 3).

Discussion

In the current study, several techniques were used to characterize spontaneous nonthymic lymphomas occurring in NFS/N mice congenic for ecotropic MuLV loci from AKR/N or C58/Lw mice. The results indicate that most of these neoplasms were lymphomas of B lymphocytes. It perhaps should be emphasized that although previous analyses with flow cytometry had been done of murine lymphoid cell lines and transplantable tumors,[10,14–17] this is the first report of a large series of spontaneous, primary tumors.

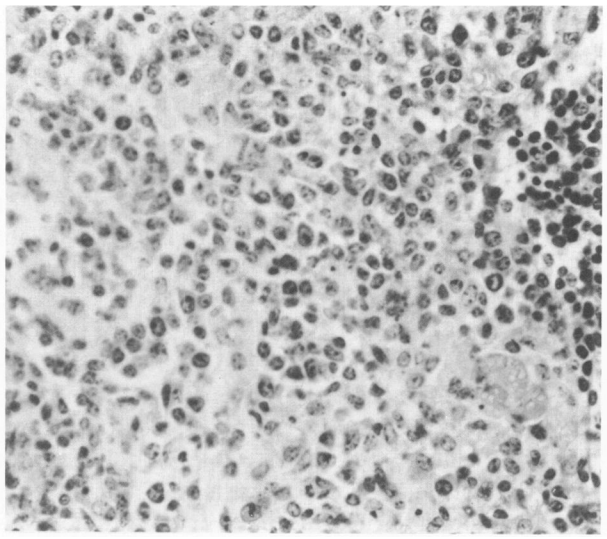
With the use of a recently developed system for classifying lymphomas on the basis of morphologic features,[6,7,10] 72% of the neoplasms were diagnosed as FCC lymphomas, mostly of the large-cell type. However, FMF analyses of spleen cells from mice with these lymphomas showed marked variation in the number of sIg^+ B cells (Table 1). In cases with less than 60% B cells there were a large number of T cells or cells which did not have any cell surface markers detectable with the antibodies used in this study. Such cells included macrophages, which sometimes appeared on histologic evaluation to be present in large numbers. In the case of SJL/J tumors, some investigators believe macrophages to be the neoplastic component.[18] Additionally, variable numbers of erythroid and myeloid cells would also be seen in the red pulp, which occasionally represented a moderate proportion of total splenic tissue. These complicating factors have made diagnosis of murine B-cell lymphomas difficult; and, indeed, only a multiparametric approach allowed their identity to be established. First, immunohistochemical demonstration of class-restricted cIg heavy- and light-chain expression in large cells (Table 1) was strongly suggestive of monoclonality. Second, two-parameter analyses of light scatter and sIg fluorescence in FMF assays permitted definitions of large, presumably malignant sIg^+ cells, even when they represented as little as 20% of the total spleen cell population (Figure 7). Finally, transplantation studies demonstrated the malignant nature of these cells. The increased homogeneity of cells in the transplant recipients (Figures 8–11; Table 3) indicates that the proliferation of myeloid, erythroid and many lymphoid cells observed in some of the primary tumors was probably a "reactive," rather than a malignant, expansion of these cell types. The basis for the proliferation of these reactive cells is unclear but may involve a T-cell response to the tumor population associated with release of lymphokines specific for cells in the T, myeloid, and erythroid lineages. Regardless of the mechanism, it is clear that the reactivity observed in murine spleens containing B-cell lymphomas occurs in excess of that seen with comparable human B-lineage lymphomas. In addition, some lymphomas have a definitely sclerotic appearance (Figure 8A), possibly also associated with splenic reactivity.

In lymphomas composed of small lymphocytes, the diagnosis based on histologic evaluation was confirmed by demonstration of high levels of sIg. In addition, all other B-cell markers were positive for these cells. A similar lymphoma of small lymphocytes has been described,[19] and from our findings it appears that they are not uncommon in older mice. Their strikingly different gross appearance, compared with other B-cell lymphomas and involvement of the spleen and liver made them easy to identify.

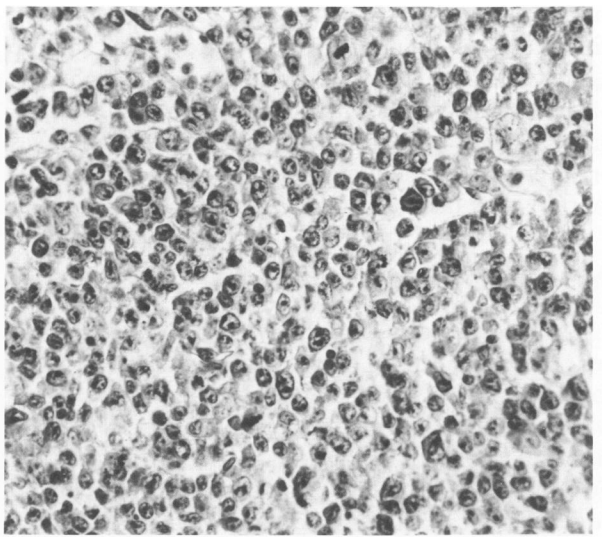
The relationship of immunoblastic lymphomas to the B-cell lineage was indicated by previous studies of BALB/c lymphomas[7] and was confirmed in the present study. Morphologic and ultrastructural features of these cells suggest that they are the immediate precursors of plasma cells. These observations may explain the relatively low frequencies of sIg^+ cells in the spleens of mice bearing these lymphomas, because many Ig-secreting plasma cells are sIg^-, and cells in the process of maturing to plasma cells may express low levels of sIg while still expressing other markers of the B cell such as Lyb-2[10,15,16] (Table 1).

More recent studies of mice with a polyclonal immunoblastic B-cell disease confirmed this suggestion in demonstrating that spleens and lymph nodes of these

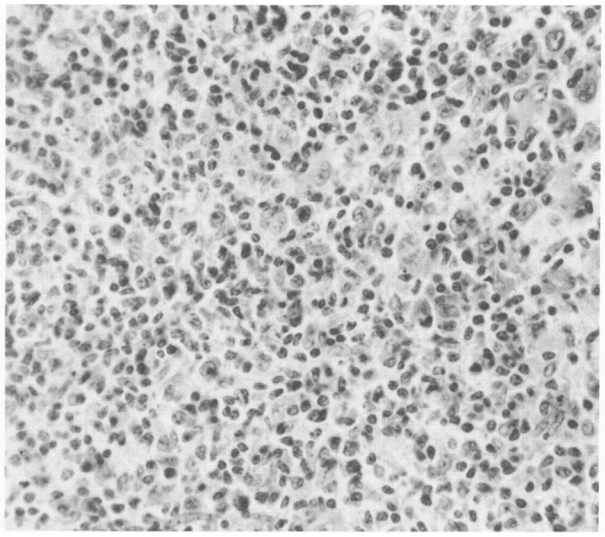
8A



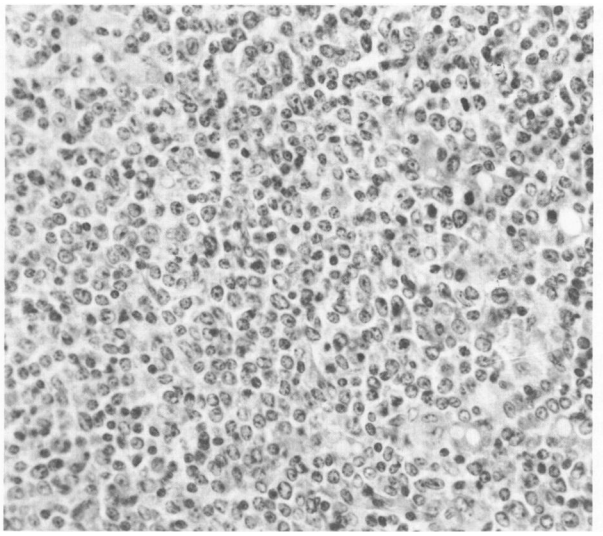
8B



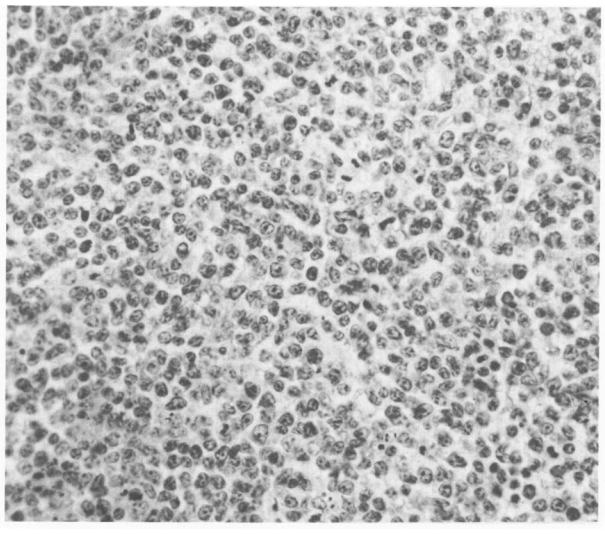
9A



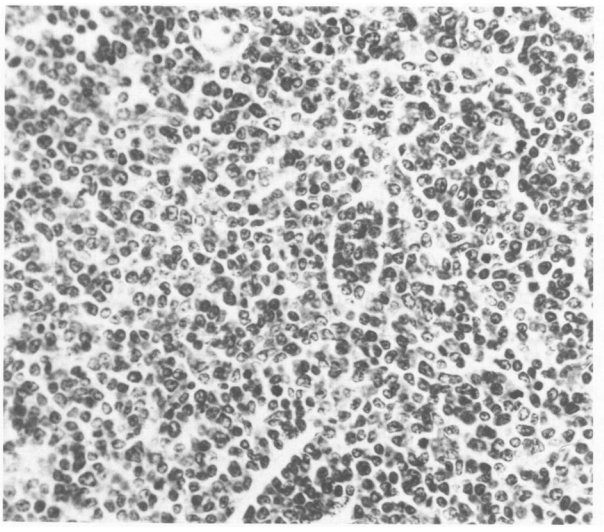
9B



10A



10B



mice contained reduced numbers of cells with most B-cell surface antigens, even though these tissues were almost completely populated with immunoblasts.[24] These results suggest that B cells, late in their progression to terminally differentiated plasma cells, rapidly shed many of their characteristic cell surface determinants in a less than orderly fashion. This suggestion would be consistent with the finding of significant discrepancies among the frequencies of sIg$^+$, Ly-5(B220)$^+$, Lyb-2$^+$, and Ia$^+$ cells in spleens of mice with FCC and immunoblastic lymphomas (Table 1).

Neoplasms diagnosed morphologically as lymphoblastic lymphomas may include T-, B-, and null-cell lymphomas.[6,7,10,13] Studies of cell-surface markers other than those tested for in the current study have shown that some of the null-cell neoplasms are pre-B-cell lymphomas.[10,16,17] Of the 4 lymphoblastic lymphomas examined in the current study, 1 (41) was of B-cell origin, 2 (39 and 40) were null for distinctive surface or cytoplasmic markers, and the fourth (42) was of T-cell origin on the basis of all surface markers.

The malignant nature of the B-lineage lymphomas observed in NFS V-congenic mice was confirmed by the observation that they can be transplanted to other animals (Figures 8–11). In addition, earlier studies showed that some of these neoplasms can be readily adapted to continuous growth *in vitro*.[16]

Because they were observed in V-congenic mice, some inferences can be made regarding the relationship between retrovirus and the induction of B-cell lymphomas. The role of replication-competent MuLV in the genesis of B-cell lymphomas, in contrast to T-cell lymphomas, remains unclear. In AKR mice, endogenous ecotropic MuLV is expressed systemically at high levels from early in life, which results in frequent recombination events with endogenous nonecotropic viral sequences. Some of these viral recombinants, in contrast to ecotropic MuLV, exhibit broadened host range specificity and the ability to induce foci in mink lung cells, which leads to their designation as dual tropic or MCF MuLV.[3,20] Amplified expression of cell-surface gp70s, including G_{IX}[21] and XenCSA,[22] on thymocytes heralds the appearance in thymus of a subset of MCFs capable of replicating in this organ. Cloned thymotropic MCFs accelerate the development of thymic lymphomas, which suggests that they are the etiologic agents in the development of this disease.[3,4,20]

Some parallels to this progression in the development of thymic lymphomas in AKR mice have been found in the development of B-cell lymphomas in NFS V-congenic mice. First, a comparison of young V-congenic mice that expressed low levels of noninfectious ecotropic MuLV with those that expressed high levels showed that only high-virus mice developed a high frequency of nonthymic lymphomas.[9] As shown in the current study, most nonthymic lymphomas were shown to be of B-cell origin (Table 1). Another indication that MuLV was involved in the develoment of lymphomas is the observation that XenCSA levels on lymphomatous spleen cells were amplified in comparison with levels on spleen cells of age-matched NFS V-congenic mice without lymphomas (Tables 1 and 2). Indeed, this antigen seems to serve almost as a tumor-specific antigen, in that the correlation between its heightened presence and a histologic diagnosis of malignancy was almost invariable. The exceptions were a few cases in which no certain diagnosis could be made histologically. In these cases, XenCSA levels were higher than for age-matched controls. Finally, MCF MuLVs have been recovered from many nonthymic and B-lineage lymphomas[3] (and J. W. Hartley, unpublished observations).

In spite of these similarities between the development of T-cell lymphomas and B-cell lymphomas, there are some major differences related to expression of MuLV. First, thymic lymphomas were not observed in studies of a large number of aged ecotropic MuLV$^-$ NFS/N mice, whereas in 7% of these mice nonthymic lymphomas developed, some of which were derived from B cells[10] (T. N. Fredrickson, P. K. Pattengale, H. C. Morse III, unpublished observations). By comparison, in approximately 10% of NFS V-congenic mice thymic lymphomas developed, and in nearly 24% nonthymic lymphomas developed. This indicates that expression of ecotropic MuLV is required for the development of thymic but not necessarily for nonthymic lymphomas. Second, although MCF MuLV recovered from AKR thymic lymphomas accelerates T-lymphoma development in AKR and some other strains of mice,[3,4,20] MCFs recovered from nonthymic lymphomas of NFS V-congenics have not been observed to induce accelerated B-lineage lymphomas in AKR or NFS V-congenic mice. Thus, replication-competent MuLV has been shown to amplify the underlying tendency for nonthymic lymphomas to develop in NFS/N mice, but its possible role

Figure 8A—Lymphoma from Mouse 12080, unlisted in Table 1, diagnosed as immunoblastic despite the presence of many macrophages, some erythroid precursors (*left*) and pink-staining connective tissue (**B**). Passage 11 of the lymphoma shown in **A**. Most cells are clearly immunoblasts. **Figure 9A**—Large FCC lymphoma from another mouse unlisted in Table 1 showing the presence of large numbers of small lymphocytes. **B**—Passage 1 of the lymphoma shown in **A** composed a much more uniform population of large FCC. Note the large number of mitotic figures. **Figure 10**—Passage 6 of another large FCC showing a similarity to the lymphoma shown in Figure 9 B. **Figure 11**—Passage 1 of another large FCC from mouse 30 showing a predominance of small FCC, although some large FCCs are present.

as an etiologic agent in the development of B-cell lymphomas remains uncertain.

References

1. Rowe WP: Genetic factors in the natural history of murine leukemia virus infection: G. H. Clowes Memorial Lecture. Cancer Res 1973, 33:3061-3068
2. Rowe WP, Pincus T: Quantitative studies of naturally occurring murine leukemia virus infections of AKR mice. J Exp Med 1972, 135:429-436
3. Hartley J, Wolford N, Old L, Rowe W: A new class of murine leukemia virus associated with development of spontaneous lymphomas. Proc Natl Acad Sci USA 1977, 74:789-792
4. Cloyd MW, Hartley JW, Rowe WP: Lymphomagenicity of recombinant mink cell focus-inducing murine leukemia viruses. J Exp Med 1980, 151:542-552
5. Dunn T: Normal and pathologic anatomy of the reticular tissue in laboratory mice with a classification and discussion of neoplasms. J Natl Cancer Inst 1954, 14:1281
6. Pattengale PK, Taylor CR: Experimental models of lymphoproliferative disease: The mouse as a model for human non-Hodgkin's lymphomas and related leukemias. Am J Pathol 1983, 113:237-265
7. Pattengale PK, Frith CH: Immunomorphologic classification of spontaneous lymphoid cell neoplasms occurring in female BALB/c mice. J Natl Cancer Inst 1983, 70: 169-179
8. Rowe WP, Kozak CA: Germ-line reinsertions of AKR murine leukemia genomes in *Akv-1* congenic mice. Proc Natl Acad Sci USA 1980, 77:4871-4874
9. Fredrickson T, Morse HC III, Rowe W: Spontaneous tumors of NFS mice congenic for ecotropic murine leukemia virus induction loci. J Natl Cancer Inst 1984, 73:521-524
10. Morse HC III: Tumors of B lymphocytes and retroviruses, Mechanisms of B Cell Neoplasia. Edited by F Melchers, M Potter, M Weigert. Workshop at the Basel Institute for Immunology, March 15-17, 1983. Roche, Basel, Switzerland, pp 108-124
11. Chan H, Bryan T, Moore J, Staal S, Rowe W, Martin M: Identification of ecotropic proviral sequences in inbred mouse strains with a cloned subgenomic DNA fragment. Proc Natl Acad Sci USA 1980, 77:5779-5783
12. Morse HC III, Chused TM, Boehm-Truitt M, Mathieson BJ, Sharrow SO, Hartley JW: XenCSA: Cell surface antigens related to the major glycoproteins (gp70) of xenotropic murine leukemia viruses. J Immunol 1979, 122:443-454
13. Fredrickson TN, Langdon WY, Hoffman PM, Hartley JW, Morse HC III: Histologic and cell-surface antigen studies of hematopoietic tumors induced by Cas-Br-M murine leukemia virus. J Natl Cancer Inst 1984, 72:447-454
14. Kim K, Kanellopoulos-Langevin C, Merwin R, Sachs D, Asofsky R: Establishment and characterization of BALB/c lymphoma lines with B cell properties. J Immunol 1979, 122:549-554
15. Lanier L, Warner N, Ledbetter J, Herzenberg L: Quantitative immunofluorescent analysis of surface phenotypes of murine B cell lymphomas and plasmacytomas with monoclonal antibodies. J Immunol 1981, 127:1691-1697
16. Davidson WF, Fredrickson TN, Rudikoff EK, Coffman RL, Hartley JW, Morse HC III: A unique series of lymphomas related to the Ly-1$^+$ pathway of B lymphocyte differentiation. J Immunol 1984, 133:744-753
17. Warner NL, Daley MJ, Richey J, Spellman C: Flow cytometry analysis of murine B cell lymphoid differentiation. Immunol Rev 1979, 48:197-243
18. Ford RJ, Ruppert B, Maizel AL: SJL tumor: A neoplasm involving macrophages. Lab Invest 1981, 45:111-119
19. Warnke RA, Slavin S, Coffman RL, Butcher EC, Knapp R, Strober S, Weissman IL: The pathology and homing of a transplantable murine B cell leukemia (BCL_1) J Immunol 1979, 123:1181-1188
20. Green N, Hiai H, Elder J, Schwartz R, Khiroya R, Thomas C, Tsichlis R, Coffin J: Expression of leukemogenic recombinant viruses associated with a recessive gene in HRS/J mice. J Exp Med 1980, 52:249-264
21. Kawashima K, Ikeda H, Hartley J, Stockert E, Rowe W, Old L: Changes in expression of murine leukemia virus antigens and production of xenotropic virus in the late preleukemic period in AKR mice. Proc Natl Acad Sci 1976, 75:4680-4684
22. Yetter RA, Morse HC III: Cell surface antigen phenotypes of MCF-induced thymic lymphomas in AKR mice. J Immunol 1984, 132:2644-2648
23. Rowe WP, Hartley JW: Genes affecting mink cell focus-inducing (MCF) murine leukemia virus infection and spontaneous lymphoma in AKR F_1 hybrids. J Exp Med 1983, 158:353-364
24. Mosier DE, Yetter RA, Morse HC III: Retroviral induction of acute lymphoproliferative disease and profound immunosuppression in adult C57BL/6 mice. J Exp Med 1985, 161:766-784

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