AGE-RELATED DIFFERENCES IN SURVIVAL OF AKR/J MICE TREATED

WITH ANTI-LYMPHOCYTE GLOBULINS, ANTI-THYMOCYTE GLOBULINS,

AND RABBIT ANTI-MOUSE BRAIN SERUM

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<u>Globulins</u>, <u>Anti-thymocyte</u> <u>Globulins</u>, <u>and</u> <u>Rabbit</u> <u>Anti-</u>
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AKR mice are known to carry a single stranded RNA
tumor virus from birth, resulting in a near 100% incidence
of leukemia after six months of age. This investigation
was designed to study the age-related differences in the
survival rates of 2- to 3- and 6- to 7-month-old AKR/J
mice after continuous treatment with anti-lymphocyte
globulins (ALG), anti-thymocyte globulins (ATG), or rabbit
anti-mouse brain serum (RAMB). Splenic lymphocytes, which
were fractionated by affinity column chromatography, were
also investigated for their effect on survival rates.
Binding of ALG, ATG, and RAMB antibodies to lymphocytes
was confirmed by fluorescent antibody, radioisotope, and
autoradiographic studies. Cells isolated from the spleen,
thymus, and lymph nodes of both 2- to 3- and 6- to 7-month-
old preleukemic mice were used. Cytotoxicity of each
antibody preparation was ascertained by trypan blue
exclusion testing for cell viability <u>in</u> <u>vitro</u>. Leukemia
was confirmed by white cell counts of peripheral blood

as well as by determination of thymic and splenic weight and tissue histopathology.

Survival rates, statistically analyzed by the chi-square test, revealed significant differences between the treated and control animals. Anti-thymocyte globulins were shown to increase the survival rate of AKR/J mice if administered at 2 to 3 months of age and continued during the lifetime of the animals. Delay of ATG treatment until 6 to 7 months of age (after the appearance of transformed cells, but before leukemic symptoms were detected) lowered the survival rate during the first 21 days of treatment (7 to 8 months old).

The results of anti-lymphocyte globulin treatments on 2- to 3- and 6- to 7-month-old AKR/J mice were exactly the opposite of those observed with ATG. Treatments initiated at 2 to 3 months of age decreased the survival rate during the leukemic stage in later life. In contrast, ALG treatment of 6- to 7-month-old mice increased the survival rate.

Rabbit anti-mouse brain sera decreased the survival rate in both 2- to 3-month-old and 6- to 7-month-old AKR/J mice. The survival of 6- to 7-month-old animals treated with spleen cells from 2- to 3-month-old donors was not appreciably greater than that of the controls.

An analysis of radioisotope-labeled antibody data indicated that greater amounts of ALG bound cells present in the lymphoid organs of 6- to 7-month-old mice than those of 2 to 3 months' age. Conversely, greater concentrations of ATG bound lymphocytes were present in lymphoid organs of 2- to 3-month-old mice than in the older animals.

Results of these experiments suggest that differences in the survival rates of various treated groups of mice can be attributed to either specific binding or cell destruction by antibody. Evidence which supports this view includes the observation of both ATG and ALG binding surfaces of lymphocytes in organs of 2- to 3- and 6- to 7-month-old AKR/J mice. Observations made by fluorescent antibody and autoradiographic techniques, as well as by trypan blue staining for cell viability, were indicative that both anti-globulins were cytotoxic to lymphocytes in these organs. Other modes of antibody activity which could explain these results include the chemical modification of cell surface receptors and the direct antibody influence on the production or action of the murine leukemia virus.

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CHAPTER I

INTRODUCTION

Nearly a 100% probability exists that the AKR strain

of mice will spontaneously develop a lymphoid leukemia

after six months of age. These leukemias are virtually

all of the T-lymphocyte type and originate in the thymus

or from cells derived from the thymus (56).

Gross first isolated murine leukemia virus from AKR

mice in 1951 (28). Virus production has been shown in

newborn mice, reaching a plateau in very early life (44).

Titers do not usually increase until the onset of leukemia

at 6 to 7 months of age. Although AKR mice are infected

with the virus <u>in utero</u> (the virus is vertically trans-

mitted to offspring), manifestations of the disease are

not apparent at an early age. Thus, onset of leukemia

appears to be related to age.

Neonatal thymectomy has been shown to reduce the

incidence of leukemia in the AKR strain (82). Therefore,

intrathymic events during the latent period prior to

6 months of age appear to be of prime importance in the

formation of thymoma and development of lymphatic leukemia.

A current theory suggests that differentiation of thymo-

cytes within the thymus results in the production of immunocompetent, thymus-derived lymphocytes (T-lymphocytes). The majority of thymocytes are found in the cortical regions of the thymus and have large amounts of Thy-1 antigen and lesser amounts of H-2 antigen on the cell surface (61, 130). A smaller, more differentiated population of thymocytes is found primarily in the medullary regions. These more mature thymocytes possess smaller amounts of Thy-1 and larger amounts of H-2 surface antigens.

Waksal et al. (124) have investigated the changes in thymocyte surface antigens in both the AKR and C3H mice. In contrast to the C3H mouse, which is unaffected by the virus, the 6- to 8-week-old AKR mouse demonstrates a great amount of Thy-1 antigen on thymocyte surfaces. At 16 weeks of age, however, AKR thymocytes begin to show a progressive reduction in Thy-1 that continues into the thymoma stage at 24 weeks. A similar reduction in Thy-1 is not demonstrable in C3H mice during this 16 to 24 week period. Both C3H and AKR thymocytes bear low concentrations of H-2 antigen at 6 to 8 weeks of age. However, by 16 weeks of age a significant increase occurs in the amount of H-2 antigen on AKR thymocytes, while in C3H thymocytes the increase is only slight. Filppi, Rheins, and St. Pierre (18) reported similar surface antigen decreases

when thymocytes from older AKR mice were exposed to rabbit antibodies against mouse brain (RAMB). Thymocytes from younger mice were considerably more sensitive to RAMB sera. One hypothesis put forth to account for the increased maturation of AKR thymocytes is that a basic defect exists in the thymus reticuloepithelium, its products, or both. Such a defect is thought to trigger a viral oncogene present in the precursor cells (48).

Specific antisera have been produced against cell surface antigens which appear at different times in the lifespan of the mouse (5, 110). The function and significance of cell populations with antigens that appear at various ages can be studied with these antisera. To date, a relationship between relative changes in cell surface antigens observed at 4 to 6 months of age and the triggering of leukemic symptoms has still not been shown. It is also not clear whether these changes in cell surface antigens represent changes on individual cells or changes in the proportion of cell types present at different ages.

This study was initiated to evaluate the effects of anti-lymphocyte globulin (ALG), anti-thymocyte globulin (ATG), and rabbit anti-mouse brain (RAMB) serum on the survival of AKR mice with disseminated leukemia. Emphasis was placed upon the response to these antibodies in groups of animals during late preleukemic stages. Lilly,

Duran-Reynals, and Rowe (56) have shown that AKR mice lack the H-2^d gene which is responsible for controlling immunologic rejection of leukemic cells. However, no evidence has been provided to explain why symptoms of the disease are not manifested before the age of six months. Results of such studies suggested that direct manipulation of cells of the immune system with specific antisera, in vivo, might reveal age-related differences in the effect of various treatments utilized. The resultant effects of these procedures might be manifested by a change in the ability to immunologically delay the onset of disease.

CHAPTER II

REVIEW OF THE LITERATURE

AKR Mice

The AKR strain of mice harbors the Gross murine leukemia virus (GMuLV). This virus is transmitted vertically, and usually results in death of the animals from lymphoma between 6 and 12 months of age. During much of the latent period, the morphology of the lymphoid tissues remains normal (19). The incidence of lymphoma in infected animals is high; the disease originates in the thymus but is disseminated to lymphoid and other tissues in its later stages (69). Accompanying changes in the immune status of AKR mice during the onset of this desease have been reported by various workers. These are summarized as follows: 1. Cellular immunity as measured by skin graft survival in Gross virus-carrying preleukemic AKR mice is depressed (14). 2. No impairment of humoral immune response has been demonstrated during the induction phase of the disease (69). 3. Wettstein and Hays (126) reported low gamma-globulin levels in 12-month-old AKR mice with lymphoma, although no specific

alteration in the immune response to sheep red blood cells (SRBC) was noted. 4. No inhibition of antigen stimulated macrophages can be demonstrated in preleukemic AKR mice by the macrophage migration inhibition (MMI) test. Production of MMI factor by lymphocytes normally inhibits macrophage migration. Lack of inhibition in preleukemic AKR mice is thus a highly significant finding since MMI has been shown to be a correlate of delayed hypersensitivity or cellular immunity (12, 22).

5. Nagaya (81) has shown that the development of leukemia can be delayed for 5 months in 5- to 6-month-old AKR mice receiving thymus grafts from 1-month-old AKR donors. This finding indicates that a latent period is required by the reticular epithelial cells of the thymus grafts themselves rather than by the lymphoid cells of the host migrating from bone marrow to the thymus. During this latent period epithelial cells mature and eventually generate hormones that have a maturation effect on thymic lymphocytes.

6. Hargis and Malkiel (33) reported that mice of the AKR/J strain are capable of participating in both immediate and delayed types of hypersensitivity reactions. The graft versus host reaction (GVH) was used as an index for delayed hypersensitivity or cellular

immunity and anaphylaxis as a measure of immediate hypersensitivity. 7. Martig and Tribble (65) also found AKR mice competent in the delayed hypersensitivity reactions using the GVH reaction. Normal responses to allogeneic stimulation in a mixed lymphocyte reaction were reported as well as development of contact sensitivity to picryl chloride. Sensitivity to picryl chloride has been used as one measure of the delayed type of cellular immunity. It was concluded that mice of this strain which are highly susceptible to leukemia have a competent cell-mediated immune system and that no depression of immunity occurs during the preleukemic period. 8. Zata (130), in 1975, studied the expression of Thy 1.1 antigen and the responses of leukemic and preleukemic T-cell subpopulations to the mitogens phytohemagglutinin (PHA) and concanavalin A (Con A). The ability of intravenous injection of T-cells to return to lymph nodes (homing) was also investigated. He attributed the progressive temporal loss of Con A responsiveness in the preleukemic thymus to either inactivation of depletion of Con A-reactive cells by murine leukemia virus (MuLV) infection. The progressive decline in Con A

responsiveness with age correlated with increased

MuLV titers in both spleen and thymus. The response

of spleen cells to phytohemagglutinin was diminished

in older animals, but was not further affected by

leukemogenesis. 9. Several other studies have

indicated that the cellular immune response in AKR

mice is impaired. For example, female mice have

longer rejection times of syngeneic male skin

grafts than mice of the C57BL strain (14). The

latter strain rejected all of the grafts within

40 days, while only 2 of 16 grafts had been rejected

by the AKR animals at 55 and 77 days. 10. Evidence

of normal cellular immune reactivity in the AKR

mouse is demonstrated by the rejection of allografts

of lymphoma cells from H-2 compatible mice (36).

The capacity of AKR mice to reject allogenic

skin grafts was also found to be intact. AKR

(H-2^k) mice were grafted with C3H/HeJ (H-2^k) and

C57BL (H-2^b) skin from animals of the same sex.

The rejection of both types of graft appeared

at 18 days and was completed at 20 days. Hargis

and Malkiel (33) also demonstrated the immunocompetence

of AKR lymphocytes in both immediate and delayed

hypersensitivity reactions.

The apparent disparity in assessment of cellular immune reactivity in the AKR mouse described may be the result of differences in assay conditions and possibly differences in subpopulations of cells involved in such reactions. Cellular immunity has been shown to be depressed, as noted in aforementioned items no. 1, 4, 8, and 9 by skin graft survival (14), lack of inhibition of migration by the MMI test (12,22), progressive loss of Con A responsiveness (130), and longer rejection times of syngeneic AKR/J male skin by AKR/J females than mice of the C57/BL strain (14). Cellular immunity has been demonstrated to be normal as cited in aforementioned items no. 6, 7, and 10, when measured by the graft-versus-host reaction (GVH) of Hargis and Malkiel (33), responses to allogeneic stimulation in a mixed lymphocyte reaction and contact sensitivity to picryl chloride (65), and rejection by AKR mice of lymphoma allografts from H-2 compatible mice (36).

Although it was reported in item no. 2 above that no impairment of the humoral response can be demonstrated during the induction phase of the disease (69), suppression of gamma-globulin levels in later stages (126) may occur, as detailed in item no. 3 above. These statements are

not contradictory because transformation and proliferation
of a particular T-cell may result in suppression of
humoral antibody production directed toward one or
any group of antigens as the disease progresses.

Viral Antigens

Gross Murine Leukemia Virus (GMuLV) contains gs
antigens. These antigens are internal in the virion and
are shared by all C-type RNA tumor viruses (86). The
gs-1 antigen is species specific, and the gs-3 is
interspecies specific. Viral gs-1 is found in embryonic,
normal and tumor tissue. Natural antibodies to these
antigens were not found in several mouse strains tested by
Old et al. (86), suggesting that they induce immunologic
tolerance. The type-specific envelope antigens of GMuLV
are weakly antigenic, although antibodies to them are not
found in the serum of AKR mice.

Cell Surface Antigens

Infection with GMuLV in the newborn AKR results in
the development of antigens on the surface of cells
directed by, but not a part of Gross Murine Leukemia Virus
(GMuLV). Such antigens are found on normal lymphoid cells,
as well as on lymphoma cells. One example of this is the
GCSA antigen (86). A second antigen, the GSA, is also
found in serum and tissue extracts of normal and leukemic

AKR mice (86), and could represent exfoliated GCSA.
Both of these antigens are present from birth, and since
GMuLV infection is not cytodestructive in mouse lymphoid
tissue, cells with GCSA can be considered to be continuous-
ly producing virus. Such antigens on lymphoma cells of
AKR mice will produce antibodies in allogeneic mice
which are cytotoxic for cells from lymphomas induced by
GMuLV (36). These antisera are also able to induce
specific transplantation resistance to syngeneic lymphoma
cells. Virus neutralizing antisera are not produced by
cellular immunization experiments in mice (86). It has
been shown, however, that repeated immunization of AKR
mice with syngeneic tumor cells can produce cytotoxic
antibodies reactive with the immunizing cells, and that
inoculation of allogeneic Gross virus-induced lymphoma
cells can improve survival of AKR mice challenged with
small doses of syngeneic lymphoma cells (39, 40).

Additional evidence for an immune response to the GCSA
is shown by the finding of immune complexes in the glomeruli
of AKR mice which contain immunoglobulins specific for
the GCSA (87). Therefore, AKR mice respond immunologically
to GMuLV but do not produce significant quantities of
virus neutralizing antibodies. Another GMuLV-related
cell surface antigen which is present on lymphoid cells of
normal mice is called G_{IX} (39). This antigen is

demonstrable in AKR thymocytes, as well as in all other lymphoid tissues and lymphoma cells. The G_{IX} antigen is related to GMuLV infection in that it appears _de_ _novo_ in cells that become productively infected with GMuLV. It is not clear yet whether G_{IX} is directed by a viral or cellular genome.

Induction of Immune Responses to Viral Antigens in AKR Mice

Several methods have resulted in an enhancement of the immune response of AKR mice to antigens present on lymphoma cells. Injection of AKR mice with cells treated with neuraminidase to enhance their antigenicity decreased the incidence of lymphoma and prolonged the latent period (40,44). Some lymphomatous AKR mice given neuraminidase-treated leukemic thymocytes after a course of chemotherapy, survived without evidence of disease. Pretreatment of AKR mice with BCG (Bacille Calmette-Guerin or tubercle bacillus) vaccine reduced the mortality from lymphoma induced by low doses of cells from a long transplanted syngeneic line (56). Doses of BCG given to 2.5-month-old mice, followed in 12 days by an injection of 2000 syngeneic lymphoma cells, also lowered the incidence and prolonged the latent period to spontaneous lymphoma development. A chronic infection with the protozoan, _Besnoitia_ _jellisoni_,

recently has been shown to delay the onset of spontaneous leukemia in AKR mice (84). These animals generally have a high natural resistance to infection by this protozoan.

Anti-lymphocyte Serum, Anti-thymocyte Serum, and Rabbit Anti-mouse Brain Serum

Thymus specificity of anti-lymphocyte serum (ALS) and anti-thymocyte serum (ATS) has been demonstrated in-vivo. ALS inhibits mitosis of thymus-derived cells in the spleen and depletes thymus-dependent paracortical areas in the lymph nodes (123). Schlesinger and Yron (110) observed that ATS treatment reduced the number of antigen-ically distinct thymus-derived cells in the lymph nodes. These morphological changes are similar to those found in thymectomized animals, viz., paracortical lymphocyte depletion and permanent loss of antigenically distinct thymus-derived cells. Both ALS and ATS treatment and thymectomy suppress cell-mediated immunity; the effect of ALS treatment has thus been referred to as an 'immuno-logical thymectomy' (129). Denman et al. (13) and Martin and Miller (66) have advanced the popular belief that ALS and ATS exert their immunosuppressive effect by the elimination of long-lived, thymus-derived lymphocytes from the circulation.

In 1972, Bron and Sauser (5) demonstrated by absorptive

techniques that ATS binds to an antigen on the cell membrane different from theta, the antigen commonly used for the identification of thymus-derived T-lymphocytes. However, after removal of the theta-positive cells of a normal spleen lymphocyte population by pretreatment with anti-theta serum and complement, the absorptive capacity of the residual cells for anti-thymocyte cytotoxicity was lost. This suggests that the antigen detected by anti-thymocyte serum appears only on theta positive cells. Anti-thymocyte serum was shown to remove 30-40% of AKR spleen lymphocytes (5).

The Thy 1 or theta antigen found on the surface of murine thymocytes and thymus-derived lymphocytes exists in two allelic forms, Thy 1.1 and Thy 1.2 (98). The former is found on thymocytes and T-lymphocytes of the great majority of mouse strains, including C3H and BALB/c. Thy 1.1 and Thy 1.2 are also found in brain tissue of the appropriate strains. Antisera with anti-Thy 1.2 specificity may be prepared by immunizing AKR mice with C3H thymocytes. Antisera with anti-Thy 1.1 specificity can be prepared by immunizing C3H mice with AKR thymocytes. Such antisera have been used for in vitro enumeration or elimination of T-lymphocytes by complement-dependent cytolysis. In vitro, anti-Thy 1 serum has been reported to diminish antibody responsiveness to a variety of antigens (109); however,

little is known concerning the in vivo effectiveness of
anti-Thy 1 sera on humoral or cell-mediated immune
responses (96). Gelfand and Paul (21) have shown that
the effect of anti-Thy 1 serum on prolongation of skin
allograft survival in mice appears to be mediated through
some positive effect of thymocytes or peripheral T-
lymphocytes and not by elimination of cytotoxic effector
T-cells. This effect is transferable by thymus or spleen
cells from recipients treated with anti-Thy 1. The mode
of action of anti-Thy 1 serum is not yet clear; it may
function either through the activation of suppressor T-
cells or through the facilitation of the synthesis of
enhancing antibody (antibody coating and thereby protecting
tumor cells) (21).

Filppi, Rheins, and St. Pierre (18), using rabbit
anti-mouse brain serum (RAMB) to treat leukemic AKR mice,
have shown that there are fewer RAMB-susceptible lympho-
cytes present in leukemic and overtly leukemic animals
than in nonleukemic mice. Their data further indicate
that the in vivo activity of RAMB serum in C3H and
preleukemic AKR mice is directed primarily toward the
less mature T-lymphocyte population. Determinations of
theta antigen density using in vitro cytotoxicity tests
indicated that splenic and thymic T-lymphocytes remaining
in the RAMB-treated preleukemic AKR mice bore less theta
surface antigen than those from untreated control animals.

Age-related Changes in the Immune System

As the thymus gland degenerates with age, the immune system has been shown to undergo a series of degenerative changes (61, 61, 69, 126). These include: (1) a marked reduction in antibody response to thymic dependent antigens; (2) a decline in responsiveness of mouse spleen cells to stimulation by mitogens such as phytohemagglutinin (PHA), concanavalin A (Con-A), pokeweed, and bacterial lipopolysaccharide; (3) reduced graft-versus-host reactions; (4) reduced cell-mediated lymphocytotoxic reactions; and (5) reduced ability to reject transplanted tumor cells. In particular, mixed lymphocyte culture reactions suggest that the cells of the recirculating lymphoid pools (T-2 cells) display a functional decline with age (60). It is significant to note that this same recirculating population of T-2 lymphocytes is probably the proliferating lymphoma population in the leukemic AKR mouse (18). Makinodan and Adler (61) suggest that the primary effect of aging of the immune system is a defect in T-cell proliferative capacity.

Current evidence concerning the effects of immunological aging on the AKR mouse favors the possibility of preleukemic changes in a particular thymic cell population at high risk for leukemic transformation (48). Surface markers and physical characteristics indicate that cells from 2-month-old and 6-month-old AKR thymus

glands belong to the same lineage and are predominantly cortical lymphocytes (60). Thymic cortex has been shown to be the predominant site of leukemia development in the AKR mouse as well as the site of increased MuLV-related antigens in 6-month-old mice (48).

Lymphoid cells in the thymic cortex are derived from cells that originate in the bone marrow. Under the influence of the thymic environment, the immigrant cells engage in intense mitotic activity. This activity is revealed by phenotypic changes in surface antigens that characterize thymic lymphocytes (48). Antigens G_{IX}^-, TL^-, LY^+, and Thy-1$^-$ are converted to G_{IX}^+, TL^+, LY^+, and Thy-1$^+$. Thymocytes from preleukemic 5- to 6-month-old AKR mice have been shown to have an altered pattern of cell surface antigens (39,48). Four MuLV-related antigens ($G_{IX}M$, GCSA, gp 70, and p30) on the cell surface are markedly increased on thymocytes of leukemic mice (48). Antigens of the H-2 complex and Thy-1 alloantigens show characteristic modifications as well with age and with the development of leukemia. Thymocytes in 2- to 3-month-old mice show a high Thy-1/ low H-2 phenotype, changing to a low Thy-1/high H-2 at six months of age (114).

Accessory (A) cells (macrophages) participate in a nonspecific manner in both B- and T-cell immune responses by phagocytizing opsonized particles and

"processing" the antigens so that they are recognized by T- or B-cells. Peritoneal A-cells from young and old AKR mice have been assessed for functional capacities. Activities of those from old mice were found to be equal to or better than those obtained from young mice by nitroblue tetrazolium testing for phagocytosis (61). Activity of hydrolytic enzymes increases in A-cells in older animals; however, their ability to initiate antibody responses in vivo and in vitro was unaffected by aging (61).

Representative B-cell populations in the spleen also do not seem to change appreciably. This finding was based on the number of cells bearing immunoglobulin receptors in younger versus older animals (56, 58, 61).

Properties of the two distinguishable T-cell populations are summarized in Table I.

Genetic Control of Gross Murine Leukemia Virus (GMuLV) Expression

In recent experiments by F. Lilly and W. Rowe (56) genetic studies were performed by crossing mice of the AKR, a high leukemia strain, with various low-leukemia, low-murine leukemia virus (MuLV) strains to determine how spontaneous leukemia and expression of MuLV is genetically controlled. The relationship between leukemia virus expression, H-2 type, and the occurrence of leukemia

TABLE I

PROPERTIES OF TWO T-CELL POPULATIONS

Property	T-1	T-2
1. Surface antigens		
TL (thymus leukemia)	TL +	TL-
θ (theta)	++*	+
MPLA (mouse peripheral lymphocyte antigen)	+ *	+
H-2	H-2+	H-2++
2. Peripheral tissue of highest concentration	spleen	lymph nodes, blood, thoracic duct
3. Recirculation	No	Yes
4. Sensitive to Cortisone	Yes	No
5. Sensitive to ALS in vivo	+ *	++++
6. Sensitive to ATS in vivo	No	Yes
7. Sensitive to RAMB in vivo	Yes	No
8. Removal by thoracic duct drainage	No	Yes
9. Effect of adult thymectomy	decrease in 2 to 6 weeks	decrease after 30 weeks
10. Migration in vivo	To spleen, then lymph node	To lymph node, then spleen
11. Helper cell function	Yes	No
12. Suppressor cell function	Yes	No

TABLE I (continued)

PROPERTIES OF TWO T-CELL POPULATIONS

Property	T-1	T-2
13. Present in high numbers in areas of tumor rejection	Yes	No
14. Alkaline Phosphatase staining on surface	Yes	No
15. Present in greatest number in leukemic mice as detected by RAMB serum	+ *	++++
16. Response to PHA	No	Yes
17. Response to ConA	Yes	Yes
18. Response to pokeweed mitogen (PWM)	No	Yes
19. Life Span	short (3-7 days)	long (months-years)
20. Origin in thymus	cortex	medulla
21. Radiation sensitive	Yes	No (resistant)
22. Role in graft-versus-host (GVH) mixed lymphocyte reaction	Precursor of cell inflicting injury	Amplifies T-1 activity

* The symbols +, ++, +++, ++++ denote degrees of positiveness.

was also examined in offspring of individual crosses.

It was found that the expression of infectious MuLV is governed by at least two types of genes, a pair of dominant Akv-1 and Akv-2 genes and a regulatory gene, Fv-1. The pair of dominant genes is referred to as the "inducibility" genes by Lilly and Rowe (56) because the presence of either gene permits the expression of the virus. The Fv-1 regulatory gene facilitates the spread of MuLV. The occurrence of spontaneous leukemia was also shown to be influenced by the same Akv-1 and Akv-2 dominant genes and an Rgv-1 gene, located within the complex H-2 region. The effect of the Rgv-1 gene was shown to be mediated by immune mechanisms. AKR, BALB/c, and BALB/c X AKR F_1 crosses were genetically examined and compared for MuLV expression and the incidence of leukemia. The results are summarized in Table II.

TABLE II

CHARACTERISTICS OF PARENTAL AND F_1 HYBRID MICE

OF THE CROSS, BALB/c X AKR[*]

Trait	AKR	(BALB/c X AKR) F_1	BALB/c
H-2 type	$H-2^k/H-2^k$	$H-2^k/H-2^d$	$H-2^d/H-2^d$
Fv-type	$Fv-1^n/Fv-1^n$	$Fv-1^n/Fv-1^b$	$Fv-1^b/Fv-1^b$
Akv-1 & Akv-2	Akv-1/Akv-1 Akv-2/Akv-2	Akv-1/- Akv-2/-	-/- -/-
Leukemia incidence	90%	4%	low
MuLV Expression	100%	83%	20-60% (low titer late in life)

*Lilly, Duran-Reynals & Rowe (56)

From their genetic crossing studies, Lilly and Rowe
(56) have formulated the following hypothetical scheme
for leukemogenesis in AKR mice. Because AKR mice carry
the Akv-1 and Akv-2 genes, they have access to the
information needed for the production of infectious MuLV.
However, since AKR mice lack the $Fv-1^b$ suppressor gene, the
production of infectious virus takes place with the virus
being expressed at a high titer. The virus appears to favor
the neoplastic transformation of lymphoid cells into leukemia
cells, although the exact mechanism for this activity has

yet to be determined. The H-2^d gene permits the animal to reject leukemia or potentially leukemic cells by immunologic means. This gene is lacking in AKR mice. Therefore, a high probability exists for the transformation of cells in AKR leukemic mice and extensive multiplication.

The BALB/c X AKR F_1 generation also possesses the complete viral information conferred by the Akv-1 and Akv-2 genes, but the Fv-1^b allele strongly suppresses the production of infectious virus. Also, the H-2^d allele prevents the progressive growth of leukemia cells that might occur at this low virus level. As a result, these F_1 mice show a very low incidence of leukemia.

Factors which tend to enhance the development of murine leukemia, therefore, are (1) virus production, favored by the Akv-1 and Akv-2 genes, (2) lack of the Fv-1^b gene, which suppresses production of virus, and (3) lack of the H-2^d gene, which prevents the progressive growth of leukemia cells (56). In contrast, factors which tend to suppress the development of leukemia are (1) lack of virus production, favored by absence of either or both of the Akv-1 and Akv-2 genes, (2) presence of one or both of the Fv-1^b genes, and (3) presence of the H-2^d gene (Table II).

CHAPTER III

MATERIALS AND METHODS

Animals

Male and female AKR/J mice were purchased from the Jackson Laboratory, Bar Harbor, Maine (Table III). All animals were maintained on Purina laboratory chow and were provided with water ad libitum. Mice of both sexes, ages 2 to 3 months or 6 to 7 months, were employed. Prior to their experimental use, differential white blood cell examinations and white blood cell counts were done on all mice for verification of absence of leukemia.

Leukocyte Counts and Differential Counts

Complete blood counts were performed on a Coulter Model S or F automatic electronic blood counter (Coulter Diagnostics, Hialeah, Florida). Mice were bled from the retro-orbital sinus of the eye with a heparinized capillary pipette; dilutions were made for electronic or manual cell counts. Mice with white blood counts in excess of $15,000/mm^3$ were considered to be either leukemic or unhealthy and were not used for experimental purposes. Peripheral blood smears were fixed in absolute methanol and stained in buffered Wright's stain for 4 minutes. Differential counts were performed by counting 100

TABLE III

Description of AKR/J Mice (26)

Origin: Fürth, 1928-36, high leukemic strain.

                         Random bred at Rockefeller Institute for

                         several generations, followed by 9 gen-

                         erations of inbreeding by Mrs. Rhoades and

                         21 generations by Dr. Lynch.

Source: Lynch to Jaxx in 1948 at F22

Characteristics: Very high incidence of leukemia by 6-8

                         months of age. The few non-leukemic

                         animals survive to 16-18 months of age.

Lymphatic Leukemia

Origin: Spontaneous. Spleen and nodes. AKR/J.

                         Jaxx 1954.

Transmission: Host AKR/J (100%). Gen. 607, 12/67.

                         7 days; kills host 2-4 weeks.

Appearance: Gross: white, flat plaque, often necrotic

                         in center.

                         Microscopic: tumor cells lymphocytic, uniform

                         in size, arranged in rows and sheets;

                         hemorrhagic and necrotic areas in center.

leukocytes and determining the percentage of each white blood cell type present. Mice with more than 50% polymorphonuclear white cells were suspected of being unhealthy and not suitable for experimentation.

Histological Studies

Spleen and thymus tissues were removed from leukemic mice for histological examination to confirm the presence of disease. Tissues were fixed in 10% formalin and dehydrated in increasing concentrations of ethyl alcohol. Clearing was performed in two changes of xylene, and tissues were infiltrated with "Paraplast Plus" embedding medium. After the tissues were embedded in paraffin and cooled, 5-μm sections were cut on a microtome. All sections were mounted on glass slides, dewaxed in xylene, and stained with hematoxylin and eosin. The weights of all organs were recorded prior to fixation.

Protein Electrophoresis

Protein electrophoresis of five pooled leukemic and nonleukemic sera was performed on cellulose acetate strips with a millipore protein electrophoresis chamber (Millipore Corporation, Bedford, Massachusetts). Samples were electrophoresed in a medium of barbital buffer, pH 8.6, and a current of approximately 8 milliamps per strip for

one hour. Serum proteins migrate from the cathode to the anode. After electrophoresis, cellulose acetate strips were fixed in 10% glacial acetic acid. Strips were stained in the same solution with 0.5% coomassie blue. Differentiation was performed in the same solution used for fixation and the density of individual bands was recorded on a densitometer (Helena Laboratories, Beaumont, Texas).

Anti-lymphocyte Globulins, Anti-thymocyte Globulins, and Rabbit Anti-mouse Brain Serum

Anti-lymphocyte globulins (ALG) and anti-thymocyte globulins (ATG) were purchased as lyophilized preparations from Microbiological Associates, Bethesda, Maryland. Anti-thymocyte globulins were developed in New Zealand white rabbits and directed against Swiss Webster (mouse) whole thymocytes. Anti-lymphocyte globulins were also developed in New Zealand white rabbits and directed against Swiss Webster whole lymphocytes from lymph nodes. Both ALG and ATG were pre-tested by the manufacturer for cytotoxicity and hemagglutination titers, and also for graft-versus-host reactions (Table IV). The ALG and ATG were reconstituted with 20-ml of sterile phosphate buffered saline (PBS), pH 7.2, on arrival, and 5-ml aliquots were dispensed into sterile serum bottles and frozen until needed.

Rabbit anti-mouse brain serum (anti-theta antigen) was prepared and adsorbed according to the methods of Fil-pi, Rheins, and St. Pierre (18) by immunization of New Zealand white rabbits with homogenized brain tissue. Brains from 10-week-old AKR/J mice were emulsified in 2.5-ml of Hank's balanced salt solution (HBSS), and 5-ml of this mixture was emulsified with an equal volume of Freund's complete adjuvant (Difco, Detroit, Michigan). The immunization schedule included three subdermal injections of 2.5-ml each in three different locations across the back, three weeks apart. The first injection included complete adjuvant; subsequent immunizations contained the incomplete form. The animals were bled by cardiac puncture four weeks after the last immunization. Occasionally, animals were given a booster injection and bled from the marginal ear vein seven days later, using a heparinized catheter and syringe. Rabbit anti-mouse brain sera were adsorbed with AKR/J liver, kidney, and red blood cells (18) and stored in 5-ml aliquots at -30° C.

TABLE IV

*ANTI-LYMPHOCYTE GLOBULIN AND ANTI-THYMOCYTE GLOBULIN

CYTOTOXICITY, HEMAGGLUTINATION, AND SKIN

GRAFT SURVIVAL TIME EVALUATIONS

	Cytotoxicity	Hemagglutination	Graft Survival Time (days)
Anti-lymphocyte Globulins Lot No. 13153	$1/800^+$	$1/50^+$	23.0
Anti-thymocyte Globulins Lot No. 15121	1/256	1/64	32.8

*Data collected and tests performed by Microbiological Associates, Bethesda, Maryland.

+Highest dilution of serum showing reactivity.

Latex Agglutination

A modification of the latex agglutination reaction
for mycoplasma (76) was used for titering ALG, ATG, and
RAMB sera. Latex (Difco, Detroit, Michigan), 0.81 um,
was diluted 1:5 in deionized water and stored at 4 to
10° C. Glycine-buffered saline (GBS), pH 8.2, was
prepared by dissolving 7.5 grams of glycine and 5.85
grams of sodium chloride (NaCl) in deionized distilled
water to a final volume of one liter. The solution was
adjusted to pH 8.2 by the addition of 1 N sodium
hydroxide.

A 10% (w/v) stock solution of bovine serum albumin
(BSA) was prepared in deionized water. Prior to
testing, 2-ml of the bovine serum albumin stock
solution were added to 98-ml of GBS (GBS-BSA). One-ml
of a suspension containing 5×10^7 of 2- to 3-month-old
splenic lymphocytes was added to 0.4-ml of a 1:5
latex suspension. After 15 minutes at room temperature,
8.6-ml of GBS-BSA (pH 8.2) mixture were added and the
preparation was allowed to stand for another 10 minutes.
Five-tenths-ml was added to each of several tubes
containing 0.5-ml amounts of serial two-fold dilutions of
ALG, ATG, or RAMB serum in GBS-BSA. The contents of the
tubes were thoroughly mixed on a vortex mixer and incubated

TABLE V

OUTLINE OF PROCEDURE FOR LATEX AGGLUTINATION

Add 5.0×10^7 spleen cells from 2- to 3-month old AKR/J
mice to 0.4-ml of 1:5 (0.81 micrometer) latex suspension

↓

Incubate 15 minutes at room temperature

↓

Add 8.6-ml of glycine buffered saline (pH 8.2)
containing 0.2% bovine serum albumin (GBS-BSA)

↓

Incubate 10 minutes at room temperature

↓

Add 0.5-ml to each of several test tubes containing 0.5-
ml amounts of serial two-fold dilutions of either anti-
lymphocyte globulins, anti-thymocyte globulins, or rabbit
anti-mouse brain serum in GBS-BSA

↓

Mix and incubate 2 hours in a 42°C water bath

↓

Centrifuge at 95 X g for 10 minutes and examine for
microscopic agglutination

for 2 hours in a 42° C water bath. Agglutination
titer endpoints were read microscopically (Table V).

Cytotoxicity and Trypan Blue Viability Testing

Cytotoxicity tests of ALG, ATG, and RAMB serum were
performed using equal volumes of cells (5×10^6/ml), a
serial dilution of antiserum in minimal essential medium,
pH 7.4 (Microbiological Associates, Bethesda, Md.), and
a 1:15 dilution of guinea pig complement (Difco, Detroit,
Michigan) (Table VI). Each tube was mixed and incubated
at 37° C for 45 minutes, followed by three washings and
the addition of 0.16% freshly prepared trypan blue in
0.85% saline. The percentage of non-viable cells in
each tube was estimated by counting the number of stained
cells for each 100 cells observed (94).

Injections of Globulins and Serum

Three groups of 6- to 7-month-old and 2- to 3-month-
old female mice each, containing 30 and 23 animals,
respectively, were injected intraperitoneally (i.p.) with
0.2-ml of anti-lymphocyte globulins (ALG), anti-thymocyte
globulins (ATG), or rabbit anti-mouse brain serum (RAMB)
(Table VII). Controls of each age group were injected
with 0.2-ml of phosphate buffered saline (PBS, pH 7.2).
All four groups of each age group were inspected at the
time of injections for symptoms of leukemia. Concentrations
of antisera were adjusted with PBS to levels that were

TABLE VI

PROCEDURE FOR CYTOTOXICITY AND TRYPAN

BLUE VIABILITY TESTING

Add 5.0 X 10^6 spleen cells in 0.5 ml of minimal essential

medium (MEM), pH 7.4, to tubes of serial two-fold dilutions

of ALG, ATG, or RAMB serum and 0.1 ml of 1:10 dilution of

guinea pig complement

$\downarrow$

Mix well by gentle agitation and incubate at 37°C for 45

minutes

$\downarrow$

Centrifuge for 10 minutes to obtain a pellet

$\downarrow$

Wash cells 3 times in fresh MEM,

centrifuging between washings

$\downarrow$

Add 0.16% freshly prepared trypan blue to the centrifuged

pellet in each tube and agitate gently

$\downarrow$

Count 100 lymphocytes in each tube and record the percent

of stained non-viable cells

TABLE VII

Outline of Procedure for AKR/J Mouse Passive Injections

Four groups of 30, 6- to 7-month-old AKR/J female retired breeders

Total = 120 mice

Four groups of 23, 2- to 3-month-old AKR/J female mice.

Total = 92 mice

Two groups of 40, 6- to 7-month-old AKR/J male retired breeders.

Total = 80 mice

Inject intraperitoneally (i.p.) twice weekly with 0.2-ml of either ALG, ATG, or RAMB serum (0.85% saline for controls) in a concentration cytotoxic in vitro for 20×10^6 2- to 3-month-old mouse spleen cells

Inject intravenously (i.v.) once per week with 0.2-milliliters of minimal essential medium (MEM) containing 20×10^6 ATG-sensitive cells from 2- to 3-month-old mice obtained by affinity chromatography. (Controls received 0.2-ml of MEM)

Inspect all mice on days of injections for physical symptoms of leukemia; confirm by leucocyte counts, peripheral blood smears, spleen and thymus weights, and histological organ sectioning. Record data and numbers of survivors.

cytotoxic for 2.0 X 10^7 splenic lymphocytes (approx-
imately 10%) as determined by trypan blue viability
testing in vitro. Blood from mice suspected of being
leukemic was then analyzed by differential counts of
peripheral smears and cell counts as previously described.
Histologic appearances of sectioned spleen and thymus
tissues, as well as demonstration of increased organ
weights, contributed to confirmation of leukemia.

Column Affinity Chromatography

Suspensions of spleen cells from 2- to 3-month-old
donors were teased into a uniform suspension in minimal
essential medium (MEM), pH 7.2 (Microbiological Associates,
Bethesda, Maryland), for fractionation on a cyanogen
bromide (CNBr) Sepharose 4B affinity column (Pharmacia
Fine Chemicals, Inc., Piscataway, N.J.). Two columns
were prepared for fractionation of T-cell populations
(Figure 1). The first column consisted of CNBr Sepharose
4B with bound (rabbit) anti-mouse IgG for removal of the
majority of B-lymphocytes. The method of Schlossman and
Hudson (111) was found to be most efficient. The effluent
cells from the column were then placed on a second column,
containing bound anti-lymphocyte globulins (ALG).
Repeated passage of cells through the ALG column yielded
an effluent cell population that was relatively free (less

AFFINITY COLUMN CHROMOTOGRAPHY OF
DONOR MOUSE SPLEENS
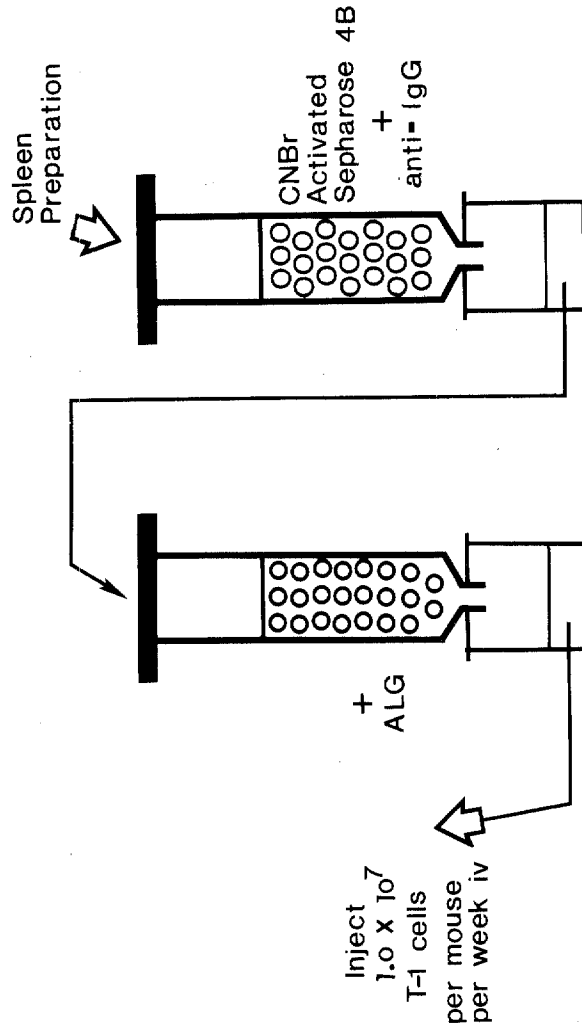
Fig. 1--Spleen cell suspensions in minimal essential media (MEM) from 2- to 3-month-old AKR/J mice were applied to columns of cyanogen bromide sepharose 4B with (rabbit) anti-mouse IgG (right), and anti-lymphocyte globulins (left). Effluent cells from the ALG column were injected into 6- to 7-month-old AKR/J mice, i.v., at a dosage of 1.0×10^7 cells per mouse each week.

than 5%) of ALG-sensitive lymphocytes for intravenous
injection into a group of forty 6 - to 7-month-old retired
breeders. Effluent cells were also tested for
reactivity with anti-thymocyte globulins (ATG), and a
population that was 80 to 85% ATG reactive was usually
obtained by only two passages through the column.
Retired breeders were injected once per week by the tail
vein with 10 X 10^6 viable cells. Controls were injected
at the same time with an equal volume (0.2 ml) of minimal
essential medium (MEM).

<div align="center">

Statistical Analysis for Comparing Sets

of Survival Pattern Data

</div>

Survival patterns of AKR/J mice treated with both
antisera and cells were analyzed using a ranked order
summary chi-square. In every case, treated groups of
animals were compared to the control group, and intervals
of one week were used for most comparisons. Intervals
of time other than one week were also analyzed in several
cases to determine if significant differences in survival
rates existed at selected intervals. The periods of most
interest were after 6 months of age, when symptoms
of leukemia were known to be manifested.

Radioimmunofluorescence and Coupling

Preleukemic 2- to 3-month-old and leukemic 7-month-old AKR/J mice were used for radioimmunofluorescence studies on thymus, spleen, and lymph node cells. Fluorescein isothiocyanate (FITC) was coupled to globulins in the antiserum by the technique of McCammon (67), with a resultant FITC-protein ratio of 1:20. The protein-FITC conjugates were then dialyzed against four changes of phosphate buffered saline (PBS), pH 7.2, and passed through a Sephadex G-25 column (Pharmacia, Uppsala, Sweden). Protein concentration at this point was approximately 0.9 mg/ml. The conjugate was then subsequently conjugated to I-125 according to the Warren and Dixon method (54). After I-125 coupling, all three double conjugates, ALG, ATG, and RAMB antiserum, were again dialyzed against four changes of distilled water at 4° C and passed through a Sephadex G-25 column.

Radioimmunofluorescence of Cells

Spleen, thymus, or cervical lymph node cells were suspended in minimal essential medium (MEM), pH 7.2, by teasing the respective organs with a needle and syringe. The cells were washed three times in MEM, resuspended in 10-ml, and counted on a hemocytometer.

Paired aliquots of approximately 10^7 lymphocytes were
pipetted into tubes for each antiserum or globulin (ALG,
ATG, or RAMB serum) and for each organ (spleen, thymus, or
lymph node). After centrifugation to obtain a pellet,
fluorescein-coupled antiserum in a concentration of
1 mg/ml was added in 0.1-ml portions to each tube. Tubes
were incubated for 30 minutes at 4° C, followed by
resuspension of cells and washing in phosphate buffered
saline (PBS), pH 7.4, five times to remove unbound
antibody. All tubes containing cell pellets were then
placed in a gamma counter for one minute to measure
radioactivity emitted. The same pellets of cells were
then smeared on coverslips for fluorescent antibody
analysis. The percent of total spleen, thymus, or lymph
node cells indicating fluorescence was determined
using a Leitz ultraviolet microscope equipped with
exciter filter II and barrier filters 50/44. Percentages
of fluorescing cells for each antiserum or globulins were
determined by counting five sets of 200 cells and recording
the mean values obtained.

Autoradiography

The same coverslips used for fluorescent antibody
observations were used for autoradiography. A Kodak
NTB-2 nuclear gel emulsion was used in combination with
Dektol developer as recommended by Neely and Combs (83).

The emulsion was first heated to 39° C and diluted 1:5

with distilled water as recommended by Kodak. Coverslips,

previously fixed in methanol, were then slowly immersed

into the well-mixed solution and carefully drained toward

one corner. Each coverslip was then placed in a plastic

"Coulter" cup (Coulter Diagnostics, Hialeah, Florida)

diagonally, and allowed to dry overnight in the dark

room with the lid removed from each cup. After drying,

cups were tightly wrapped in aluminum foil to prevent

light from entering and incubated for 5 to 6 days in a

refrigerator at 4° C. Following incubation, coverslips

could all be found firmly attached to the bottom of each

cup in the original position, and developer and fixatives

were added directly to each cup. This method was found to

be superior to individual handling of each coverslip with

forceps, resulting in very little coverslip breakage during

processing. After thorough washing, Wright's blood stain

was added to each cup, and coverslips were permanently

mounted on labeled glass slides with molten paraffin.

CHAPTER IV

RESULTS

Listed in Table VIII are the mean values and standard deviations obtained for complete blood counts (CBC) of 72 blood samples from 2- to 3-month-old preleukemic AKR/J mice. A complete blood count included white blood cell counts (WBC), red blood cell counts (RBC), hemoglobin (Hgb), hematocrit (Hct), mean cell volume (MCV), mean cell hemoglobin (MCH), and mean cell hemoglobin concentration (MCHC). Mean values and standard deviations were used to ascertain the health of each mouse and lack of leukemic symptoms prior to experimentation. Mean spleen and thymus weights of the same group of 2- to 3-month-old preleukemic AKR/J mice are also included in Table VIII. Mean white cell counts for leukemic mice are given in Table IX and their general distributions are depicted in Figure 2. No mice with leukocyte counts less than $40,000/mm^3$ were determined to be leukemic. Mean leukemic spleen and thymus weights are also given in Table IX, with distributions in Figure 3. Note both the high number of leukocytes as well as the increased weights of spleen and thymus glands of leukemic mice. Mice that appeared leukemic in terms of splenomegaly and/or enlargement of the thymus gland

50

TABLE VIII

MEAN BLOOD CELL MEASUREMENTS AND ORGAN WEIGHTS OF

2- TO 3-MONTH-OLD PRELEUKEMIC AKR/J MICE

Determination	Mean	Standard Deviation
White blood cell count (X 10^3)	7.54	3.53
Red blood cell count (X 10^6)	7.97	0.70
Hemoglobin concentration (gm/100 ml)	13.90	1.30
Hematocrit (%)	36.90	4.70
Mean cell volume (μ^3)[a]	47.00	3.70
Mean cell hemoglobin[b] concentration ($\mu\mu$g)	17.50	1.20
Mean cell hemoglobin percent[c] (%)	38.50	3.20
Spleen weight (gm)	0.12	0.02
Thymus weight (gm)	0.08	0.01

The abbreviations are: WBC, white blood cells; RBC, red blood cells; Hgb, hemoglobin per 100 ml of blood; Hct, hematocrit or percent packed cells; MCV, mean cell volume; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration.

[a] MCV in μ^3 = $\dfrac{\text{Hct (ml/100ml blood)}}{\text{RBC } (10^6/\text{mm}^3)}$

[b] MCH in $\mu\mu$g = $\dfrac{\text{Hgb (grams/1000 ml blood)}}{\text{RBC } (10^6/\text{mm}^3)}$

[c] MCHC in % = $\dfrac{\text{Hgb (grams/100 ml blood)}}{\text{Hct (ml/100 ml blood)}}$ X 100
(gm/100 ml)

LEUKEMIA WBC DISTRIBUTION
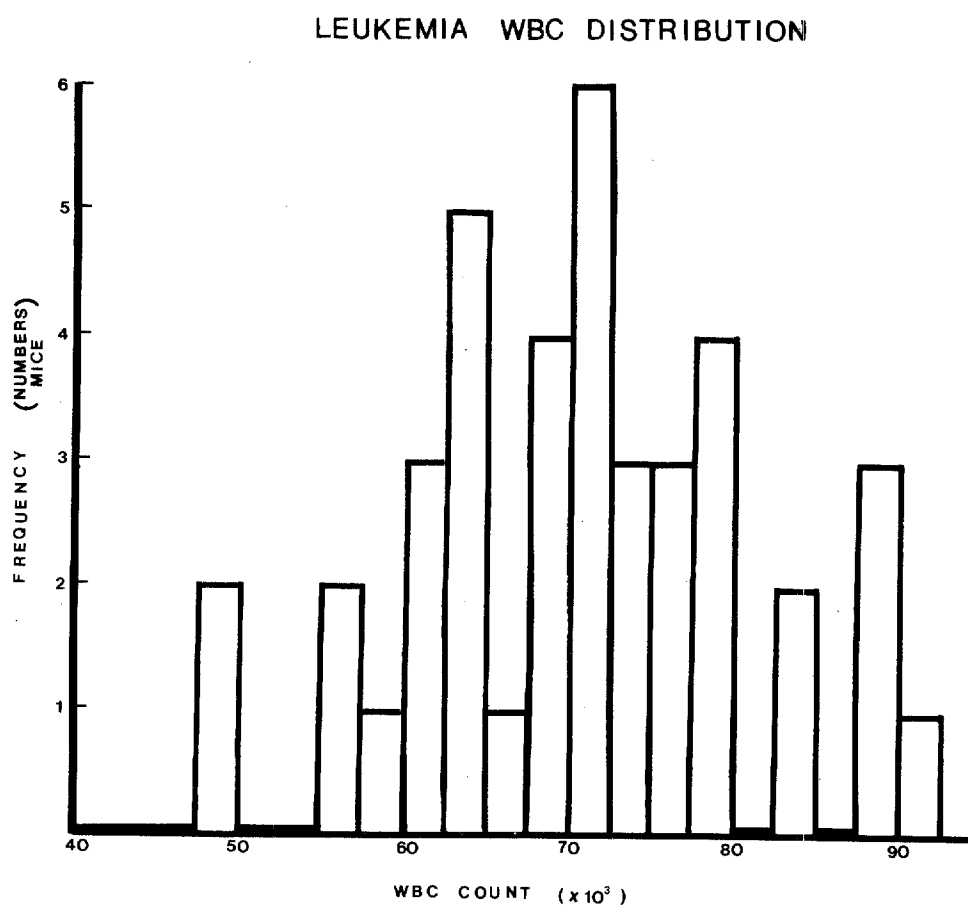
Fig. 2--Frequency of distribution of white blood
cell counts in leukemic AKR/J mice.
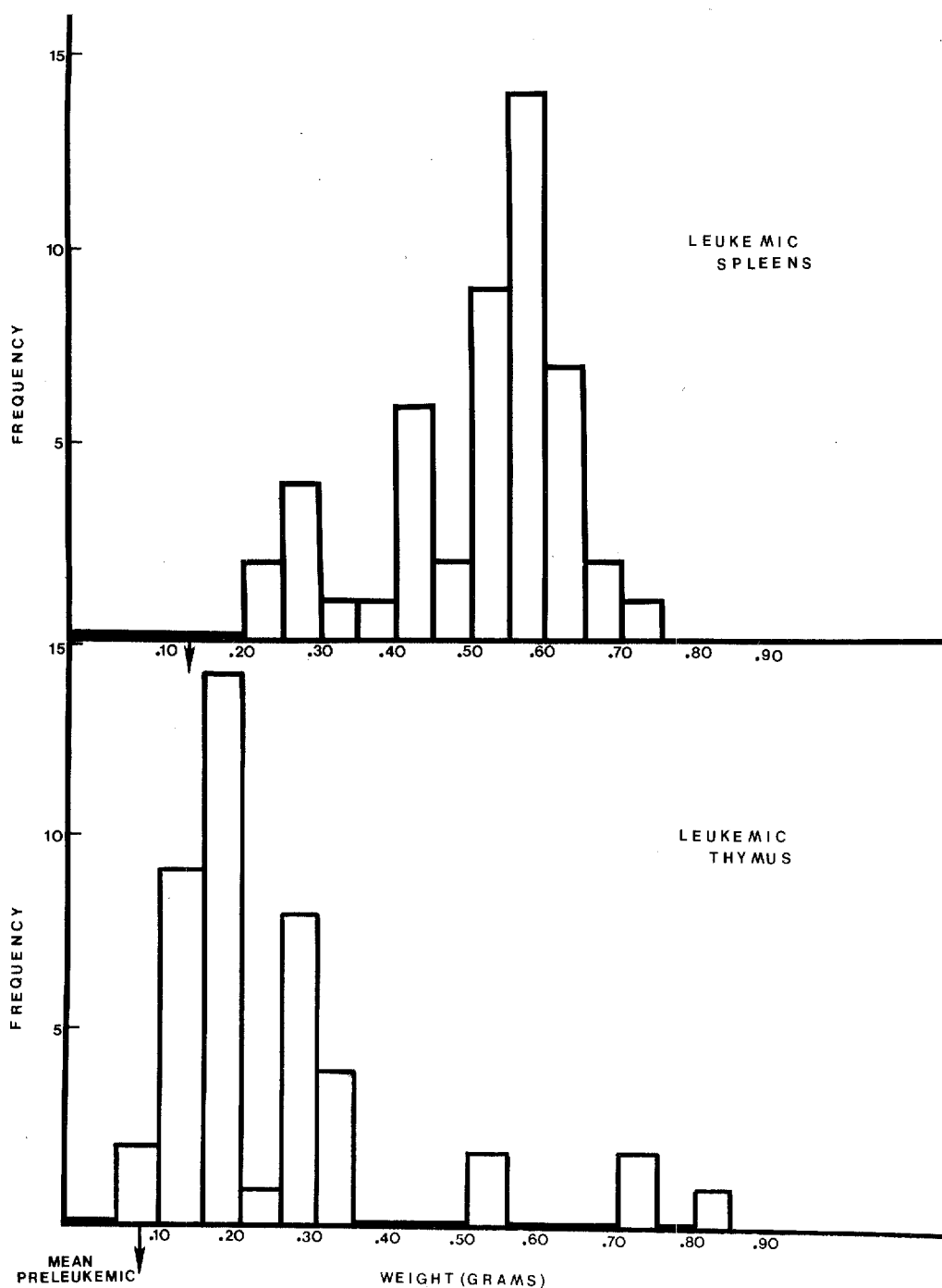
Fig. 3--Frequency of distribution of spleen and thymus weights of leukemic AKR/J mice. The arrow designates the mean weight of these organs in preleukemic mice. [a]Frequency in numbers of mice.

TABLE IX

MEAN WHITE BLOOD CELL COUNTS AND ORGAN

WEIGHTS OF LEUKEMIC AKR/J MICE

Determination	Mean	Standard Deviation*
White blood cells (X 10^3)	71.60	11.42
Spleen weight (gm)	0.51	0.12
Thymus weight (gm)	0.29	0.18

*Standard Deviation (S.D.) $= \sqrt{\dfrac{\sum (X_i - X)^2}{n - 1}}$

occasionally did not have a sufficient spillover of cells into the peripheral system to have greatly elevated leukocyte counts. These animals were suspected of having leukemia, but leukemia could not be confirmed until after death, when organs could be removed and examined.

Peripheral Blood Smears

Figures 4, 5, and 6 show some of the unusual features of cells in peripheral blood smears of AKR/J mice. In Figure 4A three lymphocytes are seen to be in contact with one another. Figure 4B shows four lymphocytes making contact. Cells in both Figures 4A and B appear morphologically to be normal peripheral lymphocytes, but the close association of cells is uncommon in non-leukemic strains. This phenomenon was commonly observed in 6- to 7-month-old preleukemic AKR/J mice and suggests recognition of receptor sites among groups of lymphocytes.

Figures 5A and B are photographs of smudge cells often observed in the peripheral blood of leukemic and late preleukemic mice. Both photographs shown are of blood smears from a leukemic animal. Figure 6A illustrates the process of cellular phagocytosis of nuclear material. Phagocytosis was often seen in blood smears from both preleukemic and leukemic mice, although the photograph shown is of a preleukemic smear. The mononuclear cell in Figure 6B morphologically appears to
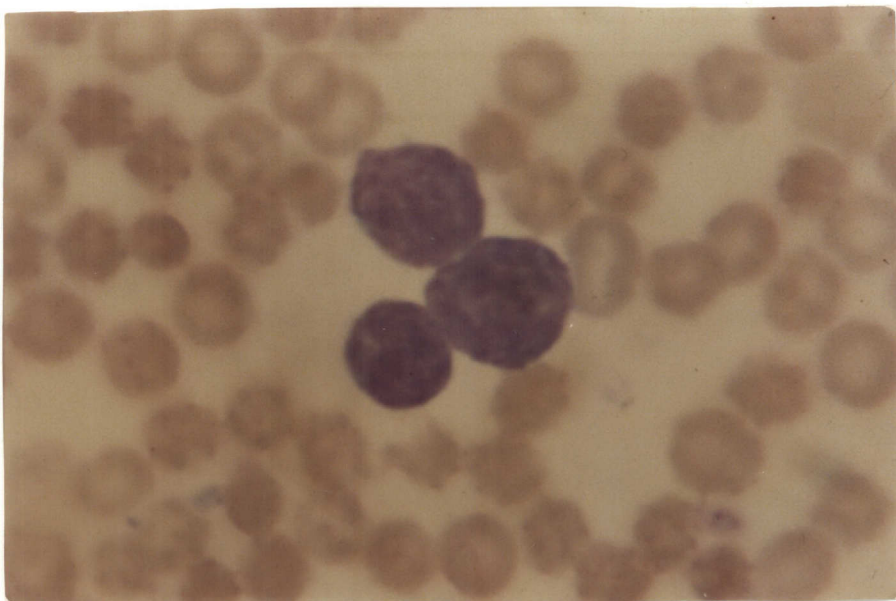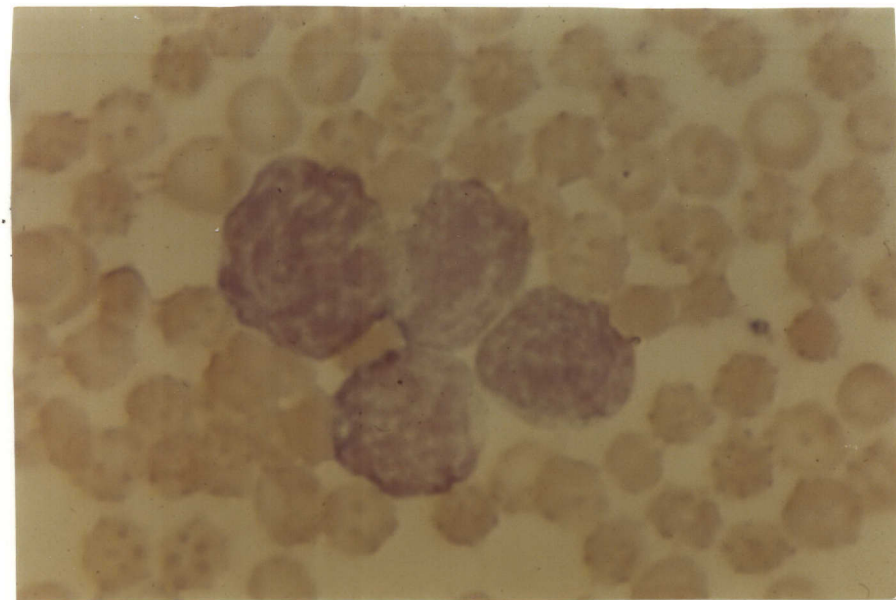
Figs. 4A and B—Wright's stain of peripheral blood from a 6- to 7-month-old preleukemic AKR/J mouse. Note cell to cell contact of peripheral lymphocytes in both A and B. Magnification 1000 X.
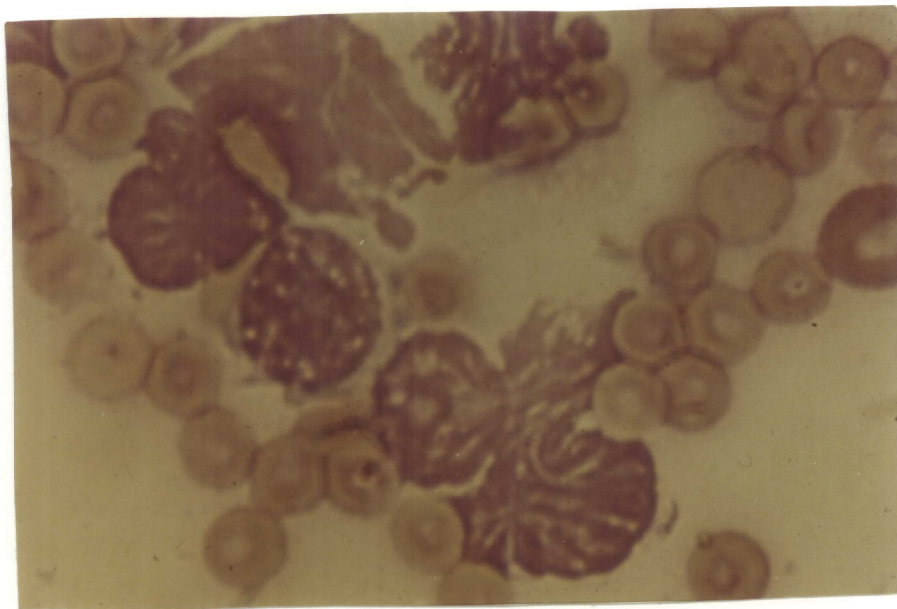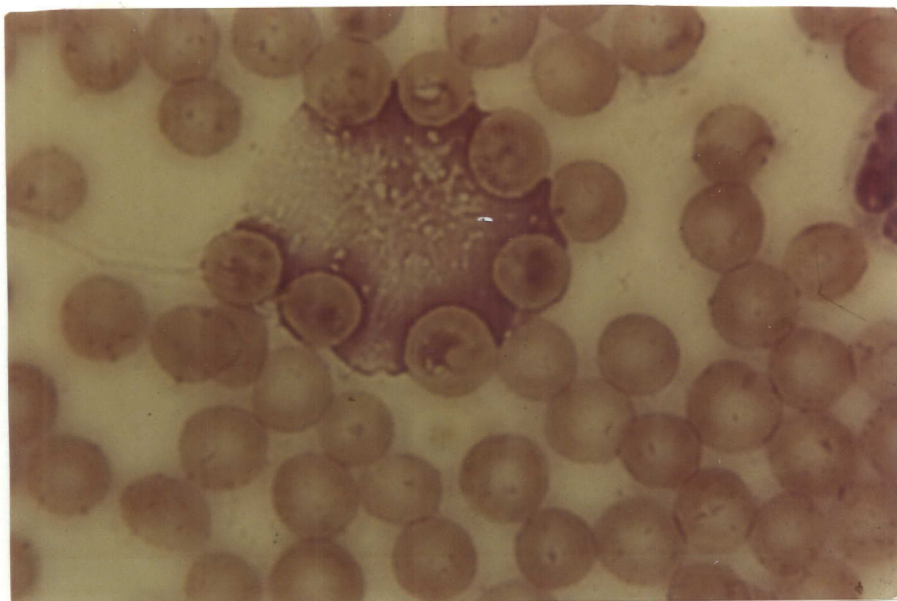
Figs. 5A and B—Wright's stain of peripheral blood from a 7- to 8-month-old leukemic AKR/J mouse. "Smudge" cells (disintegrated lymphocytes) can be noted in both leukemic smears. Magnification 1000 X.
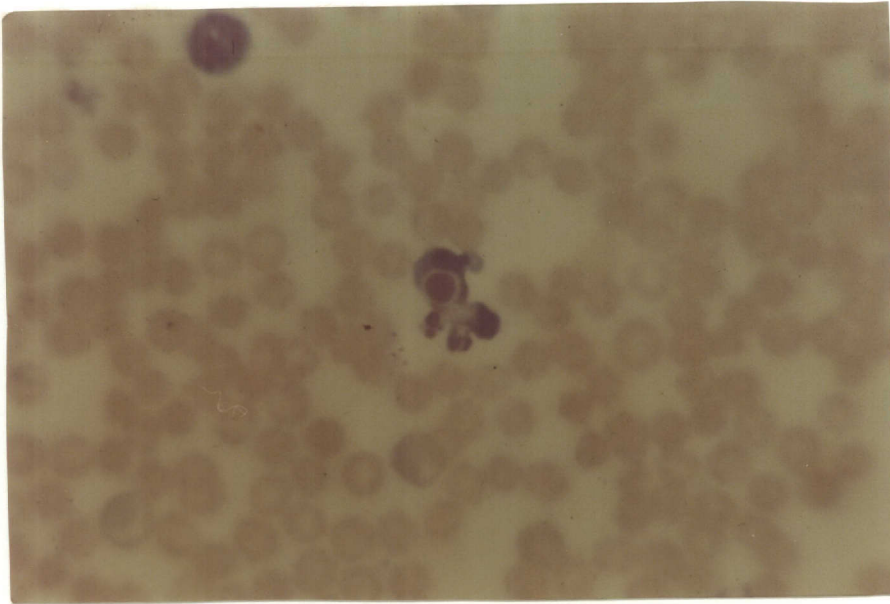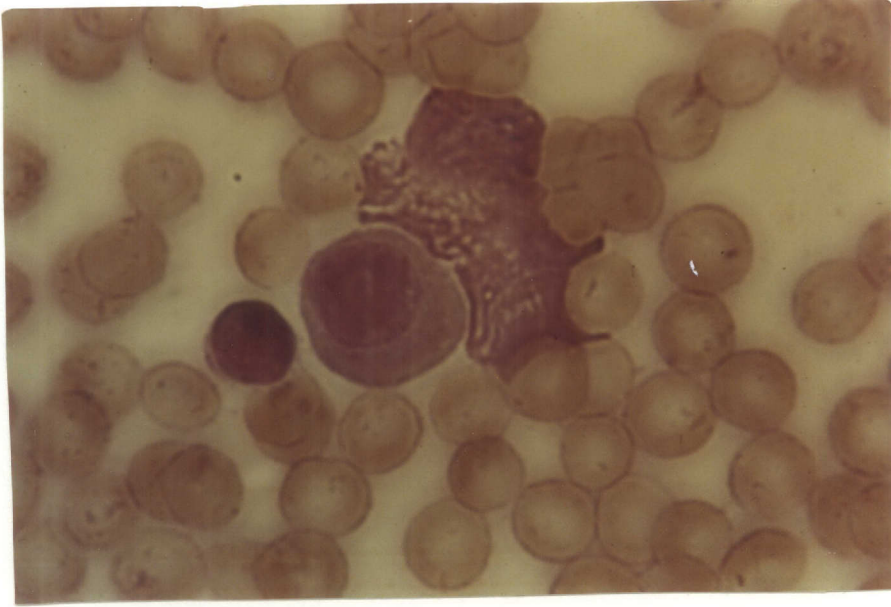
Figs. 6A and B—Wright's stain of peripheral blood smears from a 6- to 7-month-old preleukemic AKR/J mouse. Note the phagocytosis of nuclear material by the lymphocyte in A (left). Magnification 470 X. The mononuclear cell in B appears to have caused disintegration of a neighboring cell ("smudge" cell, right). Magnification 1000 X.

to be a plasma cell in close proximity to a disintegrating smudge cell. Plasma cells are infrequently seen in the peripheral blood.

Histological Sectioning of Spleens

Spleen and thymus glands of leukemic AKR/J mice were sectioned and stained for histological study. Spleens tended to lend themselves to sectioning on a microtome better than thymus glands. Figure 7A and B show hematoxylin and eosin stained sections of representative preleukemic and leukemic spleen sections, respectively. Note the lymphocyte germinal centers in the preleukemic spleen section (7A) and the complete infiltration of lymphocytes in the leukemic section (B). Complete infiltration of splenic lymphocytes was a characteristic of spleen histologic sections from leukemic AKR/J mice. Figure 8A and B shows photographs of spleen sections from 6- to 7-month-old leukemic AKR/J mice at a magnification of 470 X for A and 1000 X for B. Mitotic cells in various stages of division are visible in both, but more notably in B. Presence of numerous actively dividing cells is an additional feature of leukemic tissues.

60
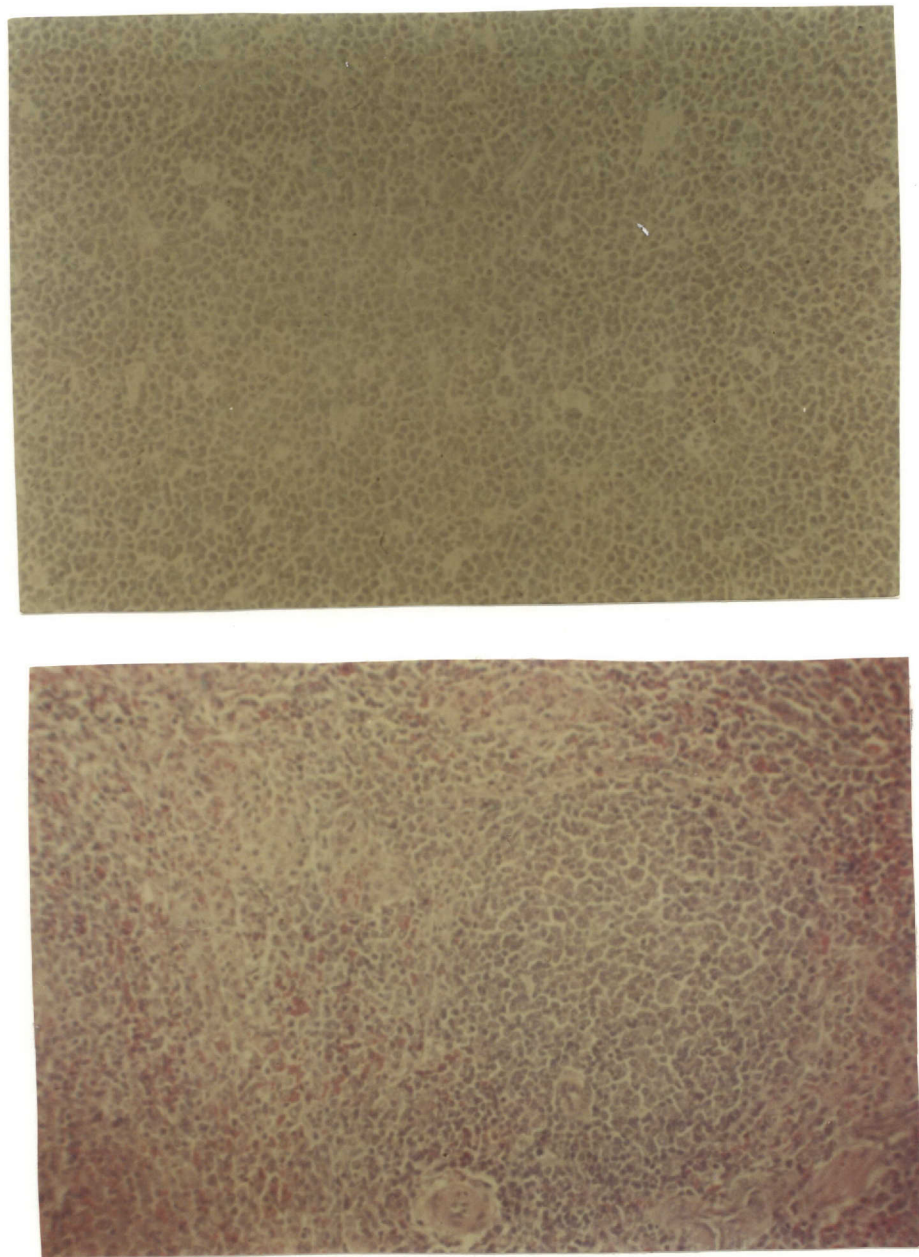
Figs. 7A and B--Sections of spleens from 6- to 7-month-old preleukemic (A) and leukemic (B) mice. Note the lymphocyte germinal centers in A and the complete infiltration of lymphocytes in the leukemic section (B). Magnification 1000 X.
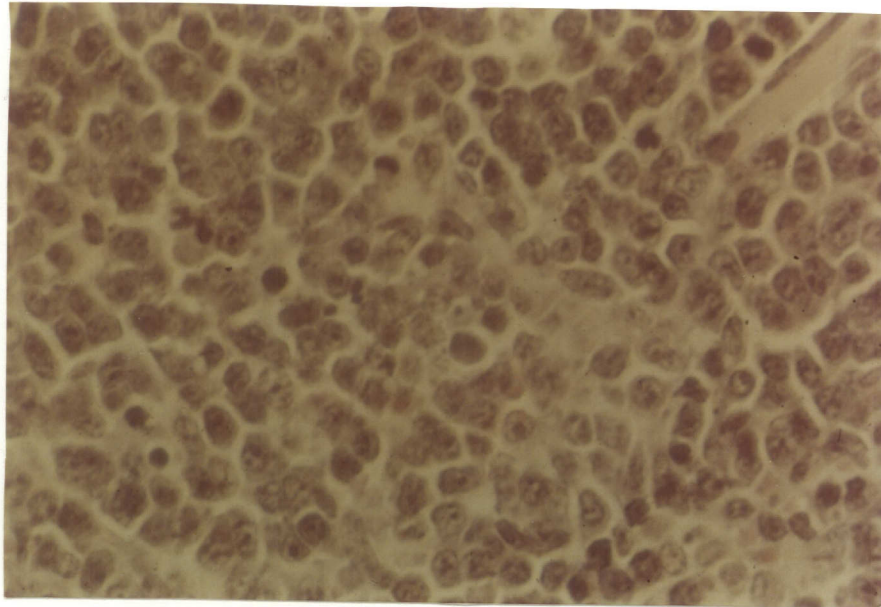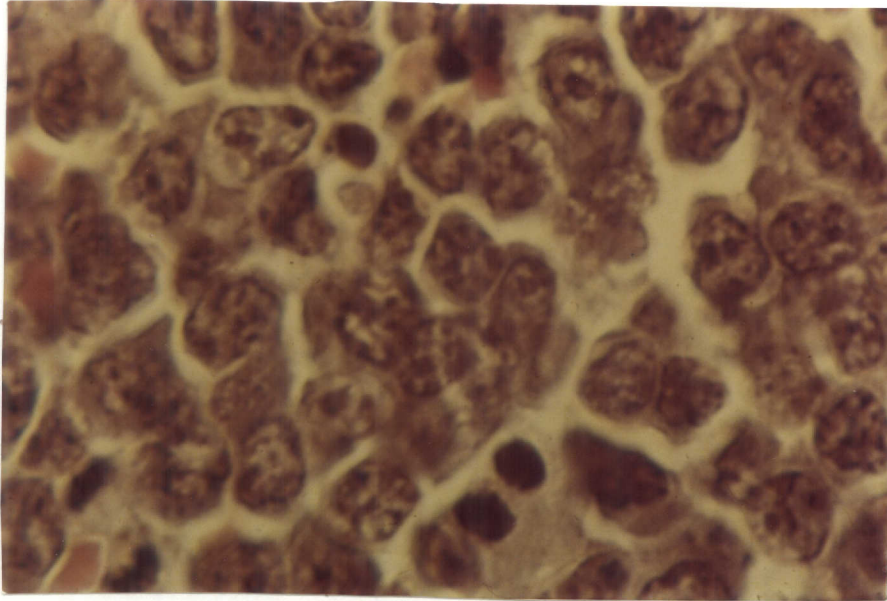
Figs. 8A and B--Photographs of spleen sections from 6- to 7-month-old leukemic AKR/J mice. Mitotic cells can be seen in both A and B, but more notably in B. Magnification 470 X (A) and 1000 X (B).

Protein Electrophoresis

Figure 9 consists of two densitometer scans of electrophoretic patterns of leukemic and preleukemic sera, respectively. Two pools containing five leukemic or preleukemic sera each were subjected to electrophoresis. Densitometer scans of the electrophoretic patterns shown in Figure IX show peaks representing from left to right: albumin, alpha 1, alpha 2, beta, and gamma proteins. On close examination, two major differences were seen between the leukemic electrophoretic pattern and the pattern from the pooled nonleukemic sera. The beta peak of the pooled leukemic animal sera is smaller in proportion to the nonleukemic, while the alpha 2 peak appearing immediately adjacent to the beta peak, is greater.

Titration of Antisera by Latex Agglutination
and Trypan Blue Staining as a Test for
Cytotoxicity of Antisera

In order to standardize the concentrations of antisera to be used in passive immunization procedures, latex agglutination and cytotoxicity titers were determined in vitro. Titers obtained with anti-thymocyte globulins, and rabbit anti-mouse brain sera by agglutination of latex particles coated onto splenic lymphocytes from a 2- to 3-month-old AKR/J mouse are shown in Table X.
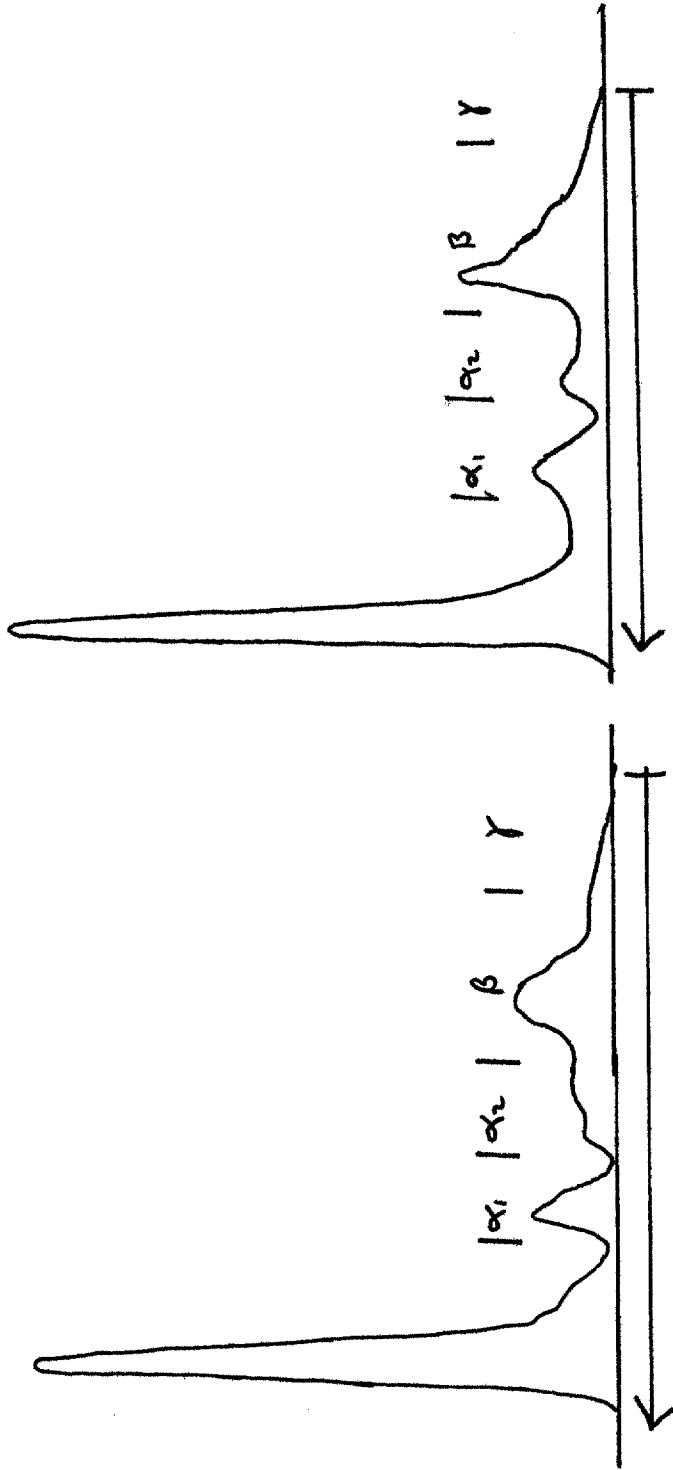
Fig. 9--Protein electrophoresis of pooled sera from nonleukemic (left) and leukemic (right) AKR/J mice. Serum protein peaks can be identified from left to right as: albumin, alpha-1, alpha-2, beta, and gamma.

TABLE X

LATEX AGGLUTINATION TITERS

Antisera	Und.*	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
Anti-lymphocyte Globulins (ALG)	3+	3+	4+	4+	4+	4+	3+	3+	-
Anti-thymocyte Globulins (ATG)	4+	4+	4+	4+	4+	3+	-	-	-
Rabbit Anti-mouse Brain Serum (RAMB)	4+	4+	4+	3+	3+	-	-	-	-

*Undiluted ALG and ATG contained 5 mg/ml protein; undiluted RAMB serum was whole rabbit serum.

Table XI lists percentages of lymphocytes staining with trypan blue after treatment of spleen cells from a 2- to 3-month-old AKR/J mouse with various dilutions of ALG, ATG, or RAMB serum and complement. Cells which are non-viable absorb stain because there is no membrane integrity; viable cells, or cells with intact membranes, do not stain.

Survival Rates of 2- to 3-Month-Old AKR/J Mice Treated with Globulins and Anti-mouse Brain Serum

As illustrated in Table XII, a sharp drop in the survival rates of 2- to 3-month-old mice occurred in all groups after 108 days of treatments with globulins and anti-mouse brain serum. Since treatments were initiated at 2 to 3 months of age, this time interval represents the age in the lifespan of the animal (6 to 7 months) when appreciable losses were expected to appear due to the onset of viral-induced leukemia. Tables XIII, XIV, and XV show statistical comparisons of survival rates of 2- to 3-month-old AKR/J mice treated with anti-thymocyte globulins, anti-lymphocyte globulins, and rabbit anti-mouse brain serum. Intervals of 49, 104, 154, and 207 days after initiation of treatment were compared to controls by the method described previously (Materials and Methods).

A significant chi-square was obtained for anti-thymocyte globulin (ATG) treatment of 2- to 3-month-old AKR/J

TABLE XI

CYTOTOXICITY ASSAYS

Antisera	*Und.	1/2	1/4	1/8	1/16	1/32	1/64
Anti-lymphocyte Globulins (ALG)	25[+]	38	28	20	14	9	8
Anti-thymocyte Globulins (ATG)	35	32	26	17	18	7	6
Rabbit Anti-mouse Brain Serum (RAMB)	20	23	18	12	3	1	–

*Undiluted = 0.2 mg of ALG or ATG protein; 1/50 dilution of RAMB

[+]Numbers represent the percentage of nonviable cells as indicated by staining with trypan blue (5.0 X 10^6 spleen cells/tube).

TABLE XII

SURVIVAL OF UNTREATED AND TREATED 2- TO 3-MONTH-OLD AKR/J MICE FOLLOWING

TREATMENT WITH ATG, ALG, OR RAMB

Days After Treatment Initiation	Untreated Control Group	Groups Treated With:[+]		
		ATG	ALG	RAMB
0	23 (100)*	23 (100)	23 (100)	23 (100)
13	23 (100)	23 (100)	23 (100)	23 (100)
49	23 (100)	22 (96)	20 (87)	19 (83)
76	23 (100)	22 (96)	18 (78)	19 (83)
86	23 (100)	22 (96)	18 (78)	19 (83)
94	22 (96)	22 (96)	18 (78)	17 (74)
104	22 (96)	21 (91)	18 (78)	17 (74)
108	22 (96)	21 (91)	17 (74)	15 (66)
116	20 (87)	20 (87)	13 (57)	12 (52)
124	17 (74)	18 (78)	8 (34)	5 (22)
132	15 (66)	18 (78)	5 (22)	2 (9)
143	11 (47)	17 (74)	0	0
154	7 (30)	14 (61)	0	0
173	5 (22)	10 (43)	0	0
187	3 (13)	5 (22)	0	0
207	0	5 (22)	0	0

*Number of survivors (percent of total)
+The abbreviations are: ATG, anti-thymocyte globulin; ALG, anti-lymphocyte globulin; RAMB, rabbit anti-mouse brain serum.

TABLE XIII

SUMMARY OF COMPARISON: ANTI-THYMOCYTE GLOBULIN
(ATG) TREATED 2-3 MONTH-OLD AKR/J MICE VERSUS CONTROLS

Period in Days	49	104	154	207
Control				
Dead*	0	1	16	23
Alive	23	22	7	0
ATG Treated				
Dead	1	2	9	18
Alive	22	21	14	5

Day	D-Dead	E-Dead	Variance	Z	Chi-Square (1 DF)	
49	1.00	0.50	0.25	1.00	1.00	
104	1.00	0.98	0.49	0.03	0.00	
154	7.00	10.74	2.75	-2.26	5.10	**
207	9.00	10.67	0.89	-1.77	3.12	
-	18.00	20.50	1.14	-2.34	5.49	**
Sum	18.00	22.89	4.3758		9.23	

Combined Chi-Square (1 degree of freedom, DF) = 5.462 **
Chi-Square for Homogeneity (3 DF) = 3.765
Standardized Risk Index (SRI) = 0.786
Hypothetical S.E. of SRI = 0.091
95% Confidence Intervals for SRI = (0.604 - 0.969)

*Number of AKR/J mice dead after each period of days

TABLE XIV

SUMMARY OF COMPARISON: ANTI-LYMPHOCYTE GLOBULIN
(ALG) TREATED 2-3 MONTH-OLD AKR/J MICE VERSUS CONTROLS

Period in Days	49	104	154	207
Control				
Dead*	0	1	16	23
Alive	23	22	7	0
ALG				
Treated				
Dead	3	5	23	23
Alive	20	18	0	0

Day	D-Dead	E-Dead	Variance	Z	Chi-Square (1 DF)
49	3.00	1.50	0.72	1.77	3.14
104	2.00	1.40	0.71	0.72	0.51
154	18.00	14.85	1.47	2.60	6.77 **
207	0.0	0.0	0.0	0.0	0.0
-	23.00	23.00	0.0	0.0	0.0
Sum	23.00	17.75	2.8934		10.42 **

Combined Chi-Square (1 degree of freedom, DF) = 9.543 **
Chi-Square for Homogeneity (3 DF) = 0.880
Standardized Risk Index (SRI) = 1.296
Hypothetical S.E. of SRI = 0.096
95% Confidence Intervals for SRI = (1.104 - 1.488)

*Number of AKR/J mice dead after each period of days

TABLE XV

SUMMARY OF COMPARISON: RABBIT ANTI-MOUSE BRAIN

(RAMB) TREATED 2-3 MONTH- OLD AKR/J MICE VERSUS CONTROLS

Period in Days	49	104	154	207
Control				
Dead*	0	1	16	23
Alive	23	22	7	0
RAMB				
Treated				
Dead	4	6	23	23
Alive	19	17	0	0

Day	D-Dead	E-Dead	Variance	Z	Chi-Square (1 DF)
49	4.00	2.00	0.93	2.07	4.29 **
104	2.00	1.36	0.71	0.76	0.58
154	17.00	13.95	1.45	2.53	6.42 **
207	0.0	0.0	0.0	0.0	0.0
--	23.00	23.00	0.0	0.0	0.0
Sum	23.00	17.31	3.0897		11.29 **

Combined Chi-Square (1 degree of freedom, DF) = 10.494 **
Chi-Square for Homogeneity (3 DF) = 0.800
Standardized Risk Index (SRI) = 1.329
Hypothetical S.E. of SRI = 0.102
95% Confidence Intervals for SRI = (1.126 - 1.532)

*Number of AKR/J mice dead after each period of days

mice between 104 and 154 days of treatment. This suggests a real increase in survival during this interval of animals treated with ATG. From Table XII it can be seen that after administering ATG for 143 days, 74% of treated mice were alive, compared to only 47% of the control animals. None of the animals treated with anti-lymphocyte globulin or anti-mouse brain serum had survived as long.

When anti-lymphocyte globulins (ALG) were used to treat 2- to 3-month-old mice, a highly significant individual chi-square value of 6.77 was obtained (see Table XIV) and a positive Z-factor of 2.60. The positive sign of the Z-factor indicates that treatment significantly accelerated the mortality during the 104- to 154-day time period.

Rabbit anti-mouse brain serum (RAMB) also resulted in a highly significant number of deaths in 2- to 3-month-old mice. The calculated individual chi-square value was 6.42 for the period between 104 and 154 days of treatment (Table XV). A significant decrease in the survival rate of RAMB treated mice was also obtained for the first 49 days of treatment ($x^2 = 4.29$).

Survival Rates of 6- to 7-Month-Old AKR/J Mice Treated
with Globulins and Anti-mouse Brain Serum

Survival rates of 6- to 7-month-old mice treated
with anti-thymocyte globulins (ATG), anti-lymphocyte
globulins (ALG), and rabbit anti-mouse brain serum
(RAMB) are shown in Table XVI. Tables XVII, XIX, and
XXI contain the statistical analyses of weekly mortalities
in mice treated with ATG, ALG, and RAMB, respectively;
Tables XVIII, XX, and XXII reveal the comparisons at
3- to 4-week intervals.

AKR/J 6- to 7-month-old mice treated with ATG
did not survive longer than controls after 7, 14, and
21 days of treatments. The chi-square values of 5.36,
9.45, and 13.19, respectively, are highly significant.
The initial sharp reduction in the survival rate
of ATG-treated animals did not continue beyond three
weeks. The sudden change from a decrease in survival
rate to an insignificant difference is indicated in
Tables XVII and XVIII after 21 days of treatments by
a sign change in the Z-factor from positive to
negative.

Six to 7-month-old AKR/J mice treated with ALG
had an increased survival rate ($x^2 = 4.51$). It can be

TABLE XVI

SURVIVAL OF UNTREATED AND TREATED 6- TO 7-MONTH-OLD AKR/J MICE

Days After Treatment Initiation	Untreated Control Group	Groups Treated With[+]		
		ATG	ALG	RAMB
0	30 (100)*	30 (100)	30 (100)	30 (100)
4	30 (100)	25 (83)	30 (100)	29 (97)
9	30 (100)	20 (67)	30 (100)	29 (97)
13	30 (100)	18 (60)	30 (100)	28 (93)
16	29 (97)	17 (57)	30 (100)	26 (87)
19	29 (97)	17 (57)	30 (100)	23 (77)
23	28 (93)	16 (53)	30 (100)	20 (67)
36	27 (90)	16 (53)	30 (100)	18 (60)
30	24 (80)	16 (53)	30 (100)	14 (47)
33	24 (80)	16 (53)	30 (100)	12 (40)
37	23 (77)	16 (53)	29 (97)	11 (37)
40	21 (70)	15 (50)	28 (93)	11 (37)
44	21 (70)	15 (50)	26 (87)	11 (37)
47	19 (63)	15 (50)	26 (87)	10 (33)
49	17 (57)	15 (50)	24 (80)	10 (33)
53	15 (50)	12 (40)	21 (70)	10 (33)
68	11 (37)	7 (23)	16 (53)	8 (27)
71	11 (37)	5 (17)	14 (47)	8 (27)
74	10 (33)	5 (17)	13 (43)	7 (23)
76	8 (27)	4 (10)	11 (37)	7 (23)
80	6 (20)	3 (10)	10 (33)	6 (20)
85	3 (10)	3 (10)	8 (27)	6 (20)
88	3 (10)	2 (7)	7 (23)	5 (17)

*Number of survivors (percent of total)
+The abbreviations are: ATG, anti-thymocyte globulin; ALG, anti-lymphocyte globulin; RAMB, rabbit anti-mouse brain serum.

TABLE XVII

SUMMARY OF COMPARISON: ANTI-THYMOCYTE GLOBULIN
(ATG) TREATED 6-7 MONTH-OLD AKR/J MICE VERSUS CONTROLS

Day	7	14	21	28	35	42	49	56	70	77	84	91
Control												
Dead*	0	0	1	3	6	9	13	15	19	22	24	27
Alive	30	30	29	27	24	21	17	15	11	8	6	3
ATG Treated												
Dead	5	12	13	14	14	15	15	18	23	26	27	28
Alive	25	18	17	16	16	15	15	12	7	4	3	2

Day	D-Dead	E-Dead	Variance	Z	Chi-Square (1 DF)	
7	5.00	2.50	1.17	2.32	5.36	**
14	7.00	3.18	1.54	3.07	9.45	**
21	1.00	0.75	0.46	0.37	0.14	
28	1.00	1.11	0.67	-0.13	0.02	
35	0.0	1.12	0.67	-1.37	1.87	
42	1.00	1.60	0.89	-0.64	0.41	
49	0.0	1.67	0.89	-1.77	3.12	
56	3.00	2.34	1.08	0.63	0.40	
70	5.00	4.00	1.54	0.81]	0.65	
77	3.00	2.33	1.01	0.66	0.44	
84	1.00	1.00	0.55	0.0	0.0	
91	1.00	1.33	0.56	-0.45	0.20	
-	28.00	27.50	1.17	0.46	0.21	
Sum	28.00	22.93	11.0077		22.05	**

Combined Chi-Square (1 degree of freedom, DF) = 2.332
Chi-Square for Homogeneity (11 DF) = 19.722
Standardized Risk Index (SRI) = 1.221
Hypothetical S.E. of SRI = 0.145
95% Confidence Intervals for SRI = (0.932 - 1.510)

*Number of AKR/J mice dead after each weekly interval

TABLE XVIII

SUMMARY OF COMPARISON: ANTI-THYMOCYTE GLOBULIN
(ATG) TREATED 6-7 MONTH-OLD AKR/J MICE VERSUS CONTROLS

Period in Days	21	42	70	91
Control				
Dead*	1	9	19	27
Alive	29	21	11	3
ATG Treated				
Dead	13	15	23	28
Alive	17	15	7	2

Day	D-Dead	E-Dead	Variance	Z	Chi-Square (1 DF)
21	13.00	7.00	2.73	3.63	13.19 **
42	2.00	3.70	1.86	-1.24	1.54
70	8.00	7.50	2.25	0.33	0.11
91	5.00	5.06	0.91	-0.06	0.00
-	28.00	27.50	1.17	0.46	0.21
Sum	28.00	23.25	7.7514		14.85 **

Combined Chi-Square (1 degree of freedom, DF) = 2.909
Chi-Square for Homogeneity (3 DF) = 11.940 **
Standardized Risk Index (SRI) = 1.204
Hypothetical S.E. of SRI = 0.120
95% Confidence Intervals for SRI = (0.965 - 1.444)

*Number of AKR/J mice dead after each period of days

TABLE XIX

SUMMARY OF COMPARISON: ANTI-LYMPHOCYTE GLOBULIN
(ALG) TREATED 6-7 MONTH-OLD AKR/J MICE VERSUS CONTROLS

Day	7	14	21	28	35	42	49	56	70	77	84	91
Control												
Dead*	0	0	1	3	6	9	13	15	19	22	24	27
Alive	30	30	29	27	24	21	17	15	11	8	6	3
ALG Treated												
Dead	0	0	0	0	0	2	6	9	14	19	20	23
Alive	30	30	30	30	30	28	24	21	16	11	10	7

Day	D-Dead	E-Dead	Variance	Z	Chi-Square (1 DF)
7	0.0	0.0	0.0	0.0	0.0
14	0.0	0.0	0.0	0.0	0.0
21	0.0	0.50	0.25	-1.00	1.00
28	0.0	1.02	0.49	-1.45	2.11
35	0.0	1.58	0.72	-1.86	3.46
42	2.00	2.78	1.14	-0.73	0.53
49	4.00	4.57	1.67	-0.44	0.20
56	3.00	2.93	1.09	0.07	0.00
70	5.00	5.25	1.69	-0.19	0.04
77	5.00	4.74	1.41	0.22	0.05
84	1.00	1.74	0.65	-0.91	0.84
91	3.00	3.75	0.94	-0.77	0.60
-	23.00	25.00	2.12	-1.37	1.89
Sum	23.00	28.85	10.0560		8.81 **

Combined Chi-Square (1 degree of freedom, DF) = 3.403
Chi-Square for Homogeneity (11 DF) = 5.409 **
Standardized Risk Index (SRI) = 0.797
Hypothetical S.E. of SRI = 0.110
95% Confidence Intervals for SRI = (0.577 - 1.017)

*Number of AKR/J mice dead after each weekly interval

TABLE XX

SUMMARY OF COMPARISON: ANTI-LYMPHOCYTE GLOBULIN
(ALG) TREATED 6-7 MONTH-OLD AKR/J MICE VERSUS CONTROLS

Period in Days	21	42	70	91
Control				
Dead*	1	9	19	27
Alive	29	21	11	3
ALG				
Treated				
Dead	0	2	14	23
Alive	30	28	16	7

Day	D-Dead	E-Dead	Variance	Z	Chi-Square (1 DF)	
21	0.0	0.50	0.25	-1.00	1.00	
42	2.00	5.08	2.11	-2.12	4.51	**
70	12.00	12.57	3.03	-0.33	0.11	
91	9.00	10.07	1.58	-0.85	0.73	
-	23.00	25.00	2.12	-1.37	1.89	
Sum	23.00	28.23	6.9706		6.35	

Combined Chi-Square (1 degree of freedom, DF) = 3.924 **
Chi-Square for Homogeneity (3 DF) = 2.421
Standardized Risk Index (SRI) = 0.815
Hypothetical S.E. of SRI = 0.094
95% Confidence Intervals for SRI = (0.628 - 1.002)

*Number of AKR/J mice dead after each period of days

TABLE XXI

SUMMARY OF COMPARISON: RABBIT ANTI-MOUSE BRAIN
(RAMB) TREATED 6-7 MONTH-OLD AKR/J MICE VERSUS CONTROLS

Day	7	14	21	28	35	42	49	56	70	77	84	91
Control												
Dead*	0	0	1	3	6	9	13	15	19	22	24	27
Alive	30	30	29	27	24	21	17	15	11	8	6	3
RAMB Treated												
Dead	1	2	7	12	18	19	20	20	22	23	24	25
Alive	29	28	23	18	12	11	10	10	8	7	6	5

Day	D-Dead	E-Dead	Variance	Z	Chi-Square (1 DF)
7	1.00	0.50	0.25	1.00	1.00
14	1.00	0.49	0.25	1.02	1.03
21	5.00	2.90	1.37	1.80	3.24
28	5.00	3.10	1.52	1.54	2.38
35	6.00	3.60	1.77	1.81	3.26
42	1.00	1.33	0.81	-0.37	0.14
49	1.00	1.72	0.98	-0.73	0.53
56	0.0	0.74	0.45	-1.11	1.22
70	2.00	2.40	1.14	-0.37	0.14
77	1.00	1.68	0.81	-0.76	0.58
84	1.00	1.40	0.64	-0.50	0.25
91	1.00	2.00	0.73	-1.17	1.37
-	25.00	26.00	1.76	-0.75	0.57
Sum	25.00	21.86	10.7209		15.14 **

Combined Chi-Square (1 degree of freedom, DF) = 0.919
Chi-Square for Homogeneity (11 DF) = 14.219 **
Standardized Risk Index (SRI) = 1.144
Hypothetical S.E. of SRI = 0.150
95% Confidence Intervals for SRI = (0.844 - 1.443)

*Number of AKR/J mice dead after each weekly interval

TABLE XXII

SUMMARY OF COMPARISON: RABBIT ANTI-MOUSE BRAIN
(RAMB) TREATED 6-7 MONTH-OLD AKR/J MICE VERSUS CONTROLS

Period in Days	21	42	70	91
Control				
Dead*	1	9	19	27
Alive	29	21	11	3
RAMB				
Treated				
Dead	7	19	23	25
Alive	23	11	7	5

Day	D-Dead	E-Dead	Variance	Z	Chi-Square (1 DF)
21	7.00	4.00	1.76	2.26	5.11 **
42	12.00	8.85	3.10	1.79	3.21
70	4.00	4.81	1.83	-0.60	0.36
91	2.00	3.89	1.12	-1.79	3.19
-	25.00	26.00	1.76	-0.75	0.57
Sum	25.00	21.55	7.8104		11.87 **

Combined Chi-Square (1 DF) = 1.526
Chi-Square for Homogeneity (3 DF) = 10.343 **
Standardized Risk Index (SRI) = 1.160
Hypothetical S.E. of SRI = 0.130
95% Confidence Intervals for SRI = (0.901 - 1.420)

seen in Table XVI that no deaths occurred in ALG-treated

mice until after the first 33 days of treatment. At this

time, a total of 6 deaths had already been recorded in the

control population, 14 of 30 mice had died of leukemia in

the ATG-treated group, and 18 of the original 30 RAMB

treated mice had died. Note in Table XIX that no signif-

icant differences were seen in the survival rate of ALG-

treated mice on a weekly basis. Instead, the increased

rate of survival was the result of ALG protection over a

continued, prolonged period of 42 days (Table XX). In

fact, it was not possible for ALG-treated animals to have

an improvement in survival rate over controls prior to 21

days of treatment because no deaths occurred in either the

treated or control groups prior to this time.

A chi-square value of 5.11 and a positive Z-factor

of 2.26 (Table XXII) indicate that significantly fewer

rabbit anti-mouse brain serum (RAMB) treated mice

survived after 21 days of treatment than controls. Upon

close examination of Table XXI, it can be seen that large

numbers of animals treated with RAMB died on a weekly

basis after 21, 28, and 35 days of treatment. This trend

did not reverse itself until 42 days of treatments. At

this time a negative Z-factor was obtained for the interval

between 35 and 42 days, indicating that the death rate was

now less than that of the control population.

Survival Rates of 6- to 7-Month-Old AKR/J Mice Receiving Lymphocytes from ALG Affinity Column Chromatography

Table XXIII lists both the numbers and percentages of surviving 6- to 7-month-old AKR/J mice treated with 20×10^6 lymphocytes, i.v., once per week. Cells used for injections were derived from 2- to 3-month-old AKR/J splenic lymphocytes after passage through both an anti-mouse immunoglobulin G (anti-IgG) and an anti-lymphocyte globulin (ALG) affinity column. No significant differences were discovered between cell-treated mice and controls for any of the intervals measured (Tables XXIV and XXV). It is interesting to note, however, that more treated animals survived than controls until treatments were terminated after 49 days. Eighty-five percent of the mice receiving splenic lymphocytes had no symptoms of leukemia at this time, compared to 75% of controls. During the next three weeks, the death rate of the previously cell-treated group appeared to decline at a rate more approximating that of the controls.

TABLE XXIII

SURVIVAL OF 6-TO 7-MONTH-OLD AKR/J MICE

RECEIVING ATG SENSITIVE LYMPHOCYTES

Days After Treatment Initiation	Controls	Cell Recipients
0	40 (100)*	40 (100)
7	40 (100)	40 (100)
14	40 (100)	40 (100)
21	39 (97)	39 (97)
28	36 (90)	37 (92)
35	34 (85)	36 (90)
42	32 (80)	35 (87)
49 treatments	30 (75)	34 (85)
← terminated → 56	28 (70)	31 (77)
72	24 (60)	29 (72)

*Number of survivors (percent of total).

TABLE XXIV

SUMMARY OF COMPARISON: 6-7 MONTH
AKR/J MICE RECEIVING ANTI-THYMOCYTE GLOBULIN (ATG)
SENSITIVE LYMPHOCYTES VERSUS CONTROLS

Day	7	14	21	28	35	42	49	56	72
Control									
Dead*	0	0	1	4	6	8	10	12	16
Alive	40	40	39	26	24	22	20	18	14
Cell Treated									
Dead	0	0	1	3	4	5	6	9	11
Alive	40	40	39	37	36	35	34	31	29

Day	D-Dead	E-Dead	Variance	Z	Chi-Square (1 DF)
7	0.0	0.0	0.0	0.0	0.0
14	0.0	0.0	0.0	0.0	0.0
21	1.00	1.00	0.49	0.0	0.0
28	2.00	2.50	1.19	-0.46	0.21
35	1.00	1.52	0.73	-0.61	0.37
42	1.00	1.54	0.73	-0.64	0.40
49	1.00	1.57	0.73	-0.67	0.44
56	3.00	2.66	1.17	0.32	0.10
72	2.00	3.15	1.37	-0.99	0.97
-	11.00	13.50	4.53	-1.17	1.38
Sum	11.00	13.94	6.3945		2.50

Combined Chi-Square (1 degree of freedom, DF) = 1.351
Chi-Square for Homogeneity (8 DF) = 1.153
Standardized Risk Index (SRI) = 0.789
Hypothetical S.E. of SRI = 0.181
95% Confidence Intervals for SRI = (0.426 - 1.152)

*Number of AKR/J mice dead for a given interval

TABLE XXV

SUMMARY OF COMPARISON: 6-7 MONTH-OLD
AKR/J MICE RECEIVING ANTI-THYMOCYTE GLOBULIN (ATG)
SENSITIVE LYMPHOCYTES VERSUS CONTROLS

Period in Days	21	42	72
Control			
Dead*	1	8	16
Alive	39	32	24
Cell Treated			
Dead	1	5	11
Alive	39	35	29

Day	D-Dead	E-Dead	Variance	Z	Chi-Square (1 DF)
21	1.00	1.00	0.49	0.0	0.0
42	4.00	5.50	2.39	-0.97	0.94
72	6.00	7.31	2.80	-0.78	0.62
-	11.00	13.50	4.53	-1.17	1.38
Sum	11.00	13.81	5.6915		1.56

Combined Chi-Square (1 degree of freedom, DF) = 1.391
Chi-Square for Homogeneity (2 DF) = 0.165
Standardized Risk Index (SRI) = 0.796
Hypothetical S.E. of SRI = 0.173
95% Confidence Intervals for SRI = (0.451 - 1.142)

*Number of AKR/J mice dead after each period of days

In vitro Radioimmunofluorescence Studies

Fluorescent Antibody

The results of fluorescent antibody studies are shown on Table XXVI. Numbers of fluorescent cells were counted to calculate the percentages of lymphocytes stained with ALG, ATG, or RAMB serum. Cells were used from both leukemic and preleukemic spleen, thymus, and lymph node tissues. A greater number of ALG-sensitive cells were seen in leukemic spleen and lymph node suspensions than in similarly treated populations of lymphocytes isolated from preleukemic mice. In contrast, preleukemic spleen and lymph node cell suspensions had a greater percentage of fluorescing ATG sensitive lymphocytes than leukemic animals. Leukemic thymocytes were found to bind ATG and RAMB serum in greater number than preleukemics, while little differences were noted using ALG with thymocytes from leukemic and preleukemic mice. Figures 10 and 11 are photographs of fluorescent antibody bound to spleen and thymus lymphocytes. The cell in Figure 10 from a 6- to 7-month-old AKR/J mouse spleen is labeled with fluorescent anti-lymphocyte globulins. The cell pictured in Figure 11 is from a 6- to 7-month-old thymus gland and is labeled with fluorescent anti-thymocyte globulins. Note the capping phenomenon in Figure 11.

TABLE XXVI

FLUORESCENT ANTIBODY STUDIES*

	Anti-lymphocyte Globulins (ALG)	Anti-thymocyte Globulins (ATG)	Rabbit Anti-mouse Brain Sera (RAMB)
Leukemic (7-8 months)			
Spleen (% fluorescent lymphocytes) - mean	18.8 ±1.35	13.7 ±3.80	15.5 ±2.35
Thymus	18.0 ±2.15	25.3 ±3.01	19.6 ±1.52
Lymph Node	17.3 ±1.68	16.1 ±3.13	15.7 ±1.60
Preleukemic (2-3 months)			
Spleen (% fluorescent lymphocytes) - mean	10.3 ±2.68	15.7 ±2.56	28.2 ±3.65
Thymus	14.5 ±1.46	14.9 ±2.77	12.0 ±1.46
Lymph Node	9.3 ±0.76	28.3 ±2.91	23.8 ±3.51

*A large number of mononuclear leukocytes (approximately 50%) did not stain with either ALG, ATG, or RAMB sera.
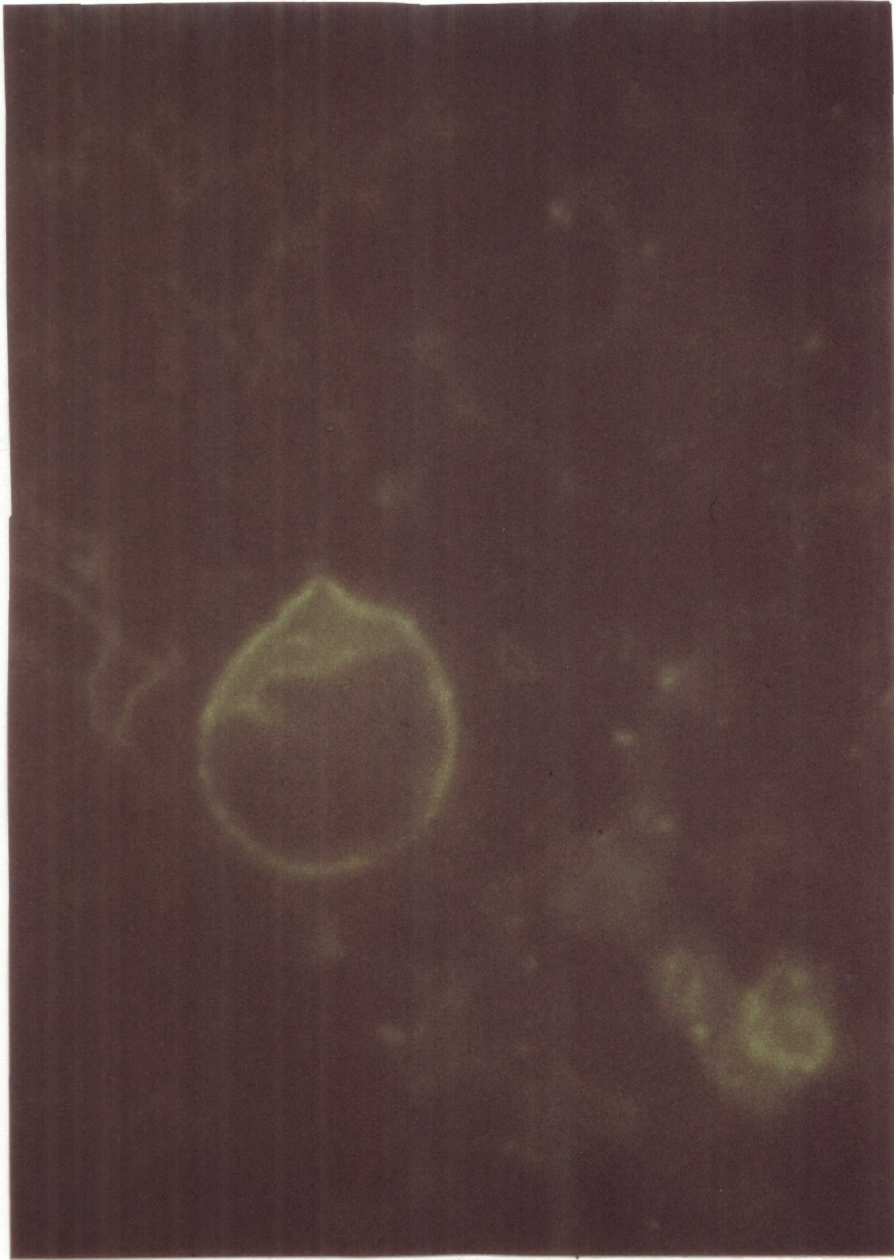
Fig. 10--Fluorescent antibody micrograph of fluorescein tagged anti-lymphocyte globulin (ALG) sensitive lymphocyte from an AKR/J 6- to 7-month-old mouse spleen. Magnification 1000 X.

Fig. 11--Fluorescent antibody micrograph of fluorescein tagged anti-thymocyte globulin (ATG) sensitive lymphocyte from AKR/J 6- to 7-month-old mouse thymus. Note capping phenomenon. Magnification 1000 X.

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^{125}I Studies

Double tagged ALG, ATG, and RAMB serum antibodies
were serially diluted and equal numbers of lymphocytes
were added to each tube from either leukemic or preleukemic
spleens, thymus glands, or lymph nodes. Following
inclubation and repeated washings, cells were counted
for radioactivity in a gamma counter. Table XXVII
lists the counts per minute (cpm) of leukemic and pre-
leukemic cell suspensions after background subtraction.
Nearly twice as much anti-lymphocyte globulin was shown
to be bound to the leukemic spleen and lymph node
suspensions as to comparable suspensions of preleukemic
cells. Anti-thymocyte globulins and rabbit anti-mouse
brain antiserum (anti-θ) gave the opposite results, however.
Leukemic spleen and lymph node cell suspensions bound nearly
half as much anti-thymocyte globulin (ATG) and rabbit anti-
mouse brain antiserum (RAMB) as those from preleukemic mice.

Autoradiography

Autoradiography was performed on fixed smears of
radioactive antibody-labeled lymphocytes by immersing
glass coverslip smears in a nuclear emulsion and
incubating in the dark at freezing temperatures.
Figures 12 and 13 are photographs of developed and
stained autoradiographic smears under the light microscope.
Using autoradiography it is possible to visualize both

TABLE XXVII

^{125}I-RADIOISOTOPE LABELED ANTIBODY STUDIES

	ALG* Globulins	ATG Globulins	RAMB Serum
Leukemic (7-8 months)			
Spleen (cpm)	1138	1080	990
Thymus (cpm)	1388	1750	1411
Lymph Node (cpm)	1211	1570	1233
Preleukemic (2-3 months)			
Spleen (cpm)	666	2083	1753
Thymus (cpm)	1265	1439	1290
Lymph Node (cpm)	542	2131	1903

*The abbreviations are: ALG, anti-lymphocyte globulins; ATG, anti-thymocyte globulins; RAMB, rabbit anti-mouse brain serum.

the location and relative number of antibody receptors
on each cell. Both labeled and unlabeled mononuclear
cells were observed in these preparations. The surface
of the labeled cells are shown to contain multiple
receptor sites, indicated by the density of silver grains
deposited on the periphery of these cells. The location
of antibody receptor sites appears to be a general
dispersion over the entire surface of the cells. Cells
were seen both individually and in groups or clusters,
but comparisons between numbers of cells exibiting silver
grain rosettes in leukemic and nonleukemic mice were not
performed because of difficulties encountered with the
technique. This difficulty centered around problems with
cells failing to adhere well to the glass slide so that
individual cells were often washed off in preparation,
and frequent cell clumps made it difficult to identify
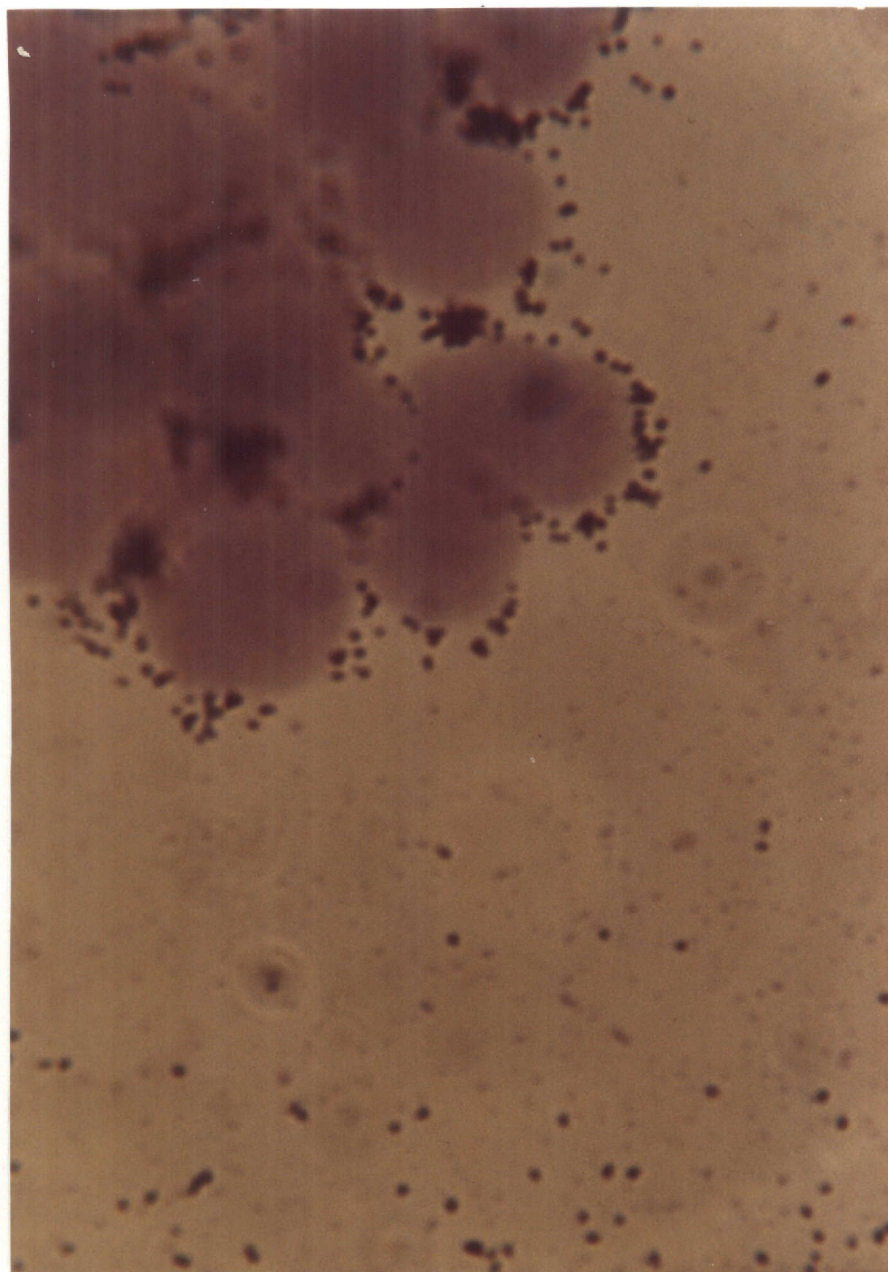individual cell grains.

Fig. 12--Photograph of a clump of spleen cells from a 6- to 7-month-old AKR/J mouse. Cells were labeled by ^{125}I-bound anti-lymphocyte globulin autoradiography techniques. Magnification 1000 X.
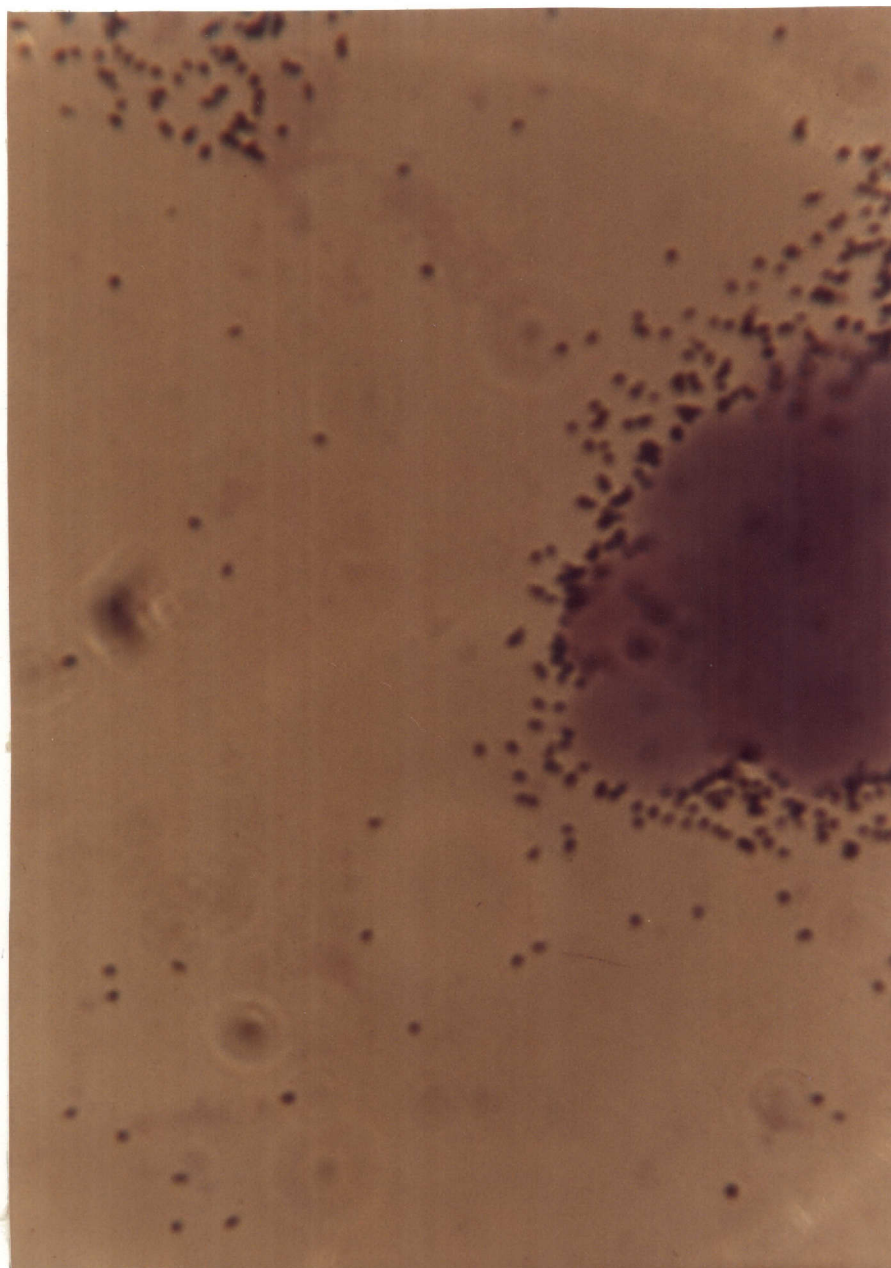
Fig. 13--Photograph of a large clump of thymocytes from a 6- to 7-month-old AKR/J mouse. Cells were labeled with ^{125}I-bound anti-thymocyte globulins for autoradiography. Magnification 1000 X.

CHAPTER V

DISCUSSION

Mice of the AKR strain transmit tumorogenic virus from mother to offspring. Lymphoma is known to develop first in the thymus; malignant cells migrate from this primary site of production to other areas of the body (3). In this study, the effect of anti-thymocyte globulins (ATG), anti-lymphocyte globulins (ALG), and rabbit anti-mouse brain (RAMB) serum was observed on lymphocytes in preleukemic and leukemic AKR/J mice. Because 6 to 7 months are required for the development of leukemia in this strain, antibodies to cell surface antigens could be studied for their ability to inhibit malignancy by in vivo administration prior to the onset of disease.

Since leukemia in the AKR strain is characterized by lymphoma, originating in the thymus and disseminating to lymphoid and other tissues (126), enlargment of one or more organs is a common observation. Numerous methods have been reported for determining the presence of leukemia, many of which are based on organ size or weight. Lilly et al. (56) diagnosed animals as being leukemic by the detection of markedly enlarged lymphoid organs, including thymus, spleen, and/or lymph nodes, prior to death and at autopsy. Zata (130) designated preleukemic AKR mice as having an enlarged thymus but a white blood cell (WBC) count less

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than 10,000/mm^3 and no enlargement of peripheral lymphoid

organs. Leukemic animals were described as having a

white blood cell count in excess of 20,000/mm^3 and

enlargement of all lymphoid organs. Wettstein and Hays

(126) defined leukemia in AKR mice as causing massive

enlargement of the thymus, often but not always accompanied

by enlargement of the spleen, lymph nodes, and liver.

In the study reported here, numerous criteria were examined

as evidence of leukemia. These included (1) observations

of physical appearances of animals, (2) white blood cell

counts, (3) spleen and thymus weights, (4) spleen

histological appearances, and (5) distributions of

leukocyte morphological types or differential blood

examinations. Although (1), (2), and (5) above were

useful as early indicators of leukemia prior to death,

increases in spleen and thymus weights were considered the

most reliable indicators.

Peripheral blood of young healthy mice has a

predominance of lymphocytes, which comprise nearly

80% of the white cells, while neutrophils represent

less than 20% of the total number. Since the proportion

of neutrophils to lymphocytes is greatly elevated in

the infectious state, and the opposite is true of the

leukemic diseased animal, cell distributions proved to be

a convenient method of confirming the onset of disease without sacrificing the animals.

As stated in the results, mice with peripheral leukocyte counts in excess of 40,000 cells/mm^3 were considered to be leukemic and data derived from them are included in the distribution graph in Figure 2. The reason for this limitation is two-fold. First, elevated white cell counts between 30,000 and 40,000/mm^3 were never observed. Secondly, the few leukocyte levels recorded between 20,000 and 30,000/mm^3 were presumed to result from infectious diseases or causes other than leukemia. This conclusion was reinforced by a proportional increase in neutrophils observed in stained peripheral blood smears. Only 3 mice were discovered to have elevated numbers of neutrophils; these were culled from the stock before treatments were initiated.

In Figure 5A and Figure 6A and B, it appears that phagocytosis of a cell or cell debris by another cell is in progress. These observations were not unusual in late preleukemic and leukemic AKR/J mice and give evidence supporting cellular and/or humoral antibody recognition of lymphoma cells. Both cellular (65,81) and humoral (33,36,126) recognition of lymphoma cells has been reported in AKR mice. Figures 4A and B contain three and four peripheral lymphocytes, respectively, in close contact.

The lack of neighboring lymphocytes in the fields suggests that these cells are not present in large numbers, negating the probability of chance association. Although no cellular destruction is visible morphologically in Figures 4A and B, the possibility exists that cell contact is a prelude to the cell destruction; thus the phagocytosis seen in Figures 5 and 6, A and B.

Figure IX consists of two densitometer scans of protein electrophoretic patterns of leukemic and preleukemic sera, respectively. The beta peak of the leukemia sera was elevated. Also, the alpha-2 peak of the sera from leukemic animals was enlarged. Serum proteins migrating within the beta peak and thus diminished in the leukemic animal are the third and fourth components of complement (C3 and C4), transferrin (an iron carrying protein decreased in liver diseases), hemopexin (specific heme-carrying protein, decreased in intravascular hemolysis), and Cl inactivator, which inhibits the activity of the Cl complement component. Serum proteins which may have increased the alpha-2 peak in leukemic sera are a_2-macroglobulin (increased in kidney diseases) and haptoglobin (increased in inflammatory and neoplastic conditions). Most of those proteins listed have been shown to be present in abnormal levels in leukemic animals (36,44,54).

Viral-induced leukemia in AKR/J mice is of a
lymphocytic type, and is characterized by differentiated,
morphologically mature thymus-derived lymphocytes. The
delay in the onset of leukemia following injection of
anti-thymocyte globulins into AKR/J mice, beginning
at 2 to 3 months of age, may be the result of specific
binding of antibodies to a pool of cells that eventually
differentiate into lymphoma cells. This conclusion is
supported by the fact that precursors of the AKR/J lymphoma
cells are thymic lymphocytes, rich in Thy-1 (theta) surface
antigens and thus are sensitive to anti-thymocyte globulins
and rabbit anti-mouse brain serum (48,60). Thymocytes in
2 to 3-month-old mice demonstrate a high Thy-1/low H-2
surface antigen phenotype, changing to a low Thy-1/high
H-2 phenotype at six months of age (114). Anti-thymocyte
globulin (ATG) is specific for the Thy-1 antigen (5).
For this reason, thymocytes from young mice would be
expected to bind more ATG than thymic lymphocytes from 6
to 7 month old, or even leukemic mice. Additional support
for the binding of ATG to a precursor of the AKR/J
leukemic cell has been provided by the experiments of
Filppi et al. (18) who used rabbit anti-mouse brain sera
(RAMB). The latter experiments have shown that RAMB
(anti-theta θ) antiserum is directed against an immature

T-lymphocyte population in the AKR/J thymus, with specific

binding decreasing progressively with age and the onset

of leukemia. Bron and Sauser (5) demonstrated, by

absorption of antisera to thymocyte membranes with

thymocytes of different theta allotypes, that ATG binds

to an antigen which is not the theta (θ) antigen, yet is

only found on theta positive T-lymphocytes. Furthermore,

Schlesinger and Yron (110) observed, by cytotoxicity

assays, that ATG reduces the number of antigenically

distinct thymus-derived cells in the lymph nodes.

Fluorescent antibody studies (Table XXVI) reported here

indicate that a greater percentage of lymph node cells

from preleukemic 2- to 3-month-old mice bound fluorescein-

tagged anti-thymocyte globulin than did similar cells

from leukemic mice. Greater amounts of ^{125}I-labeled ATG

were also shown to bind to 2- to 3-month-old mouse

spleen and lymph node cell suspensions than to equivalent

numbers of suspended cells from the same organs in

leukemic animals (Table XXVII). Additional data presented

indicate that anti-thymocyte globulins not only bind to

and agglutinate, but are cytotoxic for, a lymphocyte

population which is present in greater numbers in young,

preleukemic AKR/J mice than in older and/or leukemic

animals. This cell, identified by the Thy-1 surface

antigen, appears to be a precursor to the lymphoma
lymphocyte. Survival studies support this hypothesis
by further showing that repeated injections of antisera
specifically binding to, and presumably destroying,
Thy-1 rich precursor cells in vivo promote survival, if
initiated at 2 to 3 months of age.

A similar increase in survival rates was not shown
for 6- to 7-month-old AKR/J mice treated with ATG. Gelfand
and Paul (21) have demonstrated that ATG may function
through the activation of suppressor T-cells, and as a
result humoral and/or cellular immune responses toward
the transformed cells are suppressed. This was shown by
the capacity of anti-Thy 1.1 sera to prolong survival of
C57BL/6 (Thy 1.2) skin on AKR (Thy 1.1) mice. The effect
of anti-Thy 1 serum was transferable by thymus or spleen
cells from anti-Thy 1-treated recipients. Activation of
suppressor cells may be one explanation for the decrease
in survival of ATG treated 6- to 7-month-old mice following
injection of ATG. Humoral immunity significantly combats
the onset of disease in AKR/J mice as shown by the effect
of immunization of AKR/J mice with syngeneic tumor cells
in producing cytotoxic antibodies against the immunizing
cells (39). Similarly, inoculation of Gross virus-induced
lymphoma cells improves survival of AKR mice challenged

with small doses of syngeneic lymphoma cells (40).

Gershon et al. (23,24) and Kawashima et al. (48) have studied suppressor T-cells and the age-related changes in cell surface antigens of preleukemic AKR thymocytes, respectively. Gershon (23) reports that suppressor T-cells have a short lifespan (3-7 days) and have a suppressor function on B-cells in humoral antibody production. These studies have also shown that newborn mice have a larger percentage of suppressor T-cells than older animals, resulting in a suppressed humoral immune response. Normally, the percentage of suppressor cells decreases in relationship to T-2 helper cells, as the mice get older. Anti-thymocyte globulin (ATG) activation of suppressor cells in 6- to 7-month-old AKR/J mice, hypothetically, could upset a balance between suppressor and helper cells that may have taken six months or longer to develop, resulting in suppression of the immune system and increased susceptibility to viral induced leukemia. This hypothesis is further supported by work of Hatten and Dunton, who found that AKR/J mice develop an imbalance in humoral antibody responses with progressive malignant disease, and that these altered humoral responses were more apparent in older than in younger animals. Fluorescent antibody and radioisotope

studies indicated that fewer anti-thymocyte (ATG)
sensitive cells were present in the spleen and thymus
glands of luekemic animals compared to 2- to 3-month-
old preleukemic AKR/J mice. This is in agreement with the
age-related changes reported by Kawashima et al. (48)
for AKR mice and with the decrease in the number of
suppressor T-cells reported by Gershon et al. (23,24).
The data presented show that treatment of 6- to 7-month-
old AKR/J mice with ATG lowers the rate of survival
as well as the number of ATG-sensitive cells in the
leukemic spleen and lymph nodes, unlike the 2- to 3-
month-old preleukemic mice. Other experimental results
relating to the proportion of T-suppressor and T-helper
lymphocytes and to changes in cell surface antigens with
aging suggest that ATG may result in humoral suppression
by activation of suppressor T-cells. There are other
mechanisms by which ATG treatment of 6- to 7-month-old
AKR/J mice could diminish survival. One such mechanism
may be enhancement of malignant lymphocytes by binding
of ATG to the surfaces of either transformed cells or
precursors of transformed cells. Binding or "coating"
of antibody onto the surfaces of transformed cells
has been shown in some instances of viral induced
malignancies to protect the cell against the
cytotoxic effects of the organism's own cellular

defense mechanisms, notably "killer" T-cells (85).

Since in this particular case it is the T-lymphocytes themselves which become transformed, another possible mechanism by which ATG may result in decreased rates of survival is by a cytotoxic effect on "killer" T-cells directly. Without normal T-lymphocytes to eliminate transformed T-lymphocytes, the onset of leukemia may progress unchecked.

Treatment of 2- to 3-month-old mice with anti-lymphocyte globulins (ALG) resulted in a decreased survival rate. Among the roles reported for ALG is its immunosuppressive capabilities which seem to result in the elimination of long-lived, thymus derived lymphocytes from the circulation (13,66). Cytotoxicity and specific binding of ALG to lymphocytes were demonstrated in this study by trypan blue staining and by fluorescent and radioisotope antibody binding, respectively. ALG also inhibits the mitosis of thymus-dependent paracortical areas in the lymph nodes (123). The question then arises as to why anti-lymphocyte globulin (ALG) treatments lowered the survival rate of 2- to 3-month-old AKR/J mice. Although the present studies do not permit us to answer this question directly, the fact that T-1 cells are known to have a short lifespan and have a suppressor function on B-cells in humoral

antibody production (see Table 1, property 12) while

T-2 cells are long lived, recirculating cells which are

helpers in the immune response to T-dependent antigens

may provide clues to the answer. Since ALG is known to

be a specific agent against the long-lived recirculating

(T-2) population of lymphocytes (13,66), the decreased

survival rates it caused may relate to ALG's cytotoxicity

toward specific helper T-2 cells needed for normal humoral

immune development in the young mouse. Humoral immunity

is effective in combating the onset of leukemia in the

AKR/J mouse, as reviewed previously (39). Thus if the

T-2 cells which help in the production of humoral globulins

are destroyed by ALG, the T-1 cells would suppress B-cell

production of immunoglobulins. Still another explanation

for the decrease in survival rate of ALG-treated 2 - to 3-

month-old mice is based on the lack of specificity of

commercial ALG for T-2 cells. If the initial cells used

for immunization and development of ALG antibody contained

B-cells as well as T-2 cells, the ALG resulting from

immunization would also bind B-lymphocytes. After

confering with the manufacturer (Microbiological Associates)

of ATG and ALG, it is clear that this possibility cannot

be eliminated since whole spleen lymphocytes were used for

immunizations. Because B-cells ultimately differentiate

into plasma cells and produce antibody, cytotoxic activity

of ALG on B-cells would also be expected to severely

hinder the 2- to 3- month-old mouse's ability to develop

a humoral immune response against the malignancy. The

increase in survival of 6- to 7- month-old AKR/J mice

treated with ALG may be due to the direct cytotoxic

effects upon rapidly dividing tumor cells. This concept

is supported by the cytotoxicity of ALG demonstrated

in vitro (Table XI) and the specific cytotoxic effect of

such antisera on helper T-2 cells that are antigenically

identical to AKR/J tumor cells according to others (13,

18,66).

Treatment of both 2- to 3- month-old and 6- to 7- month-

old AKR/J mice with rabbit anti-mouse brain serum (RAMB)

resulted in increased death rates in both groups.

The possibility exists that repeated immunizations with

whole rabbit serum may have resulted in the production

of antibodies against rabbit serum proteins that are

immunogenic. Although no animals were visibly affected

(no kidney enlargement was noted) or died of anaphylactic

reactions as far as is known, the secondary effects of

immune complexes, complement, and other immune factors on

the survival rate of RAMB-treated groups must be considered.

Any possible contribution or influence by whole rabbit

serum on the survival rates of 2- to 3- or 6- to 7-
month-old AKR/J mice would be reflected only in the
in vivo studies and should not have influenced studies
conducted in vitro to any great degree. In vitro studies
include latex agglutination, fluorescent antibody, cytotox-
icity, and radioisotope-labeled antibody evaluations.
Development of antibody directed against an immunogen
from another source can be a problem whenever repeated
immunizations or injections are performed. The use of
anti-lymphocyte globulins (ALG) and anti-thymocyte
globulins (ATG), prepared from rabbit antisera, minimizes
the risk if specific antibodies developing because they
are much less effective antigens than the material from
which they were prepared, and can be used in smaller
quantities. Specific binding of RAMB immunoglobulins to
the surface of lymphocytes with dense amounts of theta (θ)
antigen has been shown by Filppi et al. (18). This same
theta-rich lymphocyte has been suggested by many to be the
precursor fo the AKR/J lymphoma cell (48,60), and, therefore,
based on the increased survival rate of ATG-treated 2- to
3-month-old mice, the same increase in survival was
expected with 2- to 3-month-old RAMB-treated mice. One
possible explanation for the lack of protection afforded
by RAMB antisera in 2- to 3-month-old mice may be related
to the length of time injections were administered

which could lead to secondary physically harmful effects as a result of antibody development. Six to seven month-old AKR/J mice treated with RAMB sera received only a small number of injections before the onset of leukemia and, therefore, would not be likely to have developed much of an antibody titer to rabbit serum proteins. Bron and Sauser (5) have demonstrated that Thy-1 antigen, for which ATG is specific, is only found on theta positive T-lymphocytes. Rabbit anti-mouse brain (RAMB) sera is directed against the theta antigen and would, therefore, be expected to bind to the same cells as anti-thymocyte globulins (ATG). However, this is where the similarity ends between ATG and RAMB. Anti-thymocyte globulins are xeno specific anti-globulins, prepared in rabbits against Swiss Webster whole thymocytes. Anti-globulins are directed against the basic structure common to Thy 1.1, 1.2, and brain tissues, but not the carbohydrate portion of the molecule that differs among them. Rabbit anti-mouse brain sera were prepared in rabbits injected with emulsified brain tissue from AKR/J mice and contains antibodies reactive with theta (θ) antigen (18). The possibility exists, therefore, that RAMB sera may act by recognition of an antigen or antigens unique to the AKR strain or species specific. These antigens could not be recognized by the

xeno specific ATG globulins, thus explaining the possible
difference in specificity between the two. No activation
of suppressor T-cells has been reported for RAMB sera,
as reported by Gelfand and Paul (21) for ATG. However,
since both RAMB and ATG are directed against surface
antigens present on the same cells, activation of
suppressor T-cells may be one explanation for the decrease
in survival rates demonstrated by both ATG and RAMB
treated 6- to 7-month-old AKR/J mice.

Specific findings reported here may be summarized as
follows: In vivo studies have shown that anti-thymocyte
globulins (ATG) prolong the survival rate of AKR/J mice
when initiated at 2 to 3 months of age. Literature has
reported the specificity of ATG for a young precursor of
the leukemia cell (5,18,48,60,110,114), suggesting that
viral induced leukemia in the AKR/J mouse can be delayed
by the reduction of tumor cell precursors. Treatment of
6 to 7 month-old AKR/J mice with ATG has been shown to
decrease the survival rate. It has been reported that
ATG suppresses humoral immunity by activation of suppressor
thymus derived lymphocytes (21,23,24), and this loss of
antibody defense may be one method by which leukemia
can be induced. The decreased rate of survival of 2- to 3-
month-old AKR/J mice treated with anti-lymphocyte globulins

(ALG) supports the role of humoral immunity in delaying the onset of disease. ALG has been shown to inhibit mitosis and to be cytotoxic to helper T-cells needed for B-cell development of tumor specific antibody (13,18,66,123). When 6-to 7-month-old AKR/J mice were treated with ALG, however, survival improved. It has been reported that the AKR/J tumor cells are antigenically identical to helper T-cells (18), suggesting that ALG can increase survival by the direct cytotoxic destruction of tumor cells.

Methods have been presented for both increasing and decreasing the rate of onset of leukemia in AKR/J mice by administering specific antisera in vivo. Binding of antisera and cytotoxicity to lymphocytes has also been demonstrated. Based on the reported specificities and activities of these antisera, possible mechanisms have been outlined for their influence on the survival of AKR/J mice. The age of groups of animals when treatments were initiated was shown to be a significant determinant of the rate of survival, thus, opposite effects were noted at different ages. In addition, the selection of antisera with different cell specificities was shown to result in completely opposite effects at two different ages. Results show that injection of anti-thymocyte

globulins and anti-lymphocyte globulins in AKR/J mice
are useful for their effect on tumor activation or
suppression. Therefore, onset of viral induced leukemia
in AKR/J mice is related to aging of the immune system.
Treatment with specific anti-globulins is dependent
upon both the specific anti-globulins used and the age
of the animal when treatments are initiated.

BIBLIOGRAPHY

1. Araneo, B.A., P.C. Marrack, and J.W. Kappler, 1975. Functional heterogeneity among the T-derived lymphocytes of the mouse. II. Sensitivity of subpopulations to anti-mouse thymocyte serum. Journal of Immunology 114: 747.

2. Baas, E.J. and D.E. Jasper, 1972. Agar block technique for identification of mycoplasma by use of fluorescent antibody. Applied Microbiology 23: 1097.

3. Bartholomaeus, W.N., A.E. Bray, J.M. Papadimitriou and D. Keast, 1974. Immune response to a transplantable malignant melanoma in mice. National Cancer Institute 53: 1065.

4. Bourne, H.R., L.N. Lichtenstein, K.L. Melmon, C.S. Henney, Y. Weinstein, and G.M. Shearer, 1974. Modulation of inflammation and immunity by cyclic AMP. Science 184: 19.

5. Bron, C. and D. Sauser, 1972. Heterologous antiserum to mouse thymus derived lymphocytes. Journal of Immunology 110: 384.

6. Burnet, F.M. and M.C. Holman, 1962. Immunological function of thymus and bursa of fabricius. Nature 194: 146.

7. Campbell, B.H., J.S. Garvey, N.E. Cremer, and D.H. Sussdorf, 1970. ^{125}I or ^{131}I-labeled bovine serum albumin. Methods in Immunolgoy, p. 1-454, W.A. Benjamin, Inc., New York.

8. Canton, H. and R. Asofsky, 1971. Synergy among lymphoid cells mediating the graft-versus-host response. III. Evidence for interaction between two types of thymus-derived cells. Journal of Experimental Medicine 135: 764.

9. Claman, H.N., 1973. The new cellular immunology. BioScience 23: 576.

10. Claman, H.N. and D.E. Mosier, 1972. Cell-cell interactions in antibody production. Progr. Allergy 16: 40.

11. Clark, H.F. and C.C. Shepard, 1963. A dialysis technique for preparing fluorescent antibody. Virology 20: 642.

12. David, J.R., S. Al-Askari, H.S. Lawrence, and L. Thomas, 1964. Delayed hypersensitivity in vitro. I. The specificity of inhibition of cell migration by antigens. Journal of Immunology 93: 264.

13. Denman, A.M., E.J. Denman, and P.H. Embling, 1968. Changes in the life span of circulating small lymphocytes in mice after treatment with anti-lymphocyte globulin. Lancet 1: 321.

14. Doell, R.G., C. deVaux St. Cyr, and P. Grabar, 1967. Immune reactivity prior to development of thymic lymphoma in C57BL mice. International Journal of Cancer 2: 103.

15. Durie, B.G.M. and S.E. Salmon, 1975. High speed scintillation autoradiography. Science 190: 1093.

16. Epstein, L.B. and R.A. Knight, 1975. Studies on mouse maloney virus induced tumors. II. Detection of p30 in the serum of mice with maloney leukemia by in vitro blocking of complement dependent antibody mediated cytotoxicity. British Journal of Cancer 31: 513.

17. Fernandez, L.A., J.M. MacSween, and G.R. Langley, 1975. Separation of T lymphocytes from normal individuals and patients with B lymphocyte chronic lymphocyte leukemia. Immunology 28: 231.

18. Filppi, J.A., M.S. Rheins, and R.L. St. Pierre, 1975. Invivo effects of rabbit anti-mouse brain serum on theta bearing lymphocytes of AKR mice. Immunology 28: 659.

19. Furth, J. and M.C. Boon, 1945. The time and site of origin of the leukemic cell. A.A.A.S. Research Conference of Cancer, AMerican Association for the Advancement of Science, Washington, D.C., 129.

20. Galili, U. and M. Schlesinger, 1975. Subpopulations of human thymus cells differing in their capacity to form E-rosettes and in their immunologic reactivity. Journal of Immunology 115: 827.

21. Gelfand, M.C. and W.E. Paul, 1975. Prolongation of allograft survival in mice by administration of anti-thy 1 serum. I. Mediation by in vivo activation of regulatory T cells. Journal of Immunology 115: 1.

22. George, M. and J.H. Vaughan, 1962. In vitro cell migration as a model for delayed hypersensitivity. Proceedings of the Society for Experimental Biology and Medicine 111: 514.

23. Gershon, R.K., P. Cohen, R. Hencin, and S.A. Liebhaver, 1972. Suppressor T-cells. Journal of Immunology 108: 586.

24. Gershon, R.K., P.H. Maurer, and C.F. Merryman, 1973. A cellular basis for genetically controlled immunologic unresponsiveness in mice: tolerance induction in T-cells. Proceedings of the National Academy of Science 70: 250.

25. Gottlieb, A.A. and R.H. Schwartz, 1972. Review antigen-RNA interactions. Cellular Immunology 5: 341.

26. Green, E.L., 1975. Handbook on genetically standardized JAX mice. Bar Harbor Times Publishing Company, Bar Harbor, Maine.

27. Green, M., 1970. Oncogenic viruses. Annual Review of Biochemistry 39: 701.

28. Gross, L., 1951. "Spontaneous" leukemia developing in C3H mice following inoculation in infancy with AK leukemic extracts of AK embryos. Proceedings of the Society of Experimental Biology and Medicine 76: 27.

29. Gross, L., 1957. Development and serial cell-free passage of a highly potent strain of mouse leukemia virus. Proceedings of the Society of Experimental Biology and Medicine 94: 767.

30. Gross L., 1970. Oncogenic Viruses. 2nd Edition, pp. 24. Pergamon Press, Oxford.

31. Hammerling, U., C. Mack and H.G. Pickel, 1976. Immunofluorescence analysis of Ig determinants of mouse thymocytes and T cells. Immunochemistry 13: 525.

32. Hammerling, U., H.G. Pickel, C. Mack, and D. Masters, 1976. Immunochemical study of an immunoglobulin-like molecule of murine thymocytes. Immunochemistry 13, 533.

33. Hargis, B.J. and S. Malkiel, 1972. The immunocapacity of the AKR mouse. Cancer Research 32: 291.

34. Hartley, J.S., W.P. Rowe, and R.J. Huebner, 1970. Host range restrictions of murine leukemia viruses in mouse embryo cell culture. Journal of Virology 5: 221.

35. Hayry, P. and L.C. Andersson, 1975. Generation of T memory cells in one-way mixed lymphocyte culture. III. Homing and lifetime of "secondary" lymphocytes. Cellular Immunology 17: 165.

36. Hays, E.F., 1972. Immune response in AKR mice. Their relationship to the development of spontaneous leukemia in this strain. In: Virus Tumorigenesis and Immunogenesis, p. 321. Ceglowski & Friedman Eds., W.C. Benjamin, Inc., New York.

37. Hellstrom, I., 1967. A colony inhibition (CI) technique for demonstration of tumor cell destruction by lymphoid cells in vitro. International Journal of Cancer 2: 65.

38. Hellstrom, I. and K.E. Hellstrom, 1969. Colony inhibition and cytotoxicity assays. International Journal of Cancer 4: 581.

39. Hilgers, J., A. Decleve, J. Galesloot, and H.S. Kaplan, 1974. Murine leukemia virus group-specific antigen expression in AKR mice. Cancer Research 34: 2553.

40. Hodes, R.J., K.S. Hathcock, and G.M. Shearer, 1975. Synergy between subpopulations of normal mouse spleen cells in the in vitro generation of cell-mediated cytotoxicity specific for "modified self" antigens. Journal of Immunology 115: 1122.

41. Hudson, B.W., 1961. Column adsorption of fluorescein-isothiocyanate-labelled antibodies. Bulletin of World Health Organization 24: 291.

42. Humphrey, G.B., J. Lankford, S. Oleinick, and R. Nitschke, 1974. Leukemic serum inhibition of phytohemagglutinin-induced transformation. Journal of Immunology 113: 63.

43. Hunter, W.M. and F.C. Greenwood, 1962. Preparation of iodine-131 labelled human growth hormone of high specific activity. Nature 194: 495.

44. Ihle, J.N., J.J. Domotor, Jr. and K.M. Bengali, 1976. Characterization of the type and group specificities of the immune response in mice to murine leukemia viruses. Journal of Virology 18: 124.

45. Judd, K.P., C.J. Bryant, and J.J. Trentin, 1971. Organ specificity of antithymocyte globulin. Clinical Experimental Immunology 9: 499.

46. Karpf, M., M.C. Gelfand, B.S. Handwerger, and R.H. Schwartz, 1975. Lack of B lymphocyte depletion from murine spleen cell populations by a human gamma globulin, anti-human gamma-globulin column system. Journal of Immunology 114: 542.

47. Kato, S., M. Miyamoto, and K. Tsuru, 1966. Interactions of ectromelia virus with macrophages and ascites tumor cells from normal and immune mice. Biken Journal 9: 219.

48. Kawashima, K., H. Ikeda, E. Stockert, T. Takahashi, and L. Old, 1976. Age-related changes in cell surface antigens of preleukemic AKR thymocytes. Journal of Experimental Medicine 144: 193.

49. Khan, A., D. Thometz, and J.M. Hill, 1975. A scoring method for E-rosettes: enhanced E-rosette formation by hodgkin's disease lymphocytes with transfer factor. Wadley Medical Bulletin 5: 297.

50. Komuro, K., and E. Boyse, 1973. In vitro demonstration of thymic hormone in the mouse by conversion of precursor cells into lymphocytes. Lancet 740.

51. Krause, P.F., and M.K. Patterson Jr., 1974. Tissue Culture Methods and Applications, p. 346. Academic Press, Inc., New York.

52. Kruger, J. and R.K. Gershon, 1972. DNA synthetic response of thymocytes to a variety of antigens. Journal of Immunology 108: 581.

53. Kucich, U.N., J.C. Bennett, and B.J. Johnson, 1975. The protein nature of the thy-1.1 alloantigen as expressed by the murine lymphoblastoid line S-49.1TB 2-3. Journal of Immunology 115: 626.

54. Kwapinski, J.B.G., 1972. Methodology of Immunochemical and Immunological Research, pp. 594-600. John Wiley and Sons, Inc., New York.

55. Leong, S., C.M. Sutherland, and E.T. Krementz, 1977. changes in distribution of human malignant melanoma membrane antigens in the presence of human antibody by immunofluorescence. Cancer Research 37: 293.

56. Lilly, F., M.L. Duran-Reynals, and W.P. Rowe, 1974. Murine leukemia virus and spontaneous leukemia in AKR mice. In: Mechanisms of Virus Disease, 1. IGN-UCLA symposia on molecular and cellular biology, p. 245. William S. Robinson and C. Cox Eds., W.C. Benjamin, Inc., New York.

57. Lipsky, P.E. and A.B. Rosenthal, 1975. Macrophage-lymphocyte interaction II. Antigen-mediated physical interactions between immune guinea pig lymph node lymphocytes and syngeneic macrophages. Journal of Experimental Medicine 141: 138.

58. Lowy, D.R., S.K. Chattopadhyay, N.M. Teich, W.P. Rowe, and A.S. Levine, 1974. AKR murine leukemia virus genome: Frequency of sequences in DNA of high-, low-, and non-virus-yielding mouse strains. Proceedings of the National Academy of Science 71: 3555.

59. Lucan, Z.J. and S.M. Walker, 1974. Cytotoxic activity of lymphocytes III. Standardization of measurement of cell-mediated lysis. Journal of Immunology 113: 209.

60. Lukaseqycz, O.A., D. Martinez, and W.H. Murphy, 1975. Immune mechanisms in leukemia; Evaluation of immuno-competent cell populations. Journal of Immunology 114: 1491.

61. Makinodan, T. and W.H. Adler, 1975. Effects of aging on the differentiation and proliferation potentials of cells of the immune system. Federation Proceedings 34: 153.

62. Mann, D.L., G.N. Rogentine, J.L. Fahey, and S. Nathenson, 1969. Human lymphocyte membrane (HLA) alloantigens: isolation, purification and properties. Journal of Immunology 103: 282.

63. Mantani, M. and S. Kato, 1970. Virus multiplication in culture cells synchronized by excess thymidine treatment. II. Effect of viral DNA synthesis of shope fibroma virus and cowpox virus upon nuclear DNA synthesis of synchronized FL cells. Biken Journal 13: 365.

64. Mantani, Masanobu, Y. Sakaue, and S. Kato, 1970. Virus multiplication in culture cells synchronized by excess thymidine treatment. I. Effect of excess thymidine upon growth of poxviruses and chikungunya virus. Biken Journal 13: 353.

65. Martig, R.J. and J.L. Tribble, 1974. Cell-mediated immune responses of preleukemic AKR mice. Infection and Immunity 10: 1018.

66. Martin, W.J. and F.A.P. Miller, 1968. Cell to cell interaction in the immune response. IV. Site of action of antilymphocyte globulin. Journal of Experimental Medicine 128: 855.

67. Mc Cammon, J.R., 1976. Radioimmunofluorescent antibody technique for detection of reovirus antigen in cell culture. Infection and Immunity 14: 811.

68. Metcalf, D., 1966. Histologic and transplantation studies on preleukemic thymus of the AKR mouse. Journal of the National Cancer Institute 37: 425.

69. Metcalf, D. and R. Moulds, 1967. Immune responses in pre-leukemic and leukemic AKR mice. International Journal of Cancer 2: 53.

70. Miller, R.G. and M. Dunkley, 1974. Quantitative analysis of the ^{51}Cr release cytotoxicity assay for cytotoxic lymphocytes. Cellular Immunology 14: 284.

71. Miller, T.E. and E. Creaghe, 1975. Quantitation of potential T-lymphocyte function in rats. Infection and Immunity 12: 722.

72. Mills, B., L. Sen, and L. Morella, 1975. Reactivity of antihuman thymocyte serum with acute leukemic blasts. Journal of Immunology 115: 1038.

73. Milnick, J.L., 1975. Taxonomy of viruses, Progressive Medical Virology 19: 353.

74. Mohanakumar, T., D.S. Miller, and R.S. Metzgar, 1976. Human acute myelomonocytic leukemia: serologic studies with simian antisera. Blood 48: 339.

75. Mond, J.J., T. Takahashi, and G.J. Thorbecke, 1972. Thymus derived cell (T cell) activation by heterologous antigens as a replacement of specific immune T cells in the transfer of the secondary response to sheep erythrocytes. Journal of Experimental Medicine 136: 715.

76. Morton, H.E., 1966. Mycoplasma-latex agglutination reaction. Journal of Bacteriology 92: 1196.

77. Mosier, D.E. and P.L. Cohen, 1975. Ontogeny of mouse T-lymphocyte function. Federation Proceedings 34: 137.

78. Mosier, D.E. and B.M. Johnson, 1975. Ontogeny of mouse lymphocyte function. II. Development of the ability to produce antibody is modulated by T-lymphocytes. Journal of Experimental Medicine 141: 216.

79. Murmatsu, S., T. Amagai, and Y. Katsura, 1975. Tolerance induction in TxXBT and TxXB mice. Immunology 28: 943.

80. Murphy, W.H., C. Bullis, L. Dabich, R. Heyn, and C.J.D. Zarafonetis, 1970. Isolation of mycoplasma from leukemic and nonleukemic patients. Journal of National Cancer Institute 45: 243.

81. Nagaya, H., 1973. Thymus function in spontaneous lymphoid leukemia. II. In vitro response of "preleukemic" and leukemic thymus cells to mitogens. Journal of Immunology 111: 1052.

82. Nagaya, H., 1973. Thymus function in spontaneous lymphoid leukemia. I. Premature leukemogenesis in "young" thymectomized mice bearing "old" thymus grafts. Journal of Immunology 111: 1048.

83. Neely, J.E. and J.W. Combs, 1976. Variation in the autoradiographic technique: I. Emulsion-developer combinations assessed by photometric measurement of single silver grains. Journal of Histochemistry and Cytochemistry 24: 1057.

84. Notkins, A.L., 1970. Effect of virus infections on immune function. Immunopathology 6th International Symposium. Ed. P.A. Miescher, Grune & Stratton, New York.

85. Notkins, A.L. and H. Koprowski, 1973. How the immune response to a virus can cause disease. Scientific American 228: 22-31.

86. Old, L., A. Boyse, and E. Stockert, 1965. The G (gross) Leukemia antigen. Cancer Research 25: 813.

87. Oldstone, M.B.A., B.C. Del Villano, and F.J. Dixon,
     1976. Autologous immune responses to the major
     oncornavirus polypeptides in unmanipulated AKR/J
     mice. Journal of Virology 18: 176.

88. Oldstone, M.B.A., A. Tishon, J.M. Chiller, W.O.
     Weigle, and F.J. Dixon, 1973. Effect of chronic
     viral infection on the immune system. I. Comparison
     of the immune responsiveness of mice chronically
     infected with LCM virus with that of noninfected
     mice. Journal of Immunology 110: 1268.

89. Owen, F.L., G.M. Bernier, and M.W. Fanger, 1976.
     Studies on human T-lymphocyte population. V. Isolation
     of T-lymphocyte antigens from human renal failure
     serum. Immunochemistry 13: 129.

90. Owen, F.L. and M.W. Ganger, 1976. Studies on the
     human T-lymphocyte population. IV. The isolation
     of T-lymphocyte antigens from peripheral lymphocytes.
     Immunochemistry 13: 121.

91. Pattengale, P.K., R.W. Smith, and P. Gerber, 1974.
     B-cell characteristics of human peripheral and cord
     blood lymphocytes transformed by epstein-barr virus.
     Journal of the National Cancer Institute 52: 1081.

92. Peter, H.H. and J.D. Feldman, 1972. Cell mediated
     cytotoxicity during rejection and enhancement of
     allogeneic skin grafts in rats. Journal of Exper-
     imental Medicine 135: 1301.

93. Peterson, R.D., R. Henrickson, and R.A. Good, 1963.
     Reduced antibody forming capacity during incubation
     period of passage A leukemia in C3H mice. Proceedings
     of the Society for Experimental Biology and Medicine
     114: 517.

94. Phillips, H.J., 1975. Dye exclusion tests for cell
     viability. In: Tissue Culture Methods and Applications,
     p. 380. Ed. P.A. Miescher, Grune & Stratton, New York.

95. Pincus, H.I.T., T. Yoshiki, M. Strand, J.T. August,
     E.A. Boyse and R.C. Mellor, 1974. Biological
     expression of antigenic determinants of murine leukemia
     virus proteins gp69/71 and p30. Journal of Virology
     14: 1274.

96. Raff, M.C., 1971. T and B lymphocytes in mice studied by using antisera against surface antigenic markers. American Journal of Pathology 65: 467.

97. Raff, M.C., and Canton, H., 1971. Thymocytes and thymus derived lymphocytes. In: Progress in Immuno-biology, Ed. B. Ames, p. 33, Academic Press, Inc., New York.

98. Reif, A.E. and J. Allen, 1964. The AKR thymic antigen and its distribution in leukemias and nervous tissues. Journal of Experimental Medicine 120: 413.

99. Riggs, J.L., P.C. Loh, and W.C. Eveland, 1961. A simple fractionation method for preparation of fluorescein-labeled gamma globulin. Society for Experimental Biology and Medicine 105: 655.

100. Roelants, G.E., F. Loor, H. von Boehmer, J. Sprent, L.B. Hagg, K.S. Mayor and A. Ryden, 1975. Five types of lymphocytes characterized by double immuno-fluorescence and electrophoretic mobility organ distribution in normal and nude mice. European Journal of Immunology 5: 127.

101. Roman, T.J. and G.J. Thorbecke, 1975. Thymus influence on the conversion of 19S to 7S antibody formation in the response to TNP-Brucella. Journal of Immunology 115: 332.

102. Roque, A.L., Jafarey, A. Naeem, and P. Coulter, 1965. A stain for the histochemical demonstration of nucleic acids. Experimental and Molecular Pathology 4: 266.

103. Rowe, W.P., J.W. Hartley, and W.I. Capps, 1966. Tissue culture and serologic studies of mouse leukemia viruses. National Cancer Institute Monographs 22: 15.

104. Rowe, W.P. and T. Pincus, 1972. Quantitative studies of naturally occurring murine leukemia virus infection of AKR mice. Journal of Experimental Medicine 135: 429.

105. Ruuskanen, O., 1975. Subpopulations of pig thymocytes. Different distribution patterns of alkaline phos-phatase positive and negative autologous thymocytes. Cellular Immunology 15: 246.

106. Ruuskanen, O. and K. Kouvalainen, 1974. Differentia-
      tion of thymus and thymocytes. A study in foetal
      guinea-pigs using alkaline phosphatase as a label
      of thymocytes. Immunology 26: 186.

107. Ruuskanen, O.J., L.J. Pelliniemi, and K.E.
      Kouvalainen, 1975. Alkaline phosphatase in
      differentiating guinea pig thymocytes: an ultra-
      cytochemical study. Journal of Immunology 114: 1611.

108. Sautes, C.N., D. Dupuis, and W.H. Friedman, 1975.
      Specificity of Fc receptors of activated T cells
      relation with released immunoglobulin-binding
      factor. European Journal of Immunology 5: 849.

109. Schlesinger, M. and I. Yron, 1969. Antigenic changes
      in lymph-node cells with administration of antiserum
      to thymus cells. Science 164: 1412.

110. Schlesinger, M. and I. Yron, 1970. Serologic
      demonstration of a thymus dependent population of
      lymph node cells. Journal of Immunology 104: 798.

111. Schlossman, S.F. and L. Hudson, 1973. Specific
      purification of lymphocyte populations on a digest-
      able immunoabsorbent. Journal of Immunology 104: 798.

112. Schuman, G. and C. Moroni, 1976. Mitogen induction
      of murine C-type viruses. I. Analysis of lymphoid
      cell subpopulations. Journal of Immunology 116: 1145.

113. Shearer, W.T. and C.W. Parker, 1975. Humoral
      immunostimulation. V. Selection of variant cell
      lines. Journal of Experimental Medicine 142: 1133.

114. Shiki, H., P. Kisielow, M. Bean, T. Takahashi, E.
      Boyse, H. Oettgen, and L. Old, 1975. Expression of
      T-cell differentiation antigens on effector cells
      in cell-mediated cytotoxicity in vitro. Evidence
      for functional heterogeneity related to the surface
      phenotype of T cells. Journal of Experimental
      Medicine 141: 227.

115. Siegler, R. and M. Rich, 1963. Unilateral histogenesis
      of AKR thymic lymphoma. Cancer Research 23: 1669.

116. Srivastava, K.K., M. Pollard, and M. Wagner, 1976.
      Bacterial decontamination and antileukemic therapy
      of AKR mice. Infection and Immunity 14: 1179.

117. Staats, J., 1964. Standardized nomenclature for inbred strains of mice third listing. Cancer Research 24: 147.

118. Stevenson, G.T., R.P. Eady, D.W. Bough, R.D. Jurd, and F.K. Stevenson, 1974. Surface immunoglobulin of guinea pig leukaemic lymphocytes. Immunology 28: 807.

119. Stobo, J.D. and W.E. Paul, 1973. Functional heterogeneity of murine lymphoid cells. III. Differential responsiveness of T cells to phytohemagglutinin and concanavalin A as a probe for T cell subsets. Journal of Immunology 110: 362.

120. Stout, R.D. and L.A. Herzenberg, 1975. The Fc receptor on thymus-derived lymphocytes II. Mitogen responsiveness of T lymphocytes bearing the Fc receptor. Journal of Experimental Medicine 142: 1041.

121. Tanenbaum, R. and D. Sulitzeanu, 1974. Enrichment of memory cells carrying receptors for a protein antigen (HSA) II. Improved enrichment technique, using BSA density gradiant separation. Immunology 29: 409.

122. Tuffrey, M., P. Crewe, L. Dawson, J. Holliday, and R.D. Barnes, 1975. Murine leukaemia virus expression in the AKR following thymectomy. British Journal of Cancer 32: 465.

123. Turk, J.L. and D.A. Willoughby, 1967. Central and peripheral effects of ALS. Lancet 1: 249.

124. Waksal, S.D., S. Smolinsky, I.R. Cohen, R.L. St. Pierre, and M. Feldman, 1975. Thymocyte maturation in AKR leukemia. Advances in Experimental Medicine and Biology 66: 141.

125. Walter, H. and H. Nagaya, 1975. Separation of human rosette and nonrosette forming lymphoid cells by counter-current distribution in an aqueous two-phase system. Cellular Immunology 19: 158.

126. Wettstein, M.F. and E.P. Hays, 1970. Immune response in preleukemic mice. Infection and Immunity 1: 398.

127. Willers, J.M.N. and E. Sluis, 1975. The influence of cyclophosphamide on antibody formation in the mouse. Ann. Immunology (Institute Pasteur) 126C: 267.

128. Wilson, M.B. and P.K. Nakane, 1976. The covalent coupling of proteins to periodate-oxidized sephadex: a new approach immunoadsorbent preparations. Journal of Immunological Methods 12: 171-181.

129. Wolstenholme, G.E.W. and M.O. O'Connor, 1967. In anti-lymphocytic serum. Ciba Foundation Study Group No. 29, little, Brown & Co., Boston.

130. Zata, M.M., 1975. Lymphocyte populations of AKR/J mice. III. Changes in the preleukemic state. Journal of Immunology 115: 1168.