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To the Graduate Council:

I am submitting herewith a thesis written by Stephen Vernon Lair entitled "Cytotoxicity of Phytohemagglutinin (PHA) and PHA-stimulated Spleen Cells on Rauscher Leukemia Virus Infected Cells." I recommend that it be accepted for nine quarter hours of credit in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

Major Professor

We have read this thesis
and recommend its acceptance:

Arthur Brown

Carl J. Wirth

Accepted for the Council:

Hilton A. Smith
Vice Chancellor for
Graduate Studies and Research

CYTOTOXICITY OF PHYTOHEMAGGLUTININ (PHA) AND PHA-STIMULATED SPLEEN CELLS ON RAUSCHER LEUKEMIA VIRUS INFECTED CELLS

A Thesis

Presented to

the Graduate Council of

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Master of Science

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by

Stephen Vernon Lair

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ABSTRACT

The cytotoxicity of phytohemagglutinin (PHA) for mouse bone marrow colony forming cells was studied. Bacto-PHA inhibited the number of granulocytic colonies formed by bone marrow cells from normal and Rauscher leukemia virus infected animals. However, PHA was more cytotoxic for leukemic than for normal cells at most concentrations. The cytotoxicity of two partially purified preparations of phytohemagglutinin, Seeney Mitogen A and Wellcome MR 69, was also tested. Seeney Mitogen A was found to be less cytotoxic than Bacto-PHA while Wellcome MR 69 was extremely cytotoxic even at low concentrations.

The possible in vivo cytotoxic effect of PHA was also studied. To this end, Rauscher virus infected mice were given transfers of normal or PHA-stimulated spleen cells. A single transfer of PHA-stimulated cells more than tripled the survival rate and a transfer of normal spleen cells almost doubled the survival of infected mice. Survival was further extended by giving multiple rather than single transfers. Multiple transfers of sonicated normal or sonicated PHA-stimulated spleen cells also significantly extended survival. This finding indicated that cell viability and integrity are not essential for this type of treatment of Rauscher disease. Possible mechanisms of action by the transfers are discussed.

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PART I. IN VITRO CYTOTOXICITY OF PHYTOHEMAGGLUTININ (PHA) FOR MOUSE BONE MARROW COLONY FORMING CELLS FROM NORMAL AND RAUSCHER VIRUS INFECTED MICE

CHAPTER I

INTRODUCTION

Phytohemagglutinin (PHA) is a glycoprotein (18) extract of the red kidney bean, Phaseolus vulgaris, which has several biological activities. PHA causes the agglutination of erythrocytes (15) and leukocytes (30), but of more interest is its ability to cause small lymphocytes to undergo blastogenesis and mitotic division (12).

The in vivo effects of PHA have recently been reviewed by Robbins (37), Naspitz and Richter (29), and Lozzio (18). When PHA is added to leukocyte cultures, changes in small lymphocytes are noted within 24 hours and large blast cells appear within 48 and 72 hours of culture. These blast cells are quite large and have a deeply basophilic cytoplasm and a large nucleus. The chromatin pattern within the nucleus is usually finely stippled and one or more nucleoli are invariably found (8). The blast cells undergo frequent division and numerous mitotic figures are seen between 48 and 72 hours. PHA is capable of stimulating blastogenesis and mitosis in a variety of lymphoid cells from humans and animals. These include thymic lymphocytes, peripheral white blood cells, thoracic duct cells, lymph node cells, tonsillar cells, spleen cells, appendix cells, and bone marrow cells (29).

Several metabolic alterations are observed in PHA-stimulated lymphocytes. The stimulated lymphocytes show an early alteration in phospholipid metabolism (9), increased histone acetylation (34),

increased RNA synthesis (26, 34), increased protein synthesis (28), an increased rate of glycolysis (35), and an increased rate of DNA synthesis (26).

Recently it has been established that PHA also affects a variety of nucleated cells other than lymphocytes (14, 17, 18, 22, 24). In this context, the addition of PHA to leukocytes in culture causes granulocytes to degenerate while lymphocytes undergo blastogenesis (18, 29, 37). Also, PHA is known to inhibit the growth of granulocytic colonies in agar (3). Unlike PHA-stimulated lymphocytes, granulocytes (equine) exposed to PHA show diminished histone acetylation and diminished RNA synthesis (33).

The effects of in vivo administration of PHA have also been investigated (6, 16-18, 22-25, 29). The intradermic injection of PHA into unsensitized mice produces a reaction analogous to a typical delayed hypersensitivity reaction or Arthus-like phenomenon while adsorbed (hemagglutinin-free) PHA can be given intravenously without major toxic effects (18). The parenteral administration of PHA produces a depression of the reticuloendothelial phagocytic activity (17, 23), depression of antibody formation after antigenic stimulation (25, 29), a stimulation of normal mouse splenic hematopoiesis and of hematopoietic spleen colony formation after sublethal irradiation (6, 16, 18), and the transformation of a significant portion of lymphocytes to undifferentiated and immature plasmacytoid cells (18, 23, 25, 29). Adult mice given PHA also develop atrophy of the thymus and hypertrophy of the lymph nodes (6, 17, 18, 22-25). PHA produces neutrophilia and lymphopenia in mice with the majority of granulocytes

showing enlargement and hypersegmentation (18, 19, 23). The in vivo administration of PHA has also been shown to retard the onset of deaths in animals infected with Rauscher leukemia virus (20, 21, 38).

The degeneration and diminished syntheses of granulocytes exposed to PHA in vitro suggest a toxic effect of PHA on mature granulocytes. However, only recently has it been possible to determine the effect of PHA on myelopoietic precursor cells. The introduction of a technique for the culture of bone marrow cells in soft agar (2) has provided a new assay system for the effect of PHA on mouse colony forming cells. When mouse bone marrow cells are cultured using this technique, the colony forming cells proliferate and partially differentiate to form granulocytic colonies within the soft agar.

Previous studies (3) have indicated that BAIB/c mice infected with Rauscher leukemia virus form more than twice as many granulocytic colonies as non-infected control mice. The results reported herein confirm this observation. The addition of various amounts of PHA was found to inhibit the number of colonies from both normal and leukemic bone marrow. However, the inhibition was greater for leukemic than for normal bone marrow cells. Two partially purified mitogenic preparations of PHA were also tested in hopes of finding a selectively cytotoxic mitogen. A selectively cytotoxic preparation would be of interest for possible in vivo treatment of Rauscher disease.

CHAPTER II

MATERIALS AND METHODS

1. Virus and Mice

The original animal passage of Rauscher leukemia virus (RLV) contaminated with lactic dehydrogenase virus was obtained from Dr. R. L. Tyndall of the Oak Ridge National Laboratory. The virus was serially passaged in six to ten week old BALB/c mice of both sexes as a 10% cell-free spleen extract. The extract was prepared by homogenization of the spleens in Ringer's solution. The suspensions were then centrifuged at 5000 rpm for 15 minutes and the supernatant was collected. Mice were injected intraperitoneally with 0.3 ml of the leukemic spleen extract containing approximately 10^3 to 10^4 ID_{50}/ml of the virus as determined by the spleen weight assay (5). The increase in spleen weight after RLV inoculation has been shown to be directly proportional to the amount of virus given and, therefore, spleen weights are used to determine the virus titer. In this assay the ID_{50} is defined as the dose of virus that will induce palpable spleens in 50% of the inoculated animals.

The BALB/c mice used in this study were obtained from a colony maintained at the University of Tennessee Memorial Research Center. The colony had been inbred for more than 130 generations at the time of this study. Mice of both sexes were used throughout the experiments and all were between the ages of six and ten weeks when the experiments were started.

2. Mitogens

Bacto-Phytohemagglutinin (PHA-P) (Difco Lab, Detroit, Mich.) and Seeney Mitogen A (Seeney Lab, Houston, Texas) were dissolved at a concentration of 20 mg/ml in E 2020 medium (Grand Island Biological Co., Grand Island, N. Y.). Wellcome MR 69 PHA preparation (Wellcome Research Lab, Beckenham, England) was dissolved at a concentration of 2 mg/ml in E 2020 medium.

3. Bone Marrow Cell Harvesting

Bone marrow cells were harvested by flushing the left femurs of mice with Spinner salt solution (Grand Island Biological Co.). One ml of the salt solution was flushed through the marrow cavity of each femur by means of a sterile tuberculin syringe. The marrow cell suspensions were immediately placed on ice and then forced through the syringe several times to break up cell clumps. The cells were diluted 1:20 in Turk's solution before counting in a hemocytometer.

4. Cell Cultures

The method for growing bone marrow cells in soft agar was essentially that of Bradley and Metcalf (2) with slight modifications as described by Chen and Schooley (4). Colony formation is dependent on the presence of colony stimulating factor within the cultures. Conditioning medium from kidney cell monolayers was used as a source of the factor. The preparation of this conditioning medium has been described by Bradley (1). The kidneys were removed from eight to 10 day old mice, minced with scissors, trypsinized for one hour, and then five ml of a suspension containing 4×10^6 cells/ml were seeded

in 35 mm plastic petri dishes. Eagle's medium (Grand Island Biological Co.) was used in the preparation of these cultures. The cultures were incubated at 37°C in a CO_2 incubator for six days. On the sixth day the medium was collected, centrifuged at 1500 rpm for 15 minutes, and the supernatant was stored at 4°C until used.

For each cell culture 0.2 ml of conditioning medium was mixed with 0.8 ml of E 2020 medium and 1.0 ml of 1% Bacto-agar (Difco Lab) at 39°C. This mixture was then plated into a 35 mm plastic petri dish. After the agar had gelled it was covered with a mixture of 10^5 bone marrow cells, E 2020 medium, and 0.6% agar (total volume of 1.0 ml) to form a soft agar layer of 0.3% agar. In cultures with PHA, the appropriate amount of mitogen solution was mixed with the cells and medium before the soft agar layer gelled.

All cell cultures were prepared in triplicate and incubated for 10-12 days at 37°C in a humidified incubator with a 5% CO_2 flow in air. At the end of the incubation period, the number of granulocytic colonies on each plate was counted using a 40 X inverted microscope. All groups of eight or more cells were scored as colonies. Cell morphology was studied on smears of selected colonies stained with Giemsa as previously described by Burns and Lozzio (3).

CHAPTER III

RESULTS OF IN VITRO CYTOTOXICITY EXPERIMENTS

The number of colonies formed in soft agar cultures has been shown to be directly proportional to the number of bone marrow cells plated (3). When 10^5 bone marrow cells from normal mice were plated, an average of 25 $\pm$ 4.8 colonies was found as shown in Table 1. In marked contrast, an identical number of cells from RLV-infected mice yielded an average of 56 $\pm$ 6.8 granulocytic colonies. Thus leukemic mice formed more than twice as many colonies as normal mice. This is in agreement with previously reported findings (3). The granulocytic colonies developing from RLV-infected mice are termed leukemic because it has been demonstrated that the virus replicates and is present in continuous bone marrow cell lines including the myelopoietic precursor cells (10, 11).

PHA was added to cultures of normal and leukemic marrow cells in the amounts of 50, 100, 200, 400, and 800 micrograms per 10^5 cells. Seeney Mitogen A and Wellcome MR 69 were used in a series of cultures as well. Results of these experiments are shown in Table 1.

The addition of Bacto-PHA reduced the number of granulocytic colonies formed by normal bone marrow cells but at most concentrations more markedly inhibited the proliferation of leukemic colonies. The percentage inhibition of colony formation is shown in Table 2. The maximum difference in inhibition was 20% at 100 and 200 micrograms of PHA. There was only an 8% difference in

TABLE 1

NUMBER OF NORMAL AND LEUKEMIC GRANULOCYTIC COLONIES FORMED IN THE PRESENCE OF DIFFERENT PREPARATIONS OF PHYTOHEMAGGLUTININ

Mitogen	Number of Mice	Donor Cells	Micrograms of Mitogen Per 100,000 Cells					
			0	50	100	200	400	800
Bacto PHA-P	10	Normal	25 ± 4.8	24 ± 2.3	16.3 ± 2.0	13 ± 1.8	5 ± 1.9	1 ± 0.9
	10	Leukemic	56 ± 6.8	50 ± 6.5	25 ± 6.4	17.9 ± 5.6	6.7 ± 3.9	3.4 ± 1.7
Seeney Mitogen A	8	Normal	25 ± 4.8	24.8 ± 3.0	23.8 ± 4.9	16.8 ± 1.2	6.3 ± 2.3	1.5 ± 1.2
	8	Leukemic	56 ± 6.8	55.4 ± 7.8	53.2 ± 7.9	29.7 ± 4.4	12 ± 3.9	9 ± 4.5
Wellcome MR 69	4	Normal	25 ± 4.8	0	0	0	0	--
	3	Leukemic	56 ± 6.8	0	0	0	0	--

Values are means ± S. E.

TABLE 2

PERCENT INHIBITION OF MOUSE BONE MARROW GRANULOCYTIC COLONY FORMATION BY PHYTOHEMAGGLUTININ

Mitogen	Number of Mice	Donor Cells	Micrograms of Mitogen Per 100,000 Cells				
			50	100	200	400	800
Bacto PHA-P	10	Normal	4%	35%	48%	80%	96%
	10	Leukemic	9%	55%	68%	88%	94%
Seeney Mitogen A	8	Normal	1%	5%	33%	75%	94%
	8	Leukemic	1%	5%	47%	78%	84%
Wellcome MR 69	4	Normal	100%	100%	100%	100%	—
	3	Leukemic	100%	100%	100%	100%	—

inhibition at 400 micrograms and at 800 micrograms PHA-P was slightly more inhibitory for normal than leukemic cells.

Colonies from normal and leukemic cultures were stained with Giemsa and microscopically examined to determine the number and type of cells present in the colonies both in the presence and absence of PHA. The addition of PHA produced no morphological differences in the cells within the colonies. The colonies apparently arose from the proliferation of single cells and reached a density of 30-300 cells per colony. Colonies from leukemic bone marrow cells usually contained roughly twice as many granulocytic cells, indicating a higher proliferative rate. Promyelocytes and myelocytes with abundant red-violet cytoplasmic granules comprised approximately 75% of the cell populations in both normal and leukemic colonies. In the majority of the cells the nucleus was eccentric and the few myeloblasts contained two to four nucleoli. Five percent or less of the total cell population within each colony were macrophages containing engulfed agar in the cytoplasm. These macrophages may not originate from the colony forming cells. Instead the macrophages in the bone marrow cells plated may migrate into the favorable microenvironment of the colonies. Although several metamyelocytes and other transitional forms were observed, only a few mature neutrophils were seen.

Seeney Mitogen A is one of two glycoprotein fractions of PHA isolated by gel electrophoresis (31). This preparation has a mitogenic activity 50-fold greater than that of Bacto-PHA as measured by the percentage of lymphocytes transformed to blast cells. Inconsistent results were obtained when this preparation was added to cell cultures

as shown in Table 2, page 10. At 50 and 100 micrograms the inhibition was minimal and exactly the same for both normal and leukemic cells. The mitogen was only 14% more inhibitory for leukemic cells at 200 micrograms and 3% more inhibitory at 400 micrograms. However, at 800 micrograms the mitogen was 10% more inhibitory for normal cells. In general Mitogen A was less cytotoxic than Bacto-PHA and the small differences in cytotoxicity for normal and leukemic cells seem to indicate a lack of selective cytotoxicity for this preparation.

When Wellcome MR 69 was added to the cell cultures it was totally inhibitory at all of the original concentrations. However, colonies were formed when much lower concentrations were used as shown in the following table.

TABLE 3

NUMBER OF COLONIES FORMED AND PERCENTAGE INHIBITION OF COLONY FORMATION IN THE PRESENCE OF WELLCOME MR 69

Number of Mice	Donor Cells	Micrograms of Mitogen Per 100,000 Cells			
		0	5	10	20
5	Normal	25 ± 4.8	10 ± 2.9	2 ± 1.7	0
		—	(59%)	(92%)	(100%)

Values are means ± S. E.

The MR 69 is another partially purified preparation of PHA in which the mitogenic to hemagglutinating ratio has been increased

by a factor of approximately 100:1 (13). Each miligram of this preparation contains 10.9 mitogenic units (27) and less than 0.02 hemagglutinating units. The units are defined in terms of a reference standard which has a mitogenic activity of one unit per miligram.

As shown in Table 3, Wellcome MR 69 was much more cytotoxic than either Bacto-PHA or Seeney Mitogen A as only 20 micrograms of the preparation was sufficient to completely inhibit colony formation by normal cells. At the smallest concentration used, five micrograms, the MR 69 was more cytotoxic than 200 micrograms of either Bacto-PHA or Mitogen A. As concentrations below five micrograms were not tested, the possibility remains that MR 69 might exert a differential effect on leukemic versus normal cells at extremely low concentrations.

CHAPTER IV

DISCUSSION

The addition of PHA to human and animal leukocyte cultures accelerates the degeneration of mature granulocytes (39) and inhibits essential metabolic functions of these cells (33). However, the mechanisms of inhibition are as yet unknown. The inhibitory effect of PHA on myelopoietic precursor cells may be the result of: a direct cytotoxicity for colony forming cells or more differentiated forms from its incorporation onto and/or into proliferating cells; the formation of a complex with the colony stimulating factor, thus rendering it non-stimulating for colony growth; or an inhibitory effect associated with one or more of its known activities, such as the mitogenic activity.

Recently it has been reported that PHA stimulates the synthesis of nucleic acids of normal mouse splenic lymphocytes whereas it inhibits the DNA synthesis of Gross virus lymphoma and L-1210 leukemic cells in cultures (7). This suggested a selective cytotoxicity for abnormal (neoplastic) lymphoid cells. However, the results reported herein do not indicate a dramatic cytotoxicity for leukemic granulocytes since colony formation by normal cells was also significantly inhibited. The greater cytotoxicity that was observed for Bacto-PHA in particular may be due to an increased density of PHA receptor sites or greater affinity of the receptor sites on the neoplastic cell surface. The increased number or affinity of receptor sites would result in a high concentration of

PHA and cell death. Accordingly, normal cells might have fewer receptor sites or the receptor sites might have less affinity and, therefore, the amount of PHA that attaches or penetrates these cells would be more likely to fall below the critical toxic level.

Otsuka and Robinson (32) have postulated that the inhibitory effect of Concanavalin A, another phytomitogen, on human and mouse colony forming cells is due to the formation of a complex with the colony stimulating factor. The complex would thus be non-stimulating for colony growth. The possibility that PHA exerts its inhibitory effect through this mechanism has not yet been excluded.

There is some evidence that the inhibitory effect is related to the mitogenic activity of PHA. Razavi (36) has reported that a partially purified mitogenic fraction of PHA attaches and penetrates granulocytes. A further suggestive finding is that when the mitogenic activity of PHA is inactivated by treatment with sodium periodate, there is no effect on the number, size, or morphology of granulocytic colonies when the inactivated PHA is added to cultures (3). Wellcome MR 69 has a high mitogenic potency and was found to be very cytotoxic for both normal and leukemic colony forming cells. However, it remains possible that the cytotoxicity of this preparation may be related to factors other than the high mitogenic content. The MR 69 is prepared as a sterile saline extract with no added preservatives so the extreme cytotoxicity cannot be attributed to these factors.

Although the above observations support the idea of the mitogenic activity as the inhibitory factor, the experiments with Seeney Mitogen A do not support the hypothesis. This preparation also

has high mitogenic potency and would have been expected to be very cytotoxic. However, this was not the case as Mitogen A was even less cytotoxic than Bacto-PHA. Mitogen A is one of two mitogenic components isolated by electrophoresis. Perhaps both of these components are needed for maximum cytotoxicity. Since these experiments were completed, Seeney Laboratories has succeeded in fractionating their Mitogen A and B preparations into several subcomponents, one of which has no mitogenic activity. It would be of interest to test the active subcomponents alone and in combination with the inactive component. Also the inactive fraction could be tested for cytotoxicity even though it has no mitogenic activity. In any event, more experiments are needed with various purified mitogenic fractions of PHA before the inhibitory effect can be definitely assigned to the mitogenic activity of PHA.

The in vivo administration of PHA has been reported to delay the onset of deaths in mice infected with Rauscher leukemia virus (20, 21, 38). Purified fractions of PHA have not yet been tested to see if they would offer a more successful means of treatment. As previously stated, it was hoped that a preparation of PHA would be selectively cytotoxic for leukemic cells but not for normal cells. Such a preparation would be of interest for possible in vivo treatment of Rauscher and other malignant diseases. The lack of more selective cytotoxicity of Seeney Mitogen A does not seem to justify its use in vivo, particularly in view of the greater expense of these purified fractions. Since Mitogen A was less cytotoxic than Bacto-P, it might well be less effective as a means of treatment. Wellcome MR 69 was found to be extremely toxic in vitro for normal cells. If this mitogen

displays a similar cytotoxicity for normal cells in vivo, it would preclude its use as a means of treatment unless much lower levels showed a selective toxicity for leukemic cells.

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PART II. SURVIVAL OF RAUSCHER LEUKEMIA VIRUS INFECTED MICE GIVEN SYNGENEIC NORMAL OR PHA-STIMULATED SPLEEN CELL TRANSFERS

CHAPTER I

INTRODUCTION

Considerable interest has developed in phytohemagglutinin (PHA) because of its mitogenic capability when incubated in vitro with lymphocytes (29). Recently the in vivo effects of PHA have been extensively studied (20, 21, 25-30). One of the more interesting observations has been the amelioration of symptoms of rapidly progressing murine leukemias by the treatment with PHA. For example, the administration of PHA has been reported to prolong the life span and reduce the splenomegaly of mice infected with Rauscher leukemia virus (23, 40). The nature of the beneficial effect of PHA on this murine leukemia is not known and remains speculative.

Rauscher leukemia virus (RLV) induces a rapid and extreme proliferation of predominantly erythrocytic and leukocytic elements as early as seven days postparental inoculation (33). The disease is associated with enormous splenomegaly and an early mortality peak at 25-35 days after inoculation. Mice that survive this early mortality peak develop a chronic lymphocytic leukemia and usually die between 62 and 90 days. The hematology of Rauscher disease has been repeatedly studied in BAIB/c mice (3, 6, 11, 14, 41) and is characterized by highly elevated numbers of immature erythroid cells, particularly in the spleen, and immature granulopoietic cells, mainly in the bone marrow. Many of these cells have a maturation arrest and some investigators have considered the disease an ineffective hematopoiesis,

particularly an ineffective erythropoiesis (13, 35). The mice die because of splenic rupture or unknown mechanisms due to the anemia or thrombocytopenia commonly observed after infection with the virus (35).

Siegel et al. (40) inoculated six week old BALB/c mice with RLV and then administered 0.5 mg of PHA three times weekly. This treatment with PHA delayed the onset of deaths four to five weeks. At 72 days after inoculation 60% of the treated mice were still alive with little or no sign of leukemia as compared to 30% of the control mice. Similar results were obtained in four month old mice given 0.8 mg of PHA three times weekly. The injection of 1.0 mg once a week, however, had no effect on the development of the leukemia. These results suggested that continuous PHA treatment might maintain decreased numbers of virus-susceptible cells.

Lozzio et al. (24) also reported that BALB/c mice infected with RLV exhibited a marked delay in death when given twice weekly injections of 100 mg of PHA per kilogram of body weight. Twenty percent of 150 infected mice given no treatment died on or around the third week after inoculation. The mortality rose sharply on the fourth (50%), fifth (68%), and sixth (90%) weeks and all of the mice were dead by the seventh week. Of 160 mice treated with PHA, only 10% were dead on the third week and extended survival was observed as indicated by mortality rates on the fourth (20%), fifth (28%), sixth (40%), and seventh (78%) weeks. Although significantly delayed, once the deaths began the rate of death resembled that of untreated mice and none of these PHA-treated mice survived beyond the eighth week.

The treatment reduced the splenomegaly, lymphocytosis, and peripheral erythroblastosis but did not modify the terminal anemia and thrombocytopenia characteristic of Rauscher leukemia. Therefore, this type of treatment did produce a delay in deaths, however, all of the mice eventually died of the disease indicating a need for a different approach to treatment.

It has been suggested that the ineffective erythropoiesis of Rauscher leukemia operates at the stem cell level (7, 8, 13, 35, 36). Stem cells are known to be altered in Rauscher disease (8, 36) where so-called tumor colony forming units have been described. Recently evidence has been obtained that the majority of leukemic colony forming cells are killed by incubation with specific antisera against RLV (7). Apparently the stem cells bear viral antigens, suggesting that they are among the target cells for the virus.

Assuming that the stem cell is responsible for the ineffective hematopoiesis, a logical means of treatment would be to stimulate the stem cell compartment to proliferate and differentiate. Normal cells are known to produce a factor which will stimulate the maturation of normal and leukemic cells in vitro (12, 16-18, 31). A recent study by Lopez and Lozzio (19) demonstrated that the transplantation of syngeneic lymph node cells, from donors treated with PHA into sublethally irradiated recipients, enhanced the proliferative capacity of endogenous hematopoietic progenitor cells and their differentiation into granulocytic and erythrocytic cells. The enhancement of stem cell proliferation demonstrated possible interactions between blast cells developing after PHA administration and blood cell precursors in

mice that have a severe depression of hematopoiesis and immunocompetence. This situation is analogous to that of mice infected with RLV, where in addition to the ineffective hematopoiesis, these mice have been shown to have a depressed level of general immunocompetence (4, 10, 38, 39).

Therefore, in the following experiments leukemic mice were given transfers of normal or PHA-stimulated syngeneic spleen cells in an attempt to stimulate erythropoiesis and overcome the terminal anemia of Rauscher disease. The effect of the transfers on the development of the leukemia and survival of the mice was studied using several variations of treatment.

CHAPTER II

MATERIALS AND METHODS

1. Virus and Mice

The Rauscher leukemia virus and the BALB/c mice used for these experiments have been described in Chapter II of Part I. All mice were six weeks old at the start of the experiments and each group of mice consisted of approximately equal numbers of males and females.

2. Spleen Cell Suspensions

Syngeneic spleen cells were obtained from normal (untreated) donor mice or from mice given two doses of Bacto-PHA (Difco Lab, Detroit, Mich.) of 200 mg/kg with a three day interval between doses. These PHA-stimulated donors were killed six days after the first injection and their spleens removed. This amount of PHA and the schedule of injections have been found to produce the most marked effects on the lymphopoietic system of mice (20, 25, 27, 28). The spleens of each donor group were pooled separately and homogenised in Hank's balanced salt solution (Grand Island Biological Co., Grand Island, N. Y.) at pH 7.0 and containing 10,000 units of penicillin per ml. Cell viability was determined by the use of a 0.5% nigrosine solution. These procedures resulted in a cell suspension of approximately 75% viable cells. An aliquot (0.3 ml) of the cell suspension containing 10^8 viable spleen cells was filtered through an extra fine nylon mesh to remove any cell clumps

and was then injected intravenously into normal mice or those mice infected with RLV seven days previously.

3. Multiple Spleen Cell Transfers

The same procedures were followed as above but these mice were given 10^8 viable cells at seven days followed by additional transfers of 5×10^7 viable cells during the fourth and eighth weeks.

4. Sonicated Spleen Cell Suspensions

These suspensions were prepared by sonicating the appropriate number of cells (10^8 or 5×10^7 cells per mouse) for three minutes. Smears of the sonicated cell suspensions were made to insure that no intact cells remained. Mice receiving sonicated cells were given three injections according to the schedule above. The sonicated cells from normal donors were not centrifuged. These suspensions proved to be effective in extending survival. Therefore, sonicated cells from PHA-stimulated donors were centrifuged at 5000 rpm for 15 minutes and only the supernatant was used for injection to see if the "survival factor" remained in the supernatant or was associated with the larger cell fragments in the pellet. The amount of supernatant was equivalent to the volume that contained 10^8 or 5×10^7 cells prior to sonication and centrifugation. Results of the survival studies indicated that the "survival factor" remained in the supernatant. The sonicated PHA cells were almost as effective as the viable PHA cells.

5. Hematological Determinations and Spleen and Body Weights

Blood samples were taken at one and two week intervals after

the transfers and the red and white blood cells were counted electronically. Leukocyte differential counts were made by counting 100 leukocytes on blood smears. Reticulocyte counts were made by counting 1000 erythrocytes on smears stained with new methylene blue. At the time of death, the spleen and body weights were recorded for each mouse.

CHAPTER III

RESULTS OF SPLEEN CELL TRANSFERS

1. Effect of a Single Transfer of Normal or PHA-stimulated Cells

The survival of leukemic and normal mice undergoing different treatment is shown in Figure 1. The half-life, the survival time of 50% of the mice, was about four weeks in leukemic mice given no treatment. None of these mice survived beyond the seventh week after virus inoculation. The treatment of leukemic mice with the syngeneic spleen cells stimulated in vivo by PHA produced a significant ($P < 0.001$) prolongation of their half-life with 50% of the mice surviving for almost 12 weeks. The total life span of surviving mice in this group was also markedly increased. This observation ended on the seventeenth week and of the four surviving mice killed, two had normal leukocyte counts and normal sized spleens while the other two had only moderate splenomegaly and marked leukocytosis and normoblastosis. All normal mice which had received cells from donors stimulated with PHA, were still alive at the end of 17 weeks. At that time the spleen and body weights were found within the normal limits.

As a control for the effect of PHA cells, another group of leukemic mice was given spleen cells from normal donors. The mean half-life of this group was around seven weeks, a value considerably longer than the half-life (four weeks) observed in leukemic mice given no treatment. Six mice out of 18 in this group survived for 17 weeks and only two of these had splenomegaly and highly elevated

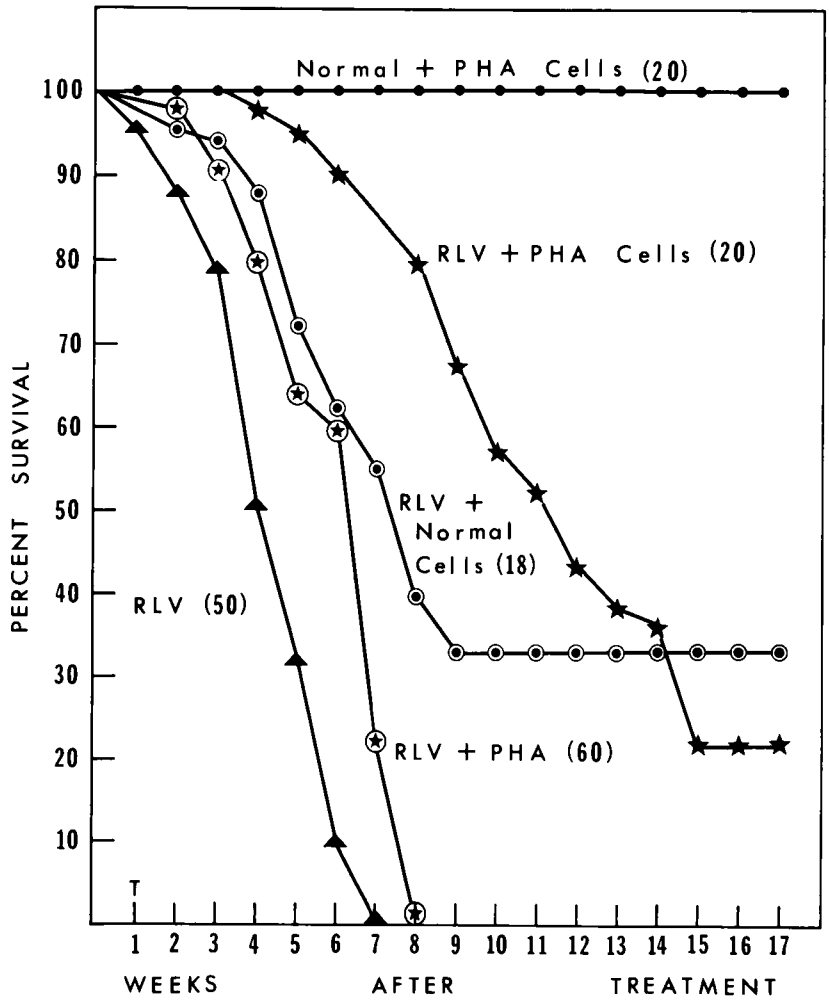


Figure 1. Survival of leukemic and normal BALB/c mice given different treatments.

Leukemic mice were inoculated with Rauscher leukemia virus (RLV) on week zero. Three groups of mice received cell transfers (T) of 10^8 spleen cells on week one, a fourth group was given phytohemagglutinin (PHA) twice weekly at a dose of 100 mg/kg, and the remaining group was untreated. The numbers in parentheses indicate the number of mice in each group.

leukocyte counts. The leukocyte counts for the other four mice were within the normal limits.

For purposes of comparison, another group of leukemic mice was injected with 100 mg/kg of PHA twice a week until their death. The mean half-life of this group was six weeks, a value nearly identical to that of leukemic mice given a transfer of normal cells. However, none of the mice in this group survived beyond the eighth week of the experiments. The administration of PHA to more than 100 normal mice produced not a single death throughout a period of eight weeks.

Splenomegaly was not present at the time of the transfers but all animals developed palpable spleens around the third week after infection. Mice dying at similar intervals after RLV inoculation had similar degrees of splenomegaly, regardless of treatment. The average spleen weight approached two, four, and five grams on the fourth, ninth, and twelfth weeks of treatment, respectively. Mice in either of the treated groups that survived beyond twelve weeks had either normal size spleens or a considerable reduction of the splenomegaly (mean = 1.5 g). As previously reported (22), normal mice given only PHA for a long period of time developed moderate splenomegaly.

As shown in Figure 2, leukocytosis was observed in all mice inoculated with RLV regardless of treatment. The results of the differential blood counts indicated that virus infection produced a progressive granulocytosis. Prior to virus inoculation the mice had a leukocyte count of about 12,000 cells/mm^3 and a lymphocyte to granulocyte ratio of 75:25. Five weeks after RLV inoculation the

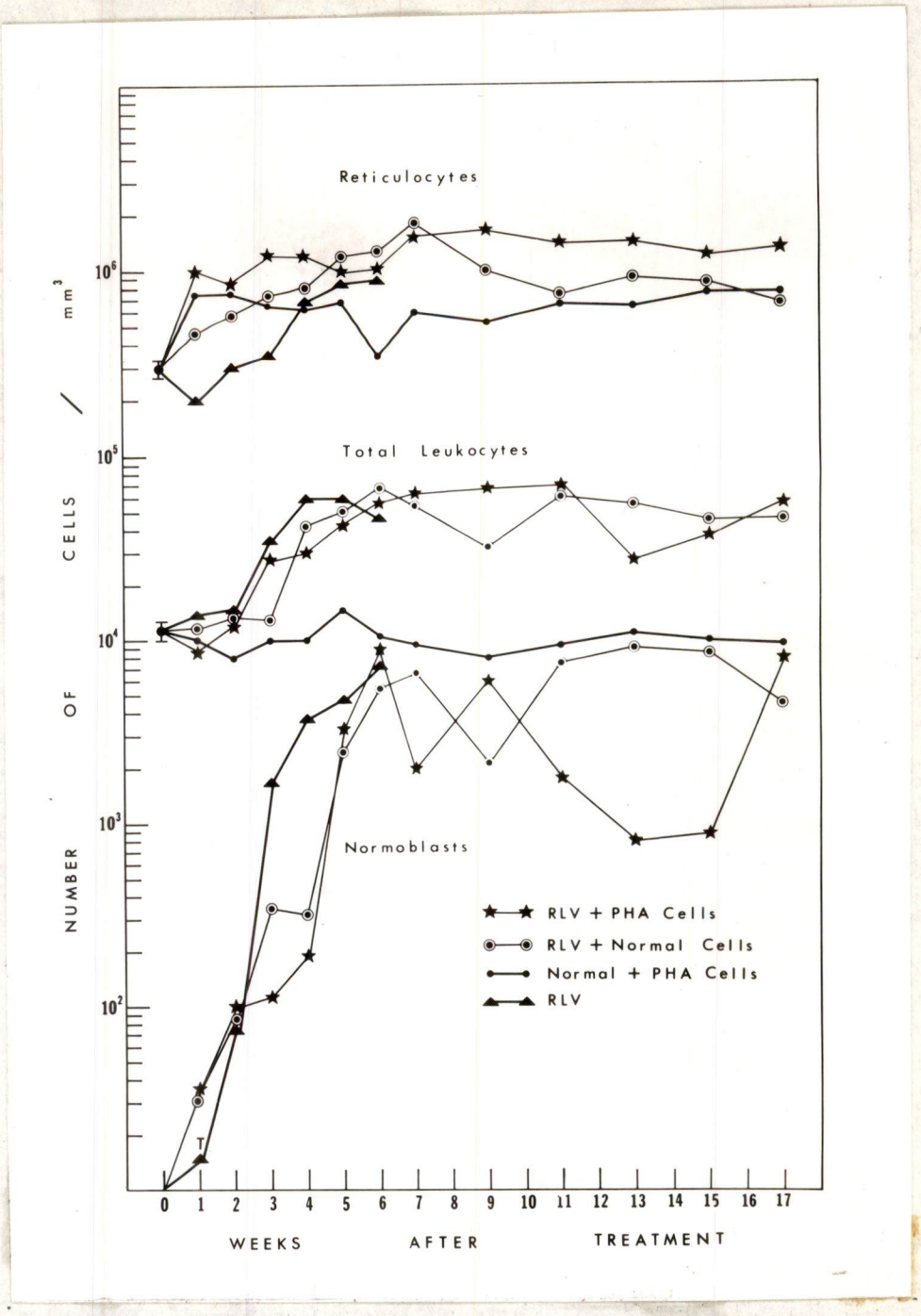


Figure 2. Peripheral leukocyte and erythroid cell counts of leukemic and normal BALB/c mice.

Leukemic mice were inoculated with Rauscher leukemia virus (RLV) on week zero. Transfers (T) of 10^8 spleen cells were given on week one. One group of leukemic mice received no treatment. Normal mice given PHA cells did not have any normoblasts in peripheral blood and thus no curve for normoblasts is shown in the figure. Values are means from six or more mice in each group.

counts ranged from 30,00 to 60,000 cells/mm^3 and the lymphocyte to granulocyte ratio was 60:40. This ratio remained virtually unchanged until the death of the mice. The cell transfers did not change the absolute cell numbers, but mature neutrophils (95%) were predominant among peripheral granulocytes of mice given a transfer. In marked contrast, myelocytes (22%) and metamyelocytes (15%) accounted for 37% of the total blood cells in leukemic mice given no treatment. Lymphocytosis (16,000 to 40,000 cells/mm^3) was also observed in all RLV inoculated mice except for two weeks following the transfers when lymphopenia (5,000 to 7,000 cells/mm^3) was observed. Mice normally have about 9,000 lymphocytes/mm^3. Normal mice given a transfer of PHA-stimulated cells did not experience leukocytosis. These mice had normal or slightly below normal leukocyte counts throughout the period of observation.

Infected mice given a transfer had as many normoblasts in the blood as leukemic mice given no treatment. Beyond the ninth week, however, mice given PHA-stimulated cells had fewer normoblasts in the circulation until the seventeenth week when their number sharply increased (Figure 2). Mice receiving a cell transfer had an early and substantial increase of the reticulocyte number of two to three times that of mice given RLV alone indicating an increased maturation of erythroid elements. The reticulocytosis persisted throughout the period of these observations with the recipients of the PHA-stimulated cells having the highest reticulocyte counts. Normal mice given PHA cells had a similar increase in the reticulocyte count.

2. Effect of Multiple Transfers of Viable or Sonicated Normal Cells

Since a single transfer of either normal or PHA-stimulated spleen cells significantly extended the survival of leukemic mice, it was of interest to see if survival could be further extended by multiple transfers. For this purpose an initial transfer of 10^8 viable cells was followed by additional transfers of 5×10^7 cells during weeks four and eight. It was also of importance to determine if viable cells were required for increased survival. Therefore, multiple transfers of sonicated cells were also given.

The survival curves for mice receiving multiple transfers of viable or sonicated normal spleen cells are shown in Figure 3. Mice given three transfers of viable cells had a half-life of 16 1/2 weeks, more than double the 7 1/2 week half-life of mice given a single transfer (Figure 1, page 32). However, there was not a significant difference in survival at 17 weeks. Mice given a single transfer had an 88% survival at four weeks and a 33% survival at 17 weeks, a difference of 55% (Figure 1, page 32). The group of mice given three transfers had a 100% survival at four weeks, the time of the second transfer, and a 42% survival at 17 weeks, a difference of 58%. Thus multiple transfers temporarily delayed deaths but the original rate of death was resumed after the last transfer.

For mice given multiple transfers of viable spleen cells there were two periods of time during which no deaths occurred. The first of these began on week five, one week after the second transfer, and lasted for three weeks. The second period began on week 10, two weeks after the third transplant and was of five weeks duration. These

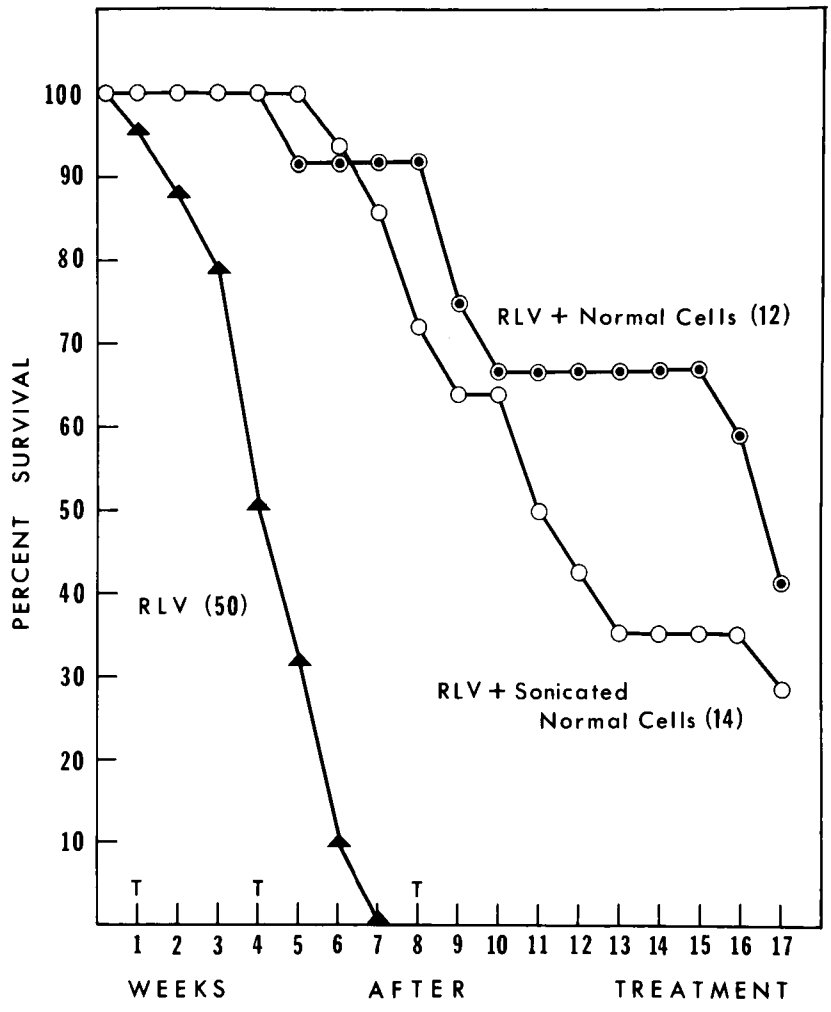


Figure 3. Survival of leukemic mice given multiple transfers of viable or sonicated normal spleen cells.

The mice were inoculated with Rauscher leukemia virus (RLV) on week zero. A transfer (T) of 10^6 cells was given on week one and transfers of 5×10^7 cells were given on weeks four and eight. The numbers in parentheses indicate the number of mice in each group.

regions on the survival curve suggest that the protection conferred by the second and third transfers took one to two weeks to become maximally effective and then maintained a high level of protection for three to five weeks. In contrast, the curve for mice given sonicated cells maintained a steep slope until week 13 when the curve leveled off.

Although the sonicated cells were less effective than multiple transfers of viable cells, the extended survival of mice in this group indicates that cell integrity and viability are not essential. The mice in this group had a half-life of 11 weeks, considerably longer than the 7 1/2 week half-life of mice given a single transfer of viable spleen cells. The mice given sonicated cells had a 29% survival at 17 weeks. It is likely that viable cells are more effective than the sonicated cells because they continue to produce the "survival factor(s)."

As in the previous experiments, all mice developed splenomegaly. The average spleen weight of the survivors at 17 weeks was 2.28 grams for mice receiving viable cells and 2.80 grams for the mice given sonicated cells.

Although the rate of increase was more gradual, leukocytosis was again observed in both groups of mice. As shown in Figure 4, the leukocytosis eventually reached levels comparable to that of the previous experiments. Mice given sonicated normal cells had an average lymphocyte to granulocyte ratio of 60:40. In contrast, mice given viable cells had an average lymphocyte to granulocyte ratio of 70:30, a near normal value. Immature granulocytes were relatively

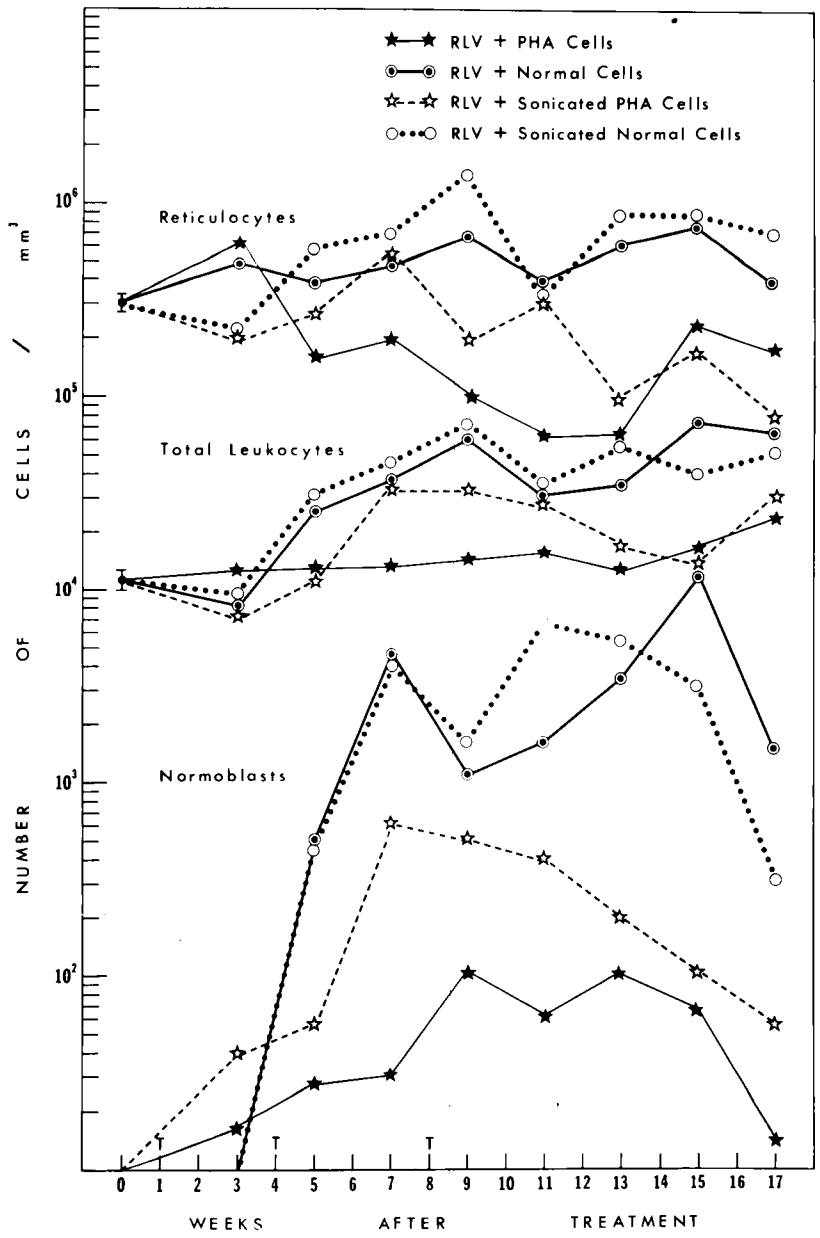


Figure 4. Peripheral leukocyte and erythroid cell counts of leukemic mice given multiple transfers of normal or PHA-stimulated spleen cells.

The mice were inoculated with Rauscher leukemia virus (RLV) on week zero. A transfer (T) of 10^6 normal or PHA-stimulated cells was given on week one and transfers of 5×10^7 cells were given on weeks four and eight. Values are means of six or more mice.

rare (less than 5%) among both groups of mice.

Normoblastosis was roughly comparable to that of the previous experiments. The reticulocytosis was more gradual and averaged about the same as RLV-inoculated mice given no treatment.

3. Effect of Multiple Transfers of Viable or Sonicated PHA-stimulated Cells

Two groups of mice were given three transfers of viable or sonicated PHA-stimulated spleen cells using the same procedures as above. The survival curves for these two groups of mice are shown in Figure 5. Once again multiple transfers were more effective than a single transfer in extending survival. Mice given a single transfer of PHA-stimulated spleen cells had a 98% survival at four weeks and a 25% survival at 15 weeks, a difference of 73% (Figure 3, page 37). Mice given multiple transfers had a 100% survival at four weeks, the time of the second transfer, and a 58% survival at 17 weeks, a difference of 42%.

There was little difference in survival between mice given multiple transfers of viable or sonicated PHA cells. This might be explained by the fact that cells exposed to PHA die after a limited period of blastogenesis and mitosis (29). Thus the transferred viable cells would not remain viable for very long after the transfer.

Unlike the sonicated normal spleen cells, the sonicated PHA cells were centrifuged at 5,000 rpm for 15 minutes and only the supernatant was used for injection. Thus the "survival factor" must be either soluble or associated with some subcellular component remaining in the supernatant.

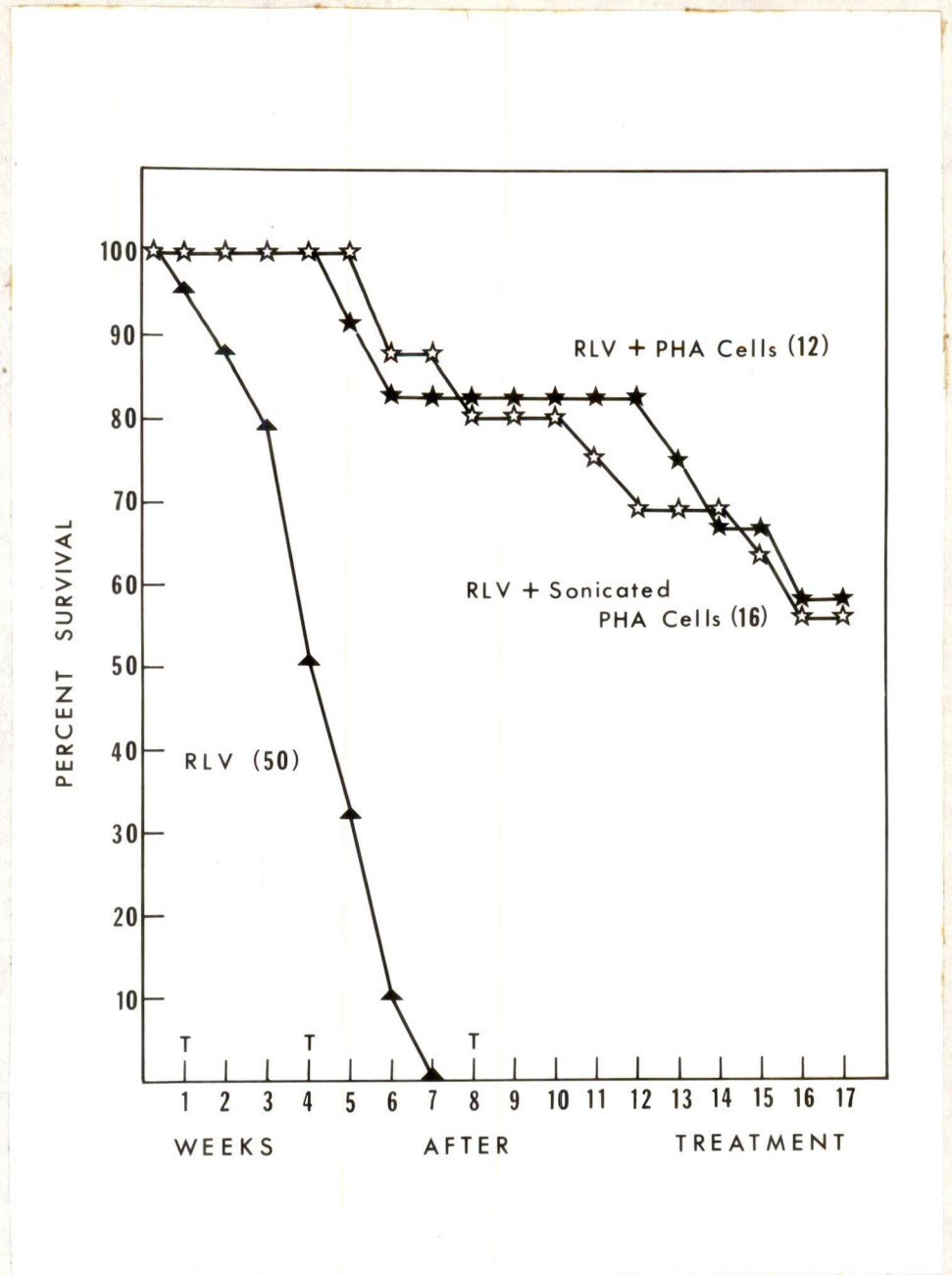


Figure 5. Survival of leukemic mice given multiple transfers of viable or sonicated PHA-stimulated spleen cells.

The mice were inoculated with Rauscher leukemia virus (RLV) on week zero. A transfer (T) of 10^8 cells was given on week one and transfers of 5×10^7 cells were given on weeks four and eight. The sonicated PHA cells were centrifuged and only the supernatant was used for injection. The numbers in parentheses indicate the number of mice in each group.

All of the mice in both groups developed splenomegaly by the third week after virus inoculation. The average spleen weight of the survivors at 17 weeks was 0.18 grams for mice given multiple transfers of viable PHA cells and 0.93 grams for the mice given sonicated cells. Thus both of these groups had a remarkable decrease in the splenomegaly.

Leukocytosis was observed (Figure 4, page 39) in the group given sonicated cells but the maximum average leukocyte count of 35,000 cells/mm^3 was considerably lower than the 72,000 cells /mm^3 observed in the group of mice given sonicated normal spleen cells. After the peak on week seven, the leukocyte count began a gradual decline. The lymphocyte to granulocyte ratio was approximately 65:35 throughout the period of observation.

The average leukocyte count for mice given viable PHA cells did not rise above 24,000 cells/mm^3 and remained at near normal values throughout most of the period of observation. However, there were two mice which died on weeks 13 and 14 that had counts of 55,000 and 90,000 cells/mm^3 shortly before their death. The average lymphocyte to granulocyte ratio for this group of mice did not change from the normal 75:25 ratio. As with all other groups of mice given a transfer, immature granulocytes constituted less than 5% of the total cell population in peripheral blood.

Normoblastosis was substantially reduced in both groups of mice. Mice given sonicated cells had a maximum of 600 normoblasts/mm^3 (Figure 4, page 39). The normoblastosis in mice given viable cells was even more curtailed. Normoblasts never exceeded

100 cells/mm^3 with the exception of one mouse which died on week 13 and had a count of 20,000 normoblasts/mm^3.

Both groups of mice had below normal reticulocyte counts throughout most of the experiment. This was most pronounced in the group given viable cells which hada minimum count of only 62,000 cells/mm^3 on week 11 (Figure 4, page 39).

The moderate leukocytosis, the substantially reduced normoblastosis, and the normal lymphocyte to granulocyte ratio of mice given viable cells indicate a remarkable amelioration of the leukemia.

4. Brief Summary of Survival Studies

The results of the survival studies of RLV-inoculated mice given syngeneic spleen cell transfers can be summarized as follows:

1. Multiple transfers were more effective than a single transfer in extending survival of the leukemic mice.
2. PHA-stimulated cells were more effective than normal spleen cells. However, multiple transfers of normal cells were more effective than a single transfer of PHA-stimulated cells.
3. Although viable cells were more effective, cell viability and integrity were not essential for prolonged survival as indicated by the effectiveness of transfers of the sonicated spleen cells.
4. Viable cells initially produced a higher survival rate than sonicated cells but by 17 weeks there was little difference in survival rates between the two groups.

5. The most successful treatment was multiple transfers of viable PHA-stimulated cells. Hematological values indicated a marked amelioration of the leukemia for mice in this group.

Possible mechanisms of action by the spleen cell transfers are discussed in the following chapter.

CHAPTER IV

DISCUSSION OF POSSIBLE MECHANISMS OF ACTION BY SPLEEN CELL TRANSFERS

The nature of the beneficial effect of syngeneic spleen cell transfers, in particular that of spleen cells stimulated in vivo by PHA is not known and remains speculative.

As previously mentioned, mice infected with RLV have a marked proliferation of immature erythroid cells, predominantly in the spleen, and immature granulopoietic cells, mainly in the bone marrow, and an elevated number of these cells are seen in the circulation (3, 6, 11, 14, 33, 35, 41). In this context it seems that the transfer of the syngeneic spleen cells, in particular those stimulated by PHA, would stimulate the maturation of granulocytic and erythroid cells. In all of the treated groups studied the maturation of granulocytic cells was markedly stimulated. Granulocytic precursors never accounted for over 5% of the cells in peripheral blood as compared with the 37% immature cells in untreated leukemic mice. The transfers did not produce as dramatic an effect on the maturation of erythroid cells. The most pronounced effect on the normoblastosis was seen in the groups of mice receiving multiple transfers of viable or sonicated PHA cells. The normoblastosis of these two groups was significantly reduced with the group given viable cells having a maximum count approximately 100-fold less than that of leukemic mice given no treatment.

The possibility that normal and PHA-stimulated cells may be

cytotoxic for neoplastic cells in vivo has not yet been explored. It is now well established that normal lymphocytes from unsensitized animals stimulated by PHA are cytotoxic for a variety of neoplastic and normal cultured cells including erythrocytes (1, 32, 34, 35). As shown in Part I of this thesis, PHA itself is cytotoxic for RLV-infected cells grown on agar. Bortin et al. (5) have reported that the transplantation of immunocompetent cells from unprimed donors is capable of killing or inactivating leukemia cells in immunosuppressed AKR mice. Thus the possible in vivo cytotoxicity of the transferred cells warrants further investigation.

Blamey (2) reported that the transplantation of unsensitized isologous lymphoid cells retarded the growth of primary methylcholanthrene-induced sarcomas in rats. He proposed two possible mechanisms for the retardation of tumor growth. First, transplantation may restore depleted immune competence. A second possibility is that transplantation may provide lymphoid cells not tolerant of the tumor-specific antigen. Either of these mechanisms could explain the results of the present investigation. The depressed general immunocompetence following RLV inoculation has previously been mentioned (4, 10, 38, 39). The cell transfers may restore immunocompetence by providing more functional non-infected cells. Studies on the extent of the viremia and whether or not antiviral antibody is formed after the transfers would lend insight to this possibility. It is also possible that the viral replication might establish a state of tolerance. In this context, the transferred non-tolerant spleen cells would be capable of recognizing the virus-infected cells as

foreign with the subsequent development of a graft-versus-leukemia reaction.

The success in extending survival with the sonicated spleen cells was not anticipated. Ultracentrifugation would establish if the "survival factor" is soluable or associated with some subcellular components. Hoffier and Donner (15) reported that PHA-stimulated lymphocytes release a soluble factor. In this study allogeneic and syngeneic spleen cells stimulated by PHA were placed in one side of a double compartmented diffusion chamber and were found to release a substance which inhibited the growth of tumor cells in the other compartment.

The "survival factor" may be similar or identical to a differentiation factor produced by various human and mouse cells in tissue culture (12, 16-18, 31). This factor has been partially characterized as a protein of approximately 70,000 molecular weight and is required for the development of macrophage and granulocytic colonies on agar arising from human and mouse myelocytic leukemic cells. The factor is known to be released into the conditioning medium of normal mouse fibroblasts and spleen cells. The differentiation factor could function in vivo to overcome the maturation arrest of granulocytic and erythroid cells observed in Rauscher disease. Attempts at purification and characterization of the "survival factor" and its suitability as a means of treatment of Rauscher and other viral leukemias are areas for possible future investigation. For example it would be of interest to test in vitro and in vivo for the differentiation promoting effect of the "survival factor" on

normal and leukemic cells. It is possible that the factor may have more than one effect or there may be several factors which can cause a variety of effects.

Whatever the mechanism(s) may be, the results of this investigation suggest that the relationship of this type of immunologic reaction to the pathogenesis of murine leukemias may lead to successful treatment of the disease.

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VITA

Stephen Vernon Lair was born in Miami, Florida, on June 14, 1949. He attended elementary schools in Knoxville, Tennessee, and was graduated from East High School in 1967. In June of 1967 he entered The University of Tennessee and received a Bachelor of Science degree in Microbiology in June, 1971.

He entered the Graduate School of The University of Tennessee in September, 1971, and received the Master of Science degree with a major in Microbiology in August, 1973. After graduation he accepted a position as a research assistant at The University of Tennessee Memorial Research Center in Knoxville. He is a member of Phi Beta Kappa and Phi Kappa Phi honor fraternities.