Immunopathology of Natural and Experimental Lymphomas Induced by Wild Mouse Leukemia Virus

MARTIN L. BRYANT, PhD,
JONATHAN L. SCOTT, MS, BIJAY K. PAL, PhD,
JOHN D. ESTES, and MURRAY B. GARDNER, MD

From the Department of Pathology, University of Southern California School of Medicine, Los Angeles, California

Naturally occurring lymphomas of Lake Casitas (LC) wild mice, and the lymphomas induced by LC murine leukemia virus (MuLV) in Swiss mice from the National Institutes of Health, displayed remarkably similar gross, microscopic, and functional characteristics. They spared the thymus, arose primarily in the splenic red pulp, became leukemic, and were comprised of stem cells lacking classic T- and B-cell markers. Cytoplasmic and surface immunoglobulin were undetectable in 34 of 35 spontaneous LC lymphomas and in any of ten LC MuLV-induced lymphomas in NIH Swiss mice. Assays for immunoglobulin secretion, complement (C'3) and Fc receptors, Thy 1.1,2 antigens, Ly 1,2 antigens, and erythroid and myeloid markers were negative on all of the spontaneous and experimental lymphomas. Cell lines were derived from five spontaneous lymphomas of LC mice. Three lines were characterized as null cells, one line as B cells, and one line as macrophages. All cell lines were diploid. The wild mouse spontaneous lymphomas, and lymphomas experimentally induced by LC MuLV in laboratory mice, provide a useful model for childhood acute lymphoblastic leukemia and for study of the early steps of B-lymphocyte differentiation. (Am J Pathol 1981, 104:272-282)

SPONTANEOUSLY OCCURRING LYMPHOMAS of inbred mice, such as the AKR and C58 strains, which express high titers of endogenous murine leukemia virus (MuLV), are mostly of thymic T-cell origin.[1-3] Radiation- and virus-induced leukemia in C57BL and C3H strains of mice are also of T-lymphocytic origin.[4,5] Experimental transmission of well-characterized laboratory virus strains, such as Gross-, Kaplan-, and Moloney-MuLV, also cause lymphomas of mainly T-lymphocytic lineage.[6-8] B-lymphocytic tumors have been reported in laboratory mice as rare spontaneous events,[9,10] and infrequently following induction by chemical carcinogens[4,11] or the Abelson strain of MuLV.[12] Recently, Rauscher-MuLV was also shown to transform immature B-cells in laboratory mice[13] and the Friend helper virus was found to have selective tropism for more mature B-cells in the spleen.[14] However, each of these laboratory models represents, to some extent, an artifact of inbreeding and virus selection. For a balanced view, it would be helpful to know more about lymphoma ands MuLV as they occur under natural conditions in feral wild mice (*Mus musculus domesticus*), the progenitor of laboratory mice. Insofar as wild mice are outbred and not exposed to laboratory strains of MuLV, they represent a more relevant model for humans.

We have shown that feral mice from different trapping areas differ in their control of indigenous MuLV and proneness to spontaneous lymphoma.[15] Whereas most populations express little virus and develop only a few tumors, several populations express high levels of virus activity and display a tenfold increased incidence of lymphoma. These mice are also susceptible to a neuromotor hind leg paralysis caused by the indigenous MuLV.[16] An example of the latter mice are those inhabiting a squab farm near Lake Casitas (LC) in southern California. LC mice have as much genetic diversity as other feral mouse populations.[17] These mice thus offer a unique opportunity to examine the phenotype of spontaneous lymphomas

Supported by NCI contract NO1-CP8-1032; Dr. Bryant was supported in part by a Medical Student Research Fellowship from USC and by grant number 5 R25 CA24426 of the Clinical Education Program, NCI.
Accepted for publication May 5, 1981.
Address reprint requests to Murray B. Gardner, MD, Department of Pathology, University of California School of Medicine, Davis, CA 95616.

0002-9440/81/0909-0272$01.05 © American Association of Pathologists

caused by MuLV in a genetically heterogenous but susceptible host. The infectious MuLV of LC wild mice show either a wide *in vitro* host range with growth in cells of both murine and heterologous species ("amphotropic"), or their growth is restricted to murine cells ("ecotropic").[18-20] Most LC mice are congenitally infected with the amphotropic class of MuLV, which persists as a lifelong viremia in the absence of any detectable immune response to this virus.[21] A few LC mice are also congenitally infected with, and tolerant to, the ecotropic class of MuLV. About 18% of the naturally infected mice develop lymphoma and 12% develop paralysis after one year of age.[22] The lymphomas are mainly of splenic origin and lack thymus antigen.[23] Upon experimental transmission of purified amphotropic or ecotropic virus clones in newborn laboratory mice and rats, lymphomas are induced in more than 30% of the recipients with an average latent period of 8 months.[24,25] The paralytic disease is transmitted only by the ecotropic MuLV. In laboratory mice, the Fv^1 genetic locus is the primary determinant of susceptibility to lymphoma induction; only Fv^{1n} genotype mice are permissive to replication and tumor induction by the LC MuLV, which are N-tropic for mouse cells.[25,26] Under natural conditions, however, a dominent ecotropic MuLV restriction gene called $Akvr^{1R}$, which is distinct from Fv^1 and is polymorphic in LC wild mice, determines susceptibility to the ecotropic virus-caused lymphoma and paralysis.[27]

In this paper we present the morphologic and functional characteristics of the naturally occurring and virus-induced experimental lymphomas. Both display remarkably similar gross and microscopic features. They spare the thymus, arise primarily in the splenic red pulp and bone marrow, and progress to leukemia early in the disease process. The tumors are composed primarily of stem cells lacking classic T- and B-cell markers. A few tumors, however, give evidence of B-cell differentiation. Thus, this wild mouse model system should be helpful in elucidating the mechanism by which MuLV induces malignancy of lymphoid stem cells at a very early stage of differentiation.

Materials and Methods

Mice

LC feral mice were trapped, brought to the laboratory, and either killed for collection of tissues or individually housed in mason jars and observed for spontaneous lymphoma.[22] Lymphomas were experimentally induced in susceptible NIH Swiss mice by intraperitoneal inoculation of LC MuLV.[24] All mice were routinely observed and palpated for splenomegaly and lymphadenopathy. Complete necropsy, including microscopic study, was performed on each mouse with possible lymphoma. Peripheral blood smears and touch preparations (imprints) of enlarged spleens were used for preliminary pathological diagnosis and further histopathology. Sections of tumor and major visceral organs were prepared for diagnostic light microscopy by fixation in 10% neutral buffered formalin, and 5-μ paraffin-embedded sections were stained with hematoxylin and eosin.

Cell Suspensions

Single-cell suspensions were prepared from portions of fresh spleens removed from lymphomatous mice and normal controls. Red blood cells were lysed with 0.83% ammonium chloride or removed by Ficoll-Hypaque gradient separation.[28] A fraction of the spleen cell preparations was maintained in suspension culture, and colonies were established in semisolid agar.[29] A second fraction was used for morphologic and functional characterization of the lymphoid cell populations. Cell viability was determined by trypan blue exclusion, and cell morphology was studied in detail using Wright-Geimsa-stained cytocentrifuged preparations.[30] The remainder was frozen in RPMI-1640 medium, containing 10% DMSO and 20% fetal calf serum, and stored at −90 C.

Detection of Surface and Cytoplasmic Immunoglobulin (Ig) by Immunofluorescence

Each viable lymphocyte preparation was screened by the indirect and direct fluorescent antibody method[31] for surface membrane Ig (SmIg), using goat anti-mouse polyvalent Ig antiserum. In addition, most preparations were studied in more detail using rabbit anti-mouse heavy-chain-specific serums, including anti-mu, anti-gamma, and anti-alpha and rabbit anti-mouse light-chain-specific serums, including anti-kappa and anti-lambda (Bionetics, Kensington, Md). The same antiserums were used to detect cytoplasmic Ig in cytocentrifuged preparations of the various lymphoma preparations. In all assays, a continuous suspension cell line, derived from a T-lymphoid tumor of a cat (FL74),[32] was used as a negative control. Routine positive controls included normal wild mouse and NIH Swiss mouse spleen cell preparations.

Immunoperoxidase Detection of Cytoplasmic Ig

Paraffin-embedded sections (5 μ) of various tissues were heated at 60 C for 1 hour, passed through

xylene, and rehydrated through alcohol to water. Endogenous peroxidase activity was blocked with a fresh 0.6% solution of hydrogen peroxide in methanol for 20 minutes. Nonspecific background staining was reduced by incubation of the paraffin sections with nonimmune, nonconjugated swine serum (Accurate Chemical and Scientific Corp., Hicksville, NY), diluted 1:20 for 15-20 minutes. Sections were then treated for 30 minutes with rabbit anti-mouse polyvalent Ig antiserums or antiserums specific for heavy chains ($gamma_{1,2}$, alpha, mu) or light chains (kappa, lambda). Unlabeled swine anti-rabbit Ig antiserum, diluted 1:30, was added for 30 minutes, followed by rabbit anti-peroxidase-peroxidase complex (PAP, Cappel Labs, Cochransville, Pa) (1:100) for 30 minutes. Sections were developed by the diaminobenzidine reaction[33] for 3-5 minutes and counterstained with hematoxylin. Tris-saline buffer (pH 7.6) was used for reactions, dilutions, and washings.

Assay for Ig Secretion

A modification of the Jerne hemolytic plaque assay using protein A-coated sheep red cells (SRBC)[34] was used to detect lymphoid cells secreting Ig. A mixture of protein A-SRBC and 10^6 lymphocytes/ml were plated in agar (Difco Laboratories, Detroit, Mich) with guinea pig complement (Grand Island Biologicals, Grand Island, NY) and either goat anti-mouse polyvalent Ig antiserum or rabbit anti-mouse chain-specific serums described above. After the agar had solidified, the plates were incubated overnight at 37 C in a humidified atmosphere. Single-cell suspensions prepared from normal NIH Swiss and wild mouse spleens served as positive controls.

Detection of Thy 1.2, Ly 1,2, and T-200 Surface Antigens

Direct cytotoxicity, using rabbit anti-mouse brain (Cedarlane Laboratories, Ltd., Accurate Chemical and Scientific Corp., Hicksville, NY) and rabbit complement (1:10), was used for the detection of Thy 1.1 and Thy 1.2 antigens on the surface of lymphocytes.[23] Normal mouse thymocytes from young LC or NIH Swiss mice were used as positive controls. Briefly, 10 λ of the lymphoid cell suspension (2×10^6 cells/ml) was added to microtiter wells, containing 10 λ of serially diluted anti-Thy serum, and incubated at room temperature for 30 minutes. Following washing, the cells were incubated at 37 C with 10 λ of complement for 30 minutes. One drop of 0.4% trypan blue was added to each well, and the cytotoxicity index was calculated. In addition, indirect immunofluorescence, using monoclonal anti-Thy 1.2 serum (New England Nuclear, Boston, Mass), was used to detect this T-cell antigen. Expression of lymphocyte differentiation antigens, Ly 1,2 and T-200, was detected by direct cytotoxicity[35] and monoclonal antibody techniques.[36]

EA and EAC Rosette Assays

Washed sheep red blood cells (SRBC) were incubated for 30-45 minutes with an equal volume of rabbit anti-SRBC (1:100) (Cordis Labs, Miami, Fla). A 19S IgM anti-SRBC was used in the complement receptor assay (EAC test), and a 7S IgG anti-SRBC was used to test for the Fc receptor (EA test). Complement-coated SRBC were prepared by incubating IgM-SRBC with fresh mouse complement (1:20) for 20 minutes at room temperature. Washed and centrifuged EA or EAC reagents were resuspended in RPMI-1640 medium and incubated with an equal volume of spleen cell suspension (5×10^6 cells/ml) at 37 C for 30-45 minutes. The EA-lymphocyte suspension was centrifuged at 200*g* and allowed to remain at 4 C for 15 minutes. Cell mixtures were counted in the presence of trypan blue; viable mononuclear cells binding four or more SRBC were considered rosette-forming cells.

Derivation of Lymphoid Cell Lines

Lymphoid cell lines were derived from lymphomatous spleens of LC mice by gently teasing apart the stromal component in RPMI-1640 medium (Grand Island Biologicals) and harvesting the single cells remaining in suspension after the connective tissue had settled. Red blood cells were removed by Ficoll-Hypaque gradient separation.[27] The viable lymphoid cells were maintained at a density of $0.5-1.0 \times 10^6$ cells/ml in RPMI-1640 medium, supplemented with 2 mM glutamine, 50 μg/ml gentamicin, and 10-20% fetal calf serum (Grand Island Biologicals). Initially, 0.005 mM 2-mercaptoethanol (Eastman Kodak Co., Rochester, NY) was added to the culture medium, but it was later discontinued as no growth advantage could be detected.

Cytochemical and Cytogenetic Assays

All histochemical procedures were performed using touch imprints of lymphomatous spleens, cytocentrifuged preparations of both fresh and *in vitro* cultured lymphoid cells, and peripheral blood smears. Nonspecific esterase (α-naphthylbutyrate esterase) in monocytes and macrophages, and α-chloroacetate

esterase in neutrophilic granulocytes, were detected using the methods of Yam et al.[37] Kulenkampff's[38] modification of the esterase technique was used to detect immunoblastic cells. Also applied were a simplified myeloperoxidase stain, using benzidine dihydrochloride,[38] to identify intracellular leukocyte peroxidase activity and Sudan black B stain[40] for lipid-containing granules. Ralph's[41] benzidine technique was used for the demonstration of hemoglobin. Chromosome preparation and karyotype determination were done according to conventional trypsin-Geimsa banding techniques.[42]

Detection of Virus

The production of MuLV by the lymphoma cell lines was detected by the RNA-dependent DNA polymerase (RDP) assay, using synthetic template primer poly(rA) · oligo(dT).[43] Virus infectivity was determined using the fluorescent focus induction method on wild mouse (SC-1) and rabbit (SIRC) cells.[18]

Latex Bead Uptake

Phagocytosis by macrophages was measured by a minor modification of the method of Oren et al.[44] One drop (0.02 ml) of a 5% suspension of latex particles (1.1 μ in diameter) was added to 10^6 cells/ml in Hanks' buffered salt solution (HBSS). Following incubation at 37 C for 45 minutes, the cells were washed three times in HBSS and observed under a coverslip for cytoplasmic-ingested latex particles.

Mitogenic Stimulation

Lymphoid cells (10^5) were incubated at 37 C in RPMI-1640 medium (with and without 10% fetal calf serum) containing 10^{-2} to 10^2 μg/ml of each of the following agents: lipopolysaccharide (LPS) (*Escherichia coli* 0127:B8, Sigma Chemical Co., St. Louis, Mo, or *Salmonella typhosa* 0901, Difco Laboratories, Detroit, Mich), concanavalin A (Con A) (Pharmacia, Uppsala, Sweden), phytohemagglutinin M (PHA) (Difco Laboratories), and dextran sulphate (DxS) (Pharmacia). After 24 hours, 1 μCi ^{3}H-thymidine was added, and the cells were incubated at 37 C overnight, lysed with 5% sodium dodecyl sulfate (SDS), collected on a 0.45-μ Millipore filter by suction, and washed with 20 ml cold 5% trichloroacetic acid (TCA), containing 1% sodium pyrophosphate (NaPP). The dried filters were counted in a Beckman liquid scintillation counter for 20 minutes. Control samples were incubated for the first 24 hours in the appropriate medium without mitogen. Viable cell counts were determined by trypan blue exclusion.

Deoxynucleotidyl Terminal Transferase (TdT) and Natural Killer (NK) Cell Assays

Deoxynucleotidyl terminal transferase (TdT) assays were performed as previously described.[45] The natural killer (NK) cell activity of the lymphoma cell lines was determined using the method of Scott et al.[46]

Results

Histopathology of Lymphomas

The spontaneous lymphomas of LC wild mice and the lymphomas induced by LC MuLV in NIH Swiss mice were remarkably similar in gross and microscopic features. They had a diffuse pattern made up almost entirely of poorly differentiated mononuclear cells (Figure 1a). They showed a generalized distribution with involvement of the spleen, liver, kidneys, systemic lymph nodes, bone marrow, and an accompanying leukemia (Figure 1b). However, the thymus was spared. The earliest involvement was usually in the spleen, where malignant cells were observed in the red pulp, with sparing of the follicles and periarteriolar T-cell zones (Figures 1 and 1c,d,e). More than 90% of the cells derived from lymphomatous spleens of both LC and NIH Swiss mice were relatively monomorphic undifferentiated lymphoblastoid cells, with large round vesicular nuclei (nuclear/cytoplasmic ratio approximately 5:1) containing diffuse heterochromatin and large nucleoli (Figure 1f). By light microscopy of Zenker's fixed conventionally stained tissue sections, the tumor cells resembled hematocytoblasts or immunoblasts, which are virtually indistinguishable on the basis of morphology alone (R. Lukes, personal communication). In a previous electron microscopic study,[47] the tumor cell nuclei had regular or slightly indented contours, and the cytoplasm contained abundant free ribosomes but only sparse rough endoplasmic reticulum. Numerous budding and extracellular type C particles were present.

Immunopathology of Lymphomas

A three-step immunoperoxidase procedure was used to detect cytoplasmic immunoglobulin (heavy and light chains) in formalin-fixed, paraffin-embedded necropsy specimens. Lymphomatous spleens and lymph nodes were the primary tissues assayed; however, leukemic infiltrates of various other organs were also examined. Cytoplasmic Ig could not be

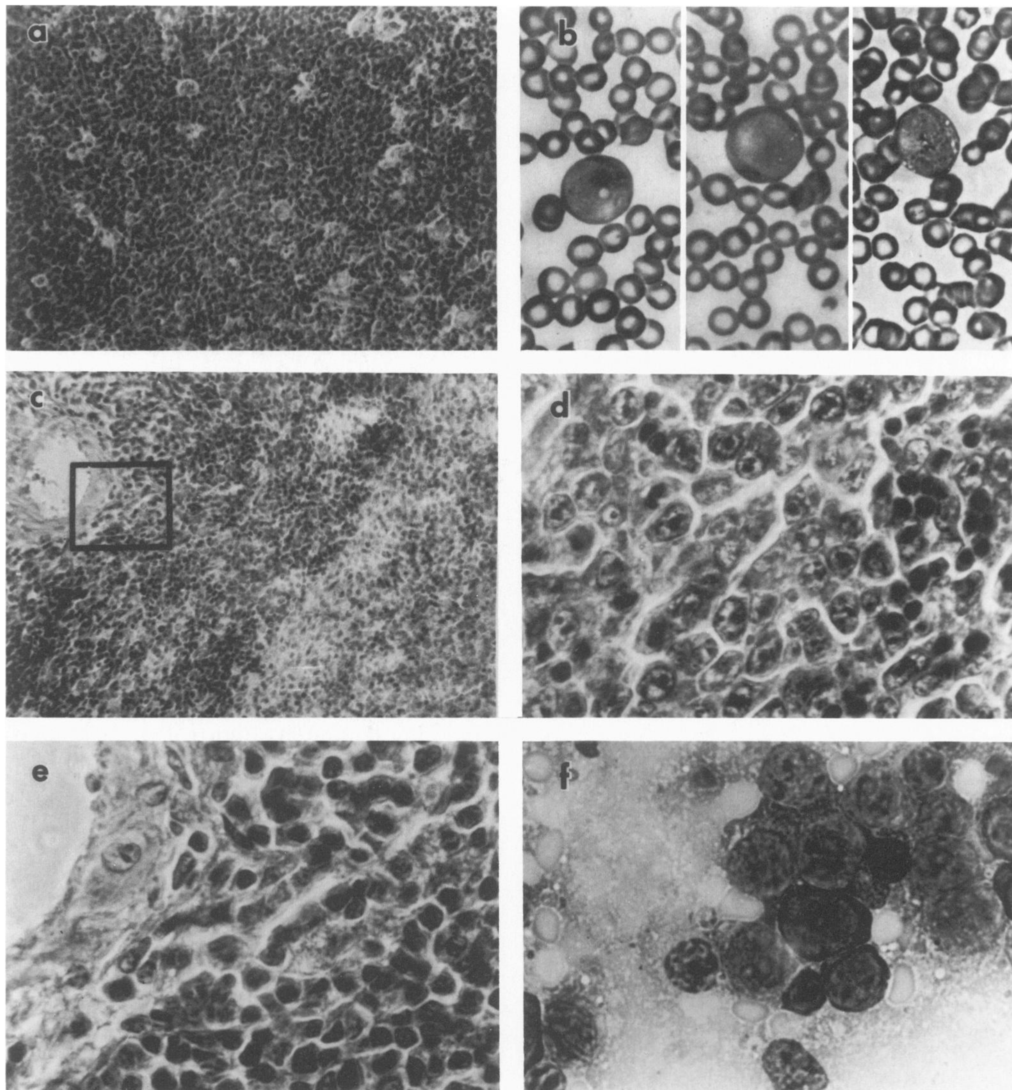

Figure 1a—Lymphomatous spleen from an LC wild mouse showing a diffuse pattern of poorly differentiated cells. The large cells are megakaryocytes. (H&E, ×160) **b**—Lymphoblastoid cells present in peripheral blood of lymphomatous wild mouse. (Wright-Geimsa, ×1000) **c**—Lymphomatous spleen from an LC wild mouse. Note sparing of the periarteriolar T-cell zone and invasion of the red pulp. The enclosed area is shown under higher magnification in **e**. (H&E, ×160) **d**—Red pulp from **c**, containing malignant lymphoid cells. (H&E, ×650) **e**—Periarteriolar area outlined in **c** showing residual T-cell zone. (H&E, ×650) **f**—Touch imprint of lymphomatous spleen from an LC wild mouse. (Wright-Geimsa, ×1000)

identified using polyvalent Ig antiserum and monospecific anti-heavy-chain (gamma$_{1,2}$, alpha, mu) and anti-light-chain (kappa, lambda) serums in any of 26 spontaneous LC lymphomas (Figure 2a), nor in any of 10 LC virus-induced lymphomas in NIH Swiss mice (Table 1). A reactive lymph node from a wild mouse, in which immunoperoxidase positive plasma cells were readily identified (Figure 2b), was used as a positive control. Direct and indirect immunofluorescence methods, using the same antiserums, also failed to detect cytoplasmic Ig in fresh fixed suspension or cyto-centrifuged preparations of splenic lymphoid cells from any of 18 LC and 25 NIH Swiss mice with lymphoma.

Surface Marker Expression and Functional Studies of the Lymphomas

The presence of SmIg on viable lymphoma and normal spleen cell preparations was studied using polyvalent Ig antiserums or antiserum specific for heavy and light Ig chains. Normal wild mouse and NIH Swiss mouse spleen cell preparations served as positive immunofluorescent controls; 5–40% of the splenic lymphocytes were stained, depending on which antiserum was employed. SmIg was detected by immunofluorescence in none of 25 experimental lymphomas and in one of 18 naturally occurring LC lymphomas (Table 1). The SmIg-positive lymphoma

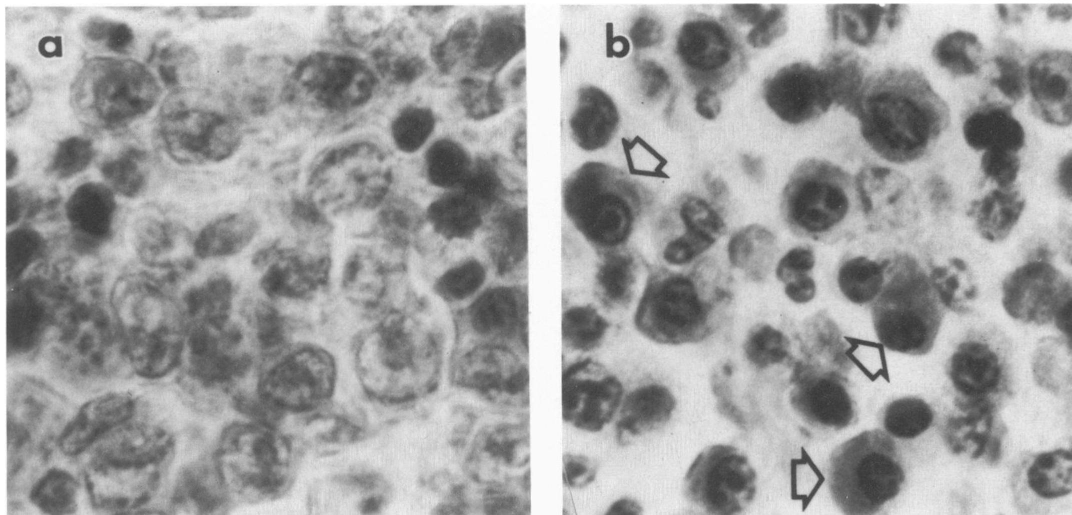

Figure 2a—Lymphomatous lymph node from an LC wild mouse stained by immunoperoxidase (IPx) technique, using rabbit anti-mouse polyvalent Ig antiserum. Note absence of any positive cells. (×1000) **b**—Reactive lymph node from an LC wild mouse stained by IPx technique using rabbit anti-mouse polyvalent Ig antiserum; arrows indicate several IPx-positive plasma cells. (×1000)

expressed kappa light chains and gamma 2b heavy chains, but appeared negative for cytoplastic Ig by immunoperoxidase and immunofluorescent staining. Complement (C'3) receptors and Fc receptors were negative on all of the spontaneous and experimental lymphoma preparations. Approximately 20–30% of the normal spleen cell populations from either wild mice or NIH Swiss mice were rosette positive for both complement receptors and Fc receptors. T-cells (LC and NIH Swiss thymocytes) were readily detected by indirect immunofluorescence, using monoclonal anti-Thy 1.2 antibody, and direct cytotoxicity, using anti-Thy 1.1,2 serum plus complement. However, these reactions were consistently negative with the wild mouse and NIH Swiss mouse lymphoma cell preparations described above (Table 1). Each of the lymphoma cell preparations was also screened for secretion of Ig, using a modification of the Jerne protein A hemolytic plaque assay and polyvalent Ig antiserum. Whereas the normal LC and NIH Swiss spleens were positive, all the lymphomas, spontaneous or experimentally induced, were negative in this *in vitro* bioassay. However, the single SmIg-positive spontaneous lymphoma was not tested. None of the spontaneous or experimental lymphomas tested showed histologic evidence of erythroid or myeloid differentiation.

Wild Mouse Lymphoma Cell Lines

We attempted to establish lymphoid cell cultures from 14 natural and ten experimental lymphomas. As determined by trypan blue exclusion, the majority (more than 90%) of the lymphoid cells in each culture remained viable for at least 1 week in suspension culture. However, in general, only about 20% were

Table 1—Immunopathologic Characteristics of Natural and Experimental Lymphomas

		Immunologic assay*						
		Cyto Ig						
Cell preparation	Number of mice tested	IPx	IFA	SmIg	Ig secretion	C'3R	FcR	Thy 1.1,2
Spontaneous LC wild mouse lymphoma†	35	0/26‡	0/18	1/18	0/9	0/18	0/18	0/18
Experimentally induced lymphoma§	25	0/10	0/25	0/25	0/25	0/25	0/25	0/25
Normal or reactive spleen; LC	5	5/5	5/5	5/5	5/5	5/5	5/5	0/5
Normal spleen; NIH Swiss	5	NT	5/5	5/5	5/5	5/5	5/5	0/5
Thymocytes; LC and NIH Swiss	5	NT	0/5	0/5	0/5	0/5	0/5	5/5

* Described in Materials and Methods.
† Lake Casitas (LC) mice trapped with spontaneous lymphoma or developing lymphoma while housed in laboratory colonies.
‡ Number positive/number tested.
§ Lymphomas induced in NIH Swiss mice using cloned MuLV derived from LC mice.
IPx = immunoperoxidase, IFA = immunofluorescence, SmIg = surface membrane immunoglobulin, Ig secretion = immunoglobulin secretion, C'3R = complement receptor, FcR = immunoglobulin Fc receptor, NT = not tested.

Table 2—Functional and Cytochemical Characteristics of Wild Mouse Lymphoma Cell Lines

Assay	Cell line designation*				
	11235	29298	29308	29292	27687
SmIg (immunofluorescence) polyvalent and monospecific	+ (alpha; kappa)	–	–	–	–
CytoIg (immunofluorescence) polyvalent and monospecific	+ (alpha; kappa)	–	–	–	–
CytoIg (RIA-mu chain)	–	–	–	NT	–
Ig secretion (polyvalent)	–	–	–	–	–
Fc receptor	–	–	–	NT	–
C'3 receptor	–	–	–	NT	–
Thy 1.1,2	–	–	–	–	–
Ly 1, Ly 2	–	–	–	NT	NT
T-200	+	+	NT	NT	NT
α-naphthylbutyrate esterase	–	–	–	–	+
α-chloroacetate esterase	–	–	–	NT	–
Myeloperoxidase	–	–	–	–	–
Sudan black B	–	–	–	–	–
Hemoglobin	–	–	–	–	–
Tdt activity	–	–	–	NT	–
NK activity	–	–	–	NT	–
Latex bead uptake	–	–	–	–	+
MuLV production	+	+	+	+	+
Mitogenic stimulation†	–	–	–	NT	–
Transplantation in nude mice	+	+	+	NT	NT
Diploid karyotype	+	+	+	+	+

* Cell line designation corresponds with the number given to the mouse developing the lymphoma. Clonal cell lines have been derived from the original lymphomas and all display characteristics identical to those presented here.

† See text for various mitogenic agents used.

SmIg = surface membrane immunoglobulin, CytoIg = cytoplasmic immunoglobulin, Fc receptor = immunoglobulin Fc receptor, C'3 receptor = complement receptor, RIA = radioimmunoassay (sensitivity, 1 ng/mg cell protein), Tdt = terminal deoxynucleotidyl transferase, NK = natural killer cell, NT = not tested.

alive after 2 weeks, and by 4 weeks most cells had died. Those cells that survived were allowed to proliferate without the addition of conditioned media or use of feeder layers. To date, cell lines from five naturally occurring LC lymphomas and none from the experimental NIH lymphomas have been adapted to continuous tissue culture. The LC lymphomas and numerous subclones derived from them in soft agar have continued to propagate as homogeneous populations for more than a year, with doubling times ranging from 12 to 48 hours. Surface marker and functional characteristics of these cell lines are summarized in Table 2.

Two of the cell lines (29298, 29308) were morphologically indistinguishable from the original wild mouse lymphoma cells and have retained the parental null (non-T, non-B) phenotype. They possessed none of the classic B-cell or T-cell surface markers, nor did they display Fc or complement receptors. Neither of these two cell lines showed any evidence of erythroid (hemoglobin synthesis) or myeloid (α-naphthylbutyrate esterase, α-chloroacetate esterase, myeloperoxidase, Sudan black B stain, phagocytosis) differentiation. One of these cell lines, however, did express the T-200 antigen, as defined by monoclonal antibodies (I. Trowbridge, personal communication), consistent with an origin from lymphoid stem cells.[36] Both cell lines were negative for mu heavy chain by a radioimmunoassay capable of detecting 1 ng of this protein (S. Aaronson, personal communication). Recently, another wild mouse lymphoma, 29292, with surface marker and functional characteristics identical to these two null cell lines has been adapted to tissue culture (Table 2).

One of the lymphoma cell lines (11235), established more than five years ago, is a monoclonal B-cell line expressing surface and cytoplasmic IgA (alpha, kappa) as determined by the immunofluorescence and immunoperoxidase methods (Figure 3). No cytoplasmic Ig was found by the immunoperoxidase technique on paraffin-embedded sections of the original tumor. Assays for additional heavy chains (mu, gamma) and light chains (lambda) have been consistently negative.

The other cell line (27687), which initially had the *in vivo* and *in vitro* appearance and immunologic characteristics of a typical LC null cell lymphoma, began, after several weeks in tissue culture, to display detectable Fc receptors. After nearly a year *in vitro*, the entire cell population, as well as cloned derivatives, expressed nonspecific esterase activity (Figure 4), Fc receptors and phagocytosed latex beads; it thus apparently consists entirely of macrophages.

Each of the lymphoid cell lines tested produced progressively growing tumors within 3 weeks after transplantation subcutaneously in weanling Balb/c

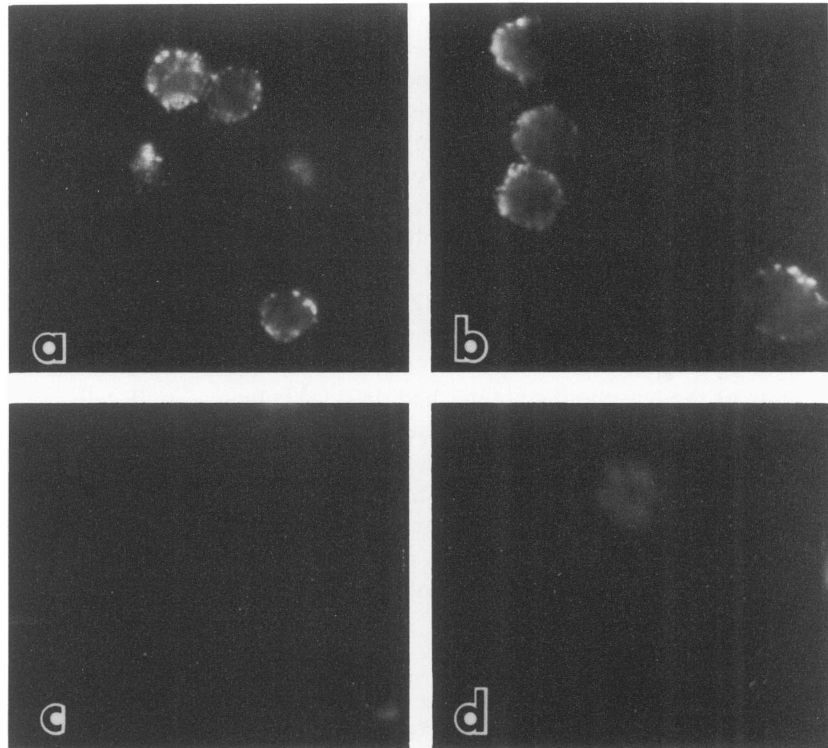

Figure 3—Indirect immunofluorescence of surface membrane immunoglobulin of lymphoma cell line 11235, rabbit anti-mouse immunoglobulin chain-specific serums. **a**—Anti-alpha. **b**—Anti-kappa. **c**—Anti-mu. **d**—Anti-lambda. (×640)

nude mice. Microscopically and immunologically, these tumors were identical to their parent cell cultures. In addition, each cell line produced amphotropic MuLV. Virus harvested from the B-lymphoid culture (11235) supernatant and null cell lymphoma cell lines induced the typical null cell lymphomas in NIH Swiss mice, after a latent period of 4 months.

The response of the wild mouse lymphoma cell lines to various mitogenic agents (LPS, PWM, PHA, Con A, and DxS at 10^{-2} to 10^{2} μg/ml, and DMSO, 200 mM), with respect to DNA synthesis, changes in surface markers, and synthesis and secretion of Ig, was examined. We observed a 50% block in growth, as measured by ^{3}H-thymidine incorporation, and viable

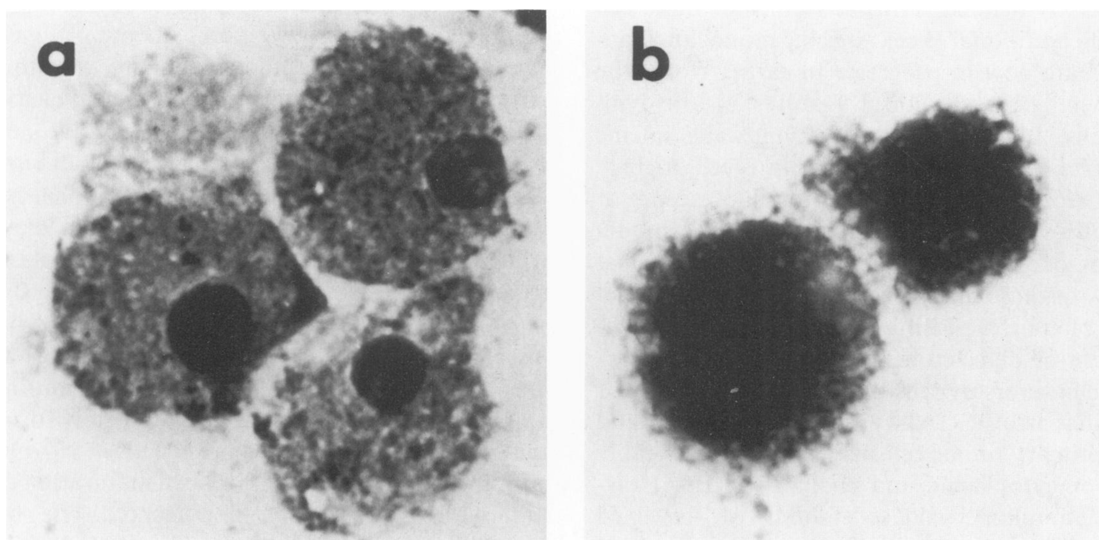

Figure 4—Nonspecific esterase stain of cytocentrifuged macrophage cell line 27687. **a**—Without substrate (alpha-napthal)-negative control. **b**—With substrate-positive reaction. (Giemsa, ×650)

cell counts when the null cell lines (29298 and 29308) were treated with LPS (10 μg/ml), but we did not detect evidence of B-cell differentiation. Cytoplasmic and surface Ig, measured by immunofluorescence, antibody secretion, and the presence of C'3 receptors and Fc receptors, remained undetectable. Additionally, the growth characteristics of the Ig-positive and macrophage lines were not detectably altered following exposure to PHA, PWM, and con A, DxS, or DMSO. The B-lymphoid culture (11235) could not be induced by these methods to secrete detectable heavy or light Ig chains.

None of the lymphoid cell lines had detectable terminal deoxynucleotidyl transferase activity, and none displayed natural killer cell activity in 4-hour ^{51}Cr release assays using YAC-1 cells as targets.[46] Preliminary chromosome analysis of the lymphoid and macrophage cell lines revealed a diploid karyotype (modal chromosome number of 40).

Discussion

Most of the LC lymphomas were apparently derived from lymphoid stem cells that lacked classic T-and B-cell markers (ie, null cells). The tumors induced by LC MuLV in newborn NIH Swiss mice were also of null cell character. The uniformity of the lymphoma phenotype suggests that the LC MuLV interacts in both feral and laboratory mice with a similar hematopoietic stem cell. The only hematopoietic determinant detected on the Ig-negative tumor cells was the T-200 antigen, indicating a lymphoid origin.[36] More deteminants than the T-200 antigen must be identified, however, before the target cells(s) can be more precisely defined. Further studies of these cell lines, using additional B-cell–specific monoclonal antibodies,[48] are now in progress. In earlier work, the cytologic and fine structural features of the lymphoma cells, and the sparing of thymus and splenic periarteriolar T-cell zones, suggested a B-cell origin.[49] The more recent detection by immunofluorescence of SmIg in one of the primary LC lymphomas, and derivation of one Ig-positive cell line, indicate that at least a few of these tumors are, indeed, of B-cell lineage. It is, of course, possible that low levels of Ig synthesis might be detected in some of the other apparent null cell tumors by sensitive metabolic labeling or radioimmunoassay (RIA). Very recently, we found that two primary tumor cell suspensions were weakly positive for cytoplasmic mu chains by RIA. However, by immunoperoxidase staining of the fixed tumor sections, we observed that they contained varying numbers of reactive plasma cells among the negative staining tumor cells. Furthermore, even careful washing might not rid these fresh cell suspensions of adsorbed mouse serum. Therefore, more study of long-term cell lines or transplant tumors derived from LC lymphomas is required to determine their potential for differentiation to pre-B cells, in which only cytoplasmic mu chains are detectable,[50] or to more mature Ig-producing B-cells.

Whether the B-cell and macrophage lines were derived by differentiation of null cells *in vitro*, or by selection for growth *in vitro* of cells that were present but not recognized in the primary tumor, is unknown. However, the latter alternative seems most likely because it has not been possible to convincingly convert null cells to pre-B cells or macrophages *in vitro*.[51] However, because LPS has been reported to block *in vitro* growth of early macrophages, as well as B-cell lymphoma cell lines,[52,53] one must consider the possibility that these null cells might represent a previously unrecognized form of macrophage precursor. Mouse lymphoid cell lines producing IgA are much less common than are those that produce IgM or IgG. Unfortunately, IgA is not released from the wild mouse lymphoma cells, and, therefore, the culture cannot serve as a source of this product. The long-term culture of pure macrophages is also unusual, although not without precedent,[54,55] and should provide a valuable source of these cells for further characterization. Productive infection of each cell line with amphotropic MuLV is consistent with our previous finding that almost all lymphomatous LC mice or virus-inoculated NIH Swiss mice are systemically infected with this virus.[15]

Both wild mouse MuLV and the Abelson strain of laboratory-derived MuLV transform hematopoietic stem cells at an early stage of maturation. The Abelson disease involves mainly bone marrow and meninges and is usually not leukemic,[56,57] whereas the LC wild mouse disease involves mainly the spleen, spares and meninges, and is usually leukemic. The Abelson virus transforms a primitive hematopoietic stem cell that grows as a null cell *in vitro* and also transforms pre-B-cells[51] or macrophages.[55] In immunologically primed mice, the virus may also transform more mature B-cells, leading to plasmacytosis *in vivo* and Ig-secreting plasma cell lines *in vitro*.[51] The wild mouse and Abelson lymphoma cells appear diploid, in contrast to the regular trisomy of chromosome 15 in certain T- and B-cell murine leukemias.[42,58,59] The Abelson virus can transform normal fibroblasts and null or pre-B cells *in vitro* but, as with the wild mouse system, B-cell maturation of true null cell lines has not been documented. The Abelson and wild mouse models thus differ from the Friend-MuLV system, in which it has been consistently possible to produce further differentiation of transformed erythroid stem cells by *in vitro* manipula-

tion.[60,61] The wild mouse MuLV differ from the Abelson virus in that they are not defective and do not transform fibroblast or lymphoid cells *in vitro*.

Spontaneous lymphomas with leukemia of null cell or, rarely, B-cell origin is a unique feature of this wild mouse retrovirus model, differing markedly in the tumor cell phenotype from the prototype high lymphoma incidence strains of laboratory mice. As a model for humans, the LC wild mouse lymphoma most closely resembles childhood acute lymphoblastic leukemia, which often consists of a leukemic proliferation of null or pre-B cells.[61–64] This natural and experimental lymphoma system also affords the opportunity to further explore the very early steps of B-lymphocyte maturation and, possibly, to devise culture conditions to induce further differentiation of the virus-transformed cells.

Note Added in Proof

The four wild mouse lymphoid cell lines were tested by Dr. Robert Coffman, Department of Pathology, Stanford University School of Medicine, with a monoclonal antibody (RA3 2C2) that specifically recognizes B cells and B cell precursors in mice (Coffman RL, Weissman IL: A monoclonal antibody that recognizes B cells and B cell precursors. J Exp Med 1981, 153:269–279). Cell lines 11235 and 29292 were strongly positive, whereas cell lines 29298 and 29308 were negative. He also confirmed that each cell line was negative by immunofluorescence for cytoplasmic mu chains. These findings indicate that the wild mouse and Abelson MuLV-associated lymphomas consist of the same or closely related two major classes of cells, viz early Ig-negative B cell precursors and null cells.

References

1. Gross L: Oncogenic Viruses. 2nd edition. Oxford, Pergamon Press, 1970
2. Law LW, Miller JH: Observations on the effect of thymectomy on spontaneous leukemias of high leukemia strains AKR and C58. J Natl Cancer Inst 1950, 11:253–262
3. Metcalf D: Thymus grafts and leukemogenesis. Cancer Res 1964, 24:1952–1957
4. Haran-Ghera N, Peled A: Thymus and bone marrow derived lymphocytic leukaemia in mice. Nature 1973, 241:396–398
5. Kaplan HS: On the natural history of the murine leukemias: presidential address. Cancer Res 1967, 27:1325–1340
6. Moloney JB: Biological studies on a lymphoid leukemia virus extracted from sarcoma 37: I. Origin and introductory investigation. J Natl Cancer Inst 1960, 24: 933–951
7. Siegler R, Rich MA: Pathogenesis of murine leukemia. Natl Cancer Inst Monogr 1966, 22:527–547
8. Shevach EM, Stobo JD, Green I: Immunoglobulin and Θ-bearing murine leukemias and lymphomas. J Immunol 1972, 108:1146–1151
9. Lanier LL, Lynes M, Haughton G, Wettstein PJ: Novel type of murine B-cell lymphoma. Nature 1978, 271:554–555
10. Slavin S, Strober S: Spontaneous murine B-cell leukaemia. Nature 1978, 272:624–626
11. Paige CJ, Kincade PW, Ralph P: Murine B-cell leukemia line with inducible surface immunoglobulin expression. J Immunol 1978, 121:641–647
12. Premkumar E, Potter M, Singer PA, Sklar MD: Synthesis, surface deposition and secretion of immunoglobulins by Abelson virus-transformed lymphosarcoma cell lines. Cell 1975, 6:149–159
13. Reddy EP, Dunn CY, Aaronson SA: Different lymphoid cell targets for transformation by replication-competent Moloney and Rauscher murine leukemia viruses. Cell 1980, 19:663–669
14. Isaak DD, Price JA, Reinisch CL, Cerny J: Target-cell heterogeneity in murine leukemia virus infection: differences in susceptibility to infection with Friend leukemia virus between B-lymphocytes from spleen, bone marrow and lymph nodes. J Immunol 1979, 123: 1822–1828
15. Gardner MB: Type C viruses of wild mice: Characterization and natural history of amphotropic, ecotropic and xenotropic MuLV. Curr Top Microbiol Immunol 1978, 79:215–259
16. Gardner MB, Henderson BE, Officer JE, Rongey RW, Parker JC, Oliver C, Estes JD, Huebner RJ: A spontaneous lower motor neuron disease apparently caused by indigenous type C RNA virus in wild mice. J Natl Cancer Inst 1973, 51:1243–1254
17. Rice MC, Gardner MB, O'Brien SJ: Genetic diversity in leukemia-prone feral house mice infected with murine leukemia virus. Biochem Genet 1980, 18:915–928
18. Bryant ML, Klement V: Clonal heterogeneity of wild mouse leukemia viruses: Host range and antigenicity. Virology 1976, 73:532–536
19. Rasheed S, Gardner MB, Chan E: Amphotropic host range of naturally occurring wild mouse leukemia viruses. J Virol 1976, 19:13–18
20. Hartley JW, Rowe WP: Naturally occurring murine leukemia viruses in wild mice: Characterization of a new "amphotropic" class. J Virol 1976, 19–25
21. Gardner MB, Chiri A, Dougherty MF, Casagrande J, Estes JD: Congenital transmission of murine leukemia virus from wild mice prone to the development of lymphoma and paralysis. J Natl Cancer Inst 1979, 62: 63–70
22. Gardner MB, Henderson BE, Estes JD, Rongey RW, Casagrande J, Pike M, Huebner RJ: The epidemiology and virology of C-type virus-associated hematological cancers and related diseases in wild mice. Cancer Res 1976, 36:574–581
23. Blankenhorn EP, Gardner MB, Estes JD: Immunogenetics of a thymus antigen in lymphoma-prone and lymphoma-resistant colonies of wild mice. J Natl Cancer Inst 1975, 54:665–672
24. Gardner MB, Klement V, Henderson BE, Casagrande J, Bryant ML, Dougherty MF, Estes JD: Lymphoma, paralysis and oncornaviruses of wild mice, Tumours of Early Life in Man and Animals. Edited by L Severi. Monteluce, Italy, Perugia University Press, 1978, pp 343–356
25. Gardner MB, Klement V, Rasheed S, Estes JD, Rongey RW, Dougherty MF, Bryant ML: Persistent MuLV infection in wild mice, Persistent Viruses. Edited by JG Stevens, GJ Todaro, CF Fox. New York, Academic Press, 1978, pp 115–132
26. Oldstone MBA, Lampert PW, Lee S, Dixon FJ: Pathogenesis of the slow disease of the central nervous

system associated with WM 1504E virus: I. Relationship of strain susceptibility and replication to disease. Am J Pathol 1977, 88:193-212
27. Gardner MB, Rasheed S, Pal BK, Estes JD, O'Brien SJ: Akvr-1, a dominant murine leukemia virus restriction gene, is polymorphic in leukemia-prone wild mice. Proc Natl Acad Sci USA 1980, 77:531-535
28. Böyum A: Separation of leukocytes from blood and bone marrow. Scand J Clin Lab Invest 1968, 21 (Suppl 97):9-109
29. Metcalf D, Nossal GJV, Warner NL, Miller J, Mandel TE, Layton JE, Gutman GA: Growth of B-lymphocyte colonies in vitro. J Exp Med 1975, 142:1534-1549
30. Watson P: A slide centrifuge: An apparatus for concentrating cells in suspension onto a microscope slide. J Lab Clin Med 1966, 68:494-501
31. Möller G: Demonstration of mouse isoantigens at the cellular level by the fluorescent antibody technique. J Exp Med 1961, 114:415-434
32. Theilen GH, Kawakami TG, Rush JD, Munn RJ: Replication of cat leukaemia virus in cell suspension cultures. Nature 1969, 222:589-590
33. Taylor CE, Burns J: The demonstration of plasma cells and other immunoglobulin containing cells in formalin fixed paraffin embedded tissues using peroxidase-labeled antibody. J Clin Pathol 1974, 27:14-20
34. Gronowicz E, Coutinho A, Melchers F: A plaque assay for all cells secreting Ig of a given type or class. Eur J Immunol 1976, 6:588-590
35. Cantor H, Boyse EA: Functional subclasses of T lymphocytes bearing different Ly antigens: I. The generation of functionally distinct T-cell sublclasses in a differentiation process independent of antigen. J Exp Med 1975, 141:1376-1389
36. Trowbridge I: Interspecies spleen-myeloma hybrid producing monoclonal antibodies against mouse lymphocyte surface glycoprotein, T200. J Exp Med 1978, 148: 313-323
37. Yam LT, Li CY, Crosby WH: Cytochemical identification of monocytes and granulocytes. Am J Clin Pathol 1971, 55:283-290
38. Kulenkampff J, Janossy G, Graves MF: Acid esterase in human lymphoid cells and leukaemic blasts: A marker for T lymphocytes. Brit J Haematol 1977, 36: 231-240
39. Kaplow LS: Simplified myeloperoxidase stain using benzidine dihydrochloride. Blood 1965, 26:215-219
40. Sheehan HL: The staining of leucocyte granules by Sudan black B. J Pathol Bacteriol 1939, 49:580-581
41. Ralph PH: The histochemical demonstration of hemoglobin in blood cells and tissue smears. Stain Technol 1941, 16:105-106
42. Klein G, Ohno S, Rosenberg N, Wiener F, Spra J, Baltimore D: Cytogenic studies in Abelson-virus-induced mouse leukemias. Int J Cancer 1980, 25:805-811
43. Rasheed S, Gardner MB, Rongey RW, Nelson-Rees WA, Arnstein P: Human bladder carcinoma: Characterization of two new tumor cell lines and search for tumor virus. J Natl Cancer Inst 1977, 58:881-890
44. Oren R, Farnham AE, Saito K, Milofsky E, Karnovsky ML: Metabolic patterns in three types of phagocytic cells. J Cell Biol 1963, 17:487-501
45. Greenwood MF, Coleman MS, Hutton JJ, Lampkin B, Krill C, Bollum FJ, Holland PJ: Terminal transferase distribution in myelocytes and hematopoietic cells. J Clin Invest 1977, 59:889-901
46. Scott J, Pal BK, Rasheed S, Gardner MB: Natural killer cell activity in a population of leukemia-prone wild mice (Mus musculus). Int J Cancer (In press)
47. Gardner MB, Henderson BE, Estes JD, Menck H, Parker JC, Huebner RJ: Unusually high incidence of spontaneous lymphoma in wild house mice. J Natl Cancer Inst 1973, 50:1571-1579
48. Raschke WC, Coffman RL: Heteroantigens of the B cell series, Differentiation Antigens of Lymphocytes. Edited by N Warner. New York, Academic Press (In press)
49. Parker JW, Taylor CR, Pattengale PK, Royston I, Tindle BH, Cain MJ, Lukes RJ: Morphologic and cytochemical comparison of human lymphoblastoid T-cell and B-cell lines: Light and electron microscopy. J Natl Cancer Inst 1978, 60:59-68
50. Ralph P: Functional subsets of murine and human B lymphocyte cell lines. Immunol Rev 1979, 48:107-121
51. Baltimore D, Rosenberg N, Witte ON: Transformation of immature lymphoid cells by Abelson murine leukemia virus. Immunol Rev 1979, 48:3-22
52. Ralph P, Nakoinz I, Raschke WC: Lymphosarcoma cell growth is selectively inhibited by B lymphocyte mitogens: LPS, Dextran Sulfate and PPD. Biochem Biophys Res Commun 1974, 61:1268-1275
53. Ralph P, Nakoinz I: Direct toxic effects of immunopotentiators on monocytic, myelomonocytic and histiocytic or macrophage tumor cells in culture. Cancer Res 1977, 37:546-550
54. Koren HS, Handwerger BS, Wunderlich JR: Identification of macrophage-like characteristics in a cultured murine tumor line. J Immunol 1975, 114:894-897
55. Raschke WC, Baird S, Ralph P, Nakoinz I: Functional macrophage cell lines transformed by Abelson leukemia virus. Cell 1978, 15:261-267
56. Abelson HT, Rabstein LS: Lymphosarcoma: Virus-induced thymic-independent disease in mice. Cancer Res 1970, 30:2213-2222
57. Abelson HT, Rabstein LS: Influence of prednisone on Moloney leukemogenic virus in Balb/c mice. Cancer Res 1970, 30:2208-2212
58. Klein G: Lymphoma development in mice and humans: Diversity of initiation is followed by convergent cytogenic evolution. Proc Natl Acad Sci USA 1979, 76: 2442-2446
59. Weiner F, Babonitis M, Spira J, Bregula U, Klein G, Merwin RM, Asofsky R, Lynes M, Haughton G: Chromosome 15 trisomy in spontaneous and carcinogen-induced lymphomas of B cell origin. Int J Cancer 1981, 27:51-58
60. Marks PA, Rifkind RA: Erythroleukemic differentiation. Annu Rev Biochem 1978, 47:419-448
61. Chess L, Levine H, MacDermott RP, Schlossman SF: Immunoglobulin functions of isolated human lymphocyte subpopulations: VI. Further characterization of the surface Ig negative E rosette negative (null cell) subset. J Immunol 1975, 115:1483-1487
62. Kaplan J, Ravindranath Y, Peterson Jr WD: T and B lymphocyte antigen-positive null cell leukemia. Blood 1977, 49:371-378
63. Brouet JC, Preud'homme JL, Penit C, Valensi F, Rouget P, Seligmann M: Acute lymphoblastic leukemia with pre-B-cell characteristics. Blood 1979, 54: 269-273
64. Foon KA, Billing RJ, Terasaki PI, Cline MJ: Immunologic classification of acute lymphoblastic leukemia. Implications for normal lymphoid differentiation. Blood 1980, 56:1120-1126

Acknowledgments

The authors thank Dr. Ian Trowbridge, Salk Institute, La Jolla, California, for assays of LC lymphoid cells with T-200 monoclonal antibody, Dr. Robert Lukes, USC School of Medicine, for consultation, and Dr. Stuart Aaronson, National Cancer Institute, Bethesda, Maryland, for performing the RIA for cytoplasmic Ig. We also thank Ann Dawson for preparation of the manuscript.