



Studies on chemotherapy of leukemia in conventionally-reared and germfree mice
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Abstract:

The purpose of these studies was to examine the effect of mixed or combined drug therapy in abrogating the leukemic process in germ free and conventionally-reared mice, and prolonging the life of the leukemic mice.

Acute lymphocytic leukemia was induced in conventionally-reared and germ free BALB/c mice by Fpiend virus. The mice were allowed to develop the disease, and then were treated with drugs. Two regimens of drugs were used in conventional reared animals, the first consisting of a combination of methotrexate, 6-mercaptopurine, and vinblastin sulfate, and the second consisting of a combination of methotrexate, 6-mercaptopurine, and hydrocortisone acetate. The first drug regimen proved to be too toxic, and treatment had to be stopped prematurely. The second regimen, however, was more effective in that it was less toxic and therefore could be maintained for long periods. The second drug regimen only was used in the germ free animals. Hematological and pathological studies, were used to assess the effects of the drugs. It was possible by using the second regimen to extend the survival time of the conventional animals by 3 times the expected survival of mice with this disease.

In the germ free mice, drug treatment was ineffective. In fact, germ free mice challenged with virus died rapidly when treated with drugs, and in general exhibited more severe symptoms of the disease than either the virus control group or the drug control group.

The progress of the disease was compared in both conventional and germ free mice, and was found to develop poorly if at all in germ free mice in contrast to a rapidly developing fatal leukoses in conventional animals. No specific answer is yet available for this observed difference, however, a postulate has been offered as a possible explanation.

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ABSTRACT

The purpose of these studies was to examine the effect of mixed or combined drug therapy in abrogating the leukemic process in germ free and conventionally-reared mice, and prolonging the life of the leukemic mice.

Acute lymphocytic leukemia was induced in conventionally-reared and germ free BALB/c mice by Friend virus. The mice were allowed to develop the disease, and then were treated with drugs. Two regimens of drugs were used in conventional reared animals, the first consisting of a combination of methotrexate, 6-mercaptopurine, and vinblastin sulfate, and the second consisting of a combination of methotrexate, 6-mercaptopurine, and hydrocortisone acetate. The first drug regimen proved to be too toxic, and treatment had to be stopped prematurely. The second regimen, however, was more effective in that it was less toxic and therefore could be maintained for long periods. The second drug regimen only was used in the germ free animals. Hematological and pathological studies were used to assess the effects of the drugs. It was possible by using the second regimen to extend the survival time of the conventional animals by 3 times the expected survival of mice with this disease.

In the germ free mice, drug treatment was ineffective. In fact, germ free mice challenged with virus died rapidly when treated with drugs, and in general exhibited more severe symptoms of the disease than either the virus control group or the drug control group.

The progress of the disease was compared in both conventional and germ free mice, and was found to develop poorly if at all in germ free mice in contrast to a rapidly developing fatal leukoses in conventional animals. No specific answer is yet available for this observed difference, however, a postulate has been offered as a possible explanation.

INTRODUCTION

Leukemia is a neoplastic disease characterized by the proliferation of hemopoietic cells in the bone marrow and other organs. The leukemogenic process, although not fully understood, most likely is promoted or is influenced by a virus or viruses and is reflected as the failure of a cell to respond to the forces which ordinarily control its reproduction and maturation. Proliferation of cells can occur anywhere in the body. The majority of the symptoms of leukemia are due to the infiltration of normal tissue by leukemic cells with the consequence of abnormal function or lack of function of that tissue. The presence of large numbers of leukemic cells in the peripheral blood is considered not to be, by itself, harmful (Miale, 1967).

Chemotherapy has become a powerful tool in the treatment of leukemia and related diseases. New drugs are constantly being investigated and some of the results obtained may be interpreted as being encouraging. Some of the drugs presently in use include 6-mercaptopurine, methotrexate[®], cyclophosphamide, busulfan[®], cortisone, vinblastin sulfate, vincristine, arabinosylcytosine, azathioprine, and a host of less well-known drugs (Sugiura, 1968; Mead et al., 1968; Tarnowski et al., 1968; Goldin et al., 1968).

Recent studies have shown that the combined use of many of these drugs for treatment has met with a great deal of success. The

symptoms of leukemia are generally suppressed more dramatically by a combination of drugs, and remissions occur for longer durations than if the drugs were used separately. Barth Hoogstraten et al., 1969, reported that the results obtained from treating patients suffering lymphosarcoma and reticulum cell sarcoma with a combination of cyclophosphamide, vincristine, and prednisone, were far better than those obtained from patients with the same diseases treated with cyclophosphamide alone. Robert W. Sidwell, et al., 1969, reported that treatment of Swiss mice given Friend leukemia virus with a combination of 6-mercaptopurine and porfiromycin was more effective than treatment with either drug alone.

Phillip George and coworkers, 1968, working at the St. Jude Childrens Hospital have been able to extend significantly the survival time of children with acute lymphocytic leukemia by using various combinations of 4 antileukemic agents in conjunction with central nervous system radiation (1200r in 11 days). The anti-leukemic agents included methotrexate, cyclophosphamide, mercaptopurine and vincristine.

In spite of the extensive research with the effect of drugs (Valdamudi et al., 1968; Colorizi et al., 1968; Quattrin and Dini, 1968; Baudo et al., 1968; DeLong et al., 1968; Dessel, 1969), and combination of drugs on leukemia, the degree of success in extending

the life expectancy, although in some cases remarkable (George et al. 1968), has been limited and the "cure" for leukemia is yet to come. This limited success can be attributed to several reasons: Patients respond differently to the same drug; many of the drugs used are extremely toxic; and not being specific to leukemia cells also destroy normal cells (Gee et al., 1969; Haggard et al., 1968). Most of the drugs used are immunosuppressive, and often the subject dies not from the leukemic process but from autoinfection with normal flora or other complications caused by toxic side effects of the drugs. In this regard George and coworkers (1968), mentioned that one child died of multiple myocardial infarctions on the third day of treatment; another died with Pseudomonas septicemia on the eleventh day of treatment with drugs. Both deaths occurred in the presence of toxicity.

Some of the problems involved in combination chemotherapy are, to find the best combination possible; to find the dosage that can be maintained for a long period of time. This would depend on the potency of the drugs, and the duration of the experiment, and it also depends on the potency of the drugs when used in combination with others. In the present study, the effect of two combinations of drugs on the leukemic process induced in female BALB/c mice by Friend leukemia virus was investigated. In accordance with this, these

studies sought to establish a combination and a dosage of drugs that were effective in the suppression of the leukemic process but were not markedly injurious to the host. In addition, parallel studies were conducted in germ-free mice to establish whether a microbe-free environment markedly altered the therapeutic regimen.

MATERIALS AND METHODS

Preliminary Preparation

Mice. For the experiments, inbred conventionally reared (CR) and germ-free (GF) BALB/c female mice ranging from 3 to 6 months of age were used. The mice were originally obtained in 1966 from the National Cancer Institute (Bethesda, Maryland) in the germ-free state and were conventionalized six months later. The mice have since been maintained by successive brother-sister matings in our laboratory. The CR mice received Purina Laboratory Chow and water ad libitum, and the GF mice received Purina Laboratory Chow 5010C. The germ-free feed was dried for 30 minutes, autoclaved for 35 minutes at 121°C and 18 pounds pressure, and dried for 30 minutes. It was cultured in thioglycollate broth and brain heart liver semi-solid (BHL) for two weeks. For the germ-free experiments both males and females were used at about 6 months of age. Fecal samples were taken once a month and cultured in thioglycollate and BHL for two weeks to insure the germ-free states of the mice.

Virus. Friend Murine Virus was used throughout the study. The virus was obtained from Dr. Fieldsteel at Stanford Research Institute in 1968 and has been maintained following a single passage in BALB/c mice, in spleen cells at -70°C.

In order to obtain sufficient virus for the study a virus stock was obtained by injecting 14 BALB/c female mice with a 0.2 ml of the virus suspension. After 30 days when splenomegaly was apparent the mice were sacrificed and the spleens aseptically removed and weighed. The spleen average weight was found to be 2.76 gms in contrast to normal spleen weights of 0.4 to 0.7 gms. The spleens were suspended in 154.4 mls of sucrose stabilizer yielding a 20% suspension. The spleens were alternately homogenized at and cooled to 4°C, 3 times for one minute periods with 3 minute rests between each homogenization. The homogenate was dispensed in 3 ml quantities into 5 ml serum vials. The vials were capped with rubber stoppers and frozen in a dry ice and acetone mixture. The vials were labelled and stored in a Revco deep freeze at -70°C.

Sucrose Diluent for Lyophylizing Viruses (2X solution) as Modified by Fieldsteel. The following formula was used to prepare the sucrose stabilizer for virus dilutions.

Formula

KOH	0.548 gms
L-glutamic acid*	1.440 gms
K ₂ HPO ₄	2.508 gms
KH ₂ PO ₄	1.034 gms
Sucrose	150.000 gms
H ₂ O	1000.000 mls

* Or Monopotassium glutamate monohydrate, 0.956 gms (Mann Research

Laboratories, Inc., New York 6, N.Y.).

As a diluent for suspending tissue preparations of viruses, the stabilizer was diluted to 1x with sterile distilled water. If the diluent was to be used to lyophilize viruses 20% hydrolyzed gelatin (autoclaved two hours at 121°C) was added to give a 2% final concentration of gelatin in the stabilizer virus mixture.

Determination of LD₅₀ for Virus. Virus titration was carried out to estimate the LD₅₀ of the virus. A total of 70 BALB/c female mice divided into 7 groups of 10 mice each were placed in separate cages. One vial of virus stock was thawed out quickly at 37° and centrifuged in an International High-Speed Refrigerated Centrifuge model HR-1, at 5000 rpm and 4°C for 5 minutes. The supernatant containing the virus was used to make ten-fold dilutions ranging from undiluted to 10⁻⁶ in 1x sucrose stabilizer. Ten mice were injected intraperitoneally (IP) using a volume of 0.1 ml per mouse. The mice were left to develop the disease for 30 days and frequently palpated for splenomegaly. If splenomegaly could not be determined by palpation, the spleens were sectioned and examined with a light microscope. The results are given in Table I where + indicates splenomegaly while - indicates no splenomegaly.

TABLE I. Titration for LD₅₀ of Virus

Titer	Und.	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶
Splenomegaly (+)	10/10	10/10	10/10	10/10	6/10	2/10	0/10
No Splenomegaly (-)	0/10	0/10	0/10	0/10	4/10	8/10	10/10

The Reed-Muench method was used to determine the LD_{50} of the virus and was calculated in Table II.

From the data in Table II it is evident that the highest dilution that will insure a 100% infection in BALB/c mice is 10^{-3} the LD_{50} being $10^{-4.32}$ this dilution contains about 20 LD_{50} units.

Drugs. The drugs used in this study were methotrexate sodium (MTX), 6-mercaptopurine (6-MP), vinblastine sulfate ($Vin-SO_4$), and hydrocortisone acetate (cortisone).

Two regimens of drugs were used, the first (Experiment I) consisting of a combination of 6-MP + $Vin-SO_4$ + MTX, and the second regimen (Experiment II) consisting of a combination of 6-MP + MTX + cortisone. The dosage of the immunosuppressive agents was 10 mg/Kg body weight or 2.5 mg/Kg body weight for cortisone and 6-MP, and 4 mg/Kg for $Vin-SO_4$ and MTX respectively. The action of these immunosuppressive agents has been discussed by Berenbaum, 1965.

For the GF mice, the same regimen as for Experiment II only was used. The drugs were suspended in sterile distilled water. Aliquots of the drug combination were cultured in thioglycollate and BHL and incubated at $37^{\circ}C$ to check for bacterial growth. The same dosage and schedule were used for the GF as for the CR.

TABLE II. Reed-Muench Method for LD₅₀ Determination

Virus dilution	Mortality ratio	Died	Survival	Total death	Total survival	Mortality ratio	%
Undiluted	10/10	10	0	48	0	48/48	100
10 ⁻¹	10/10	10	0	38	0	38/38	100
10 ⁻²	10/10	10	0	28	0	28/28	100
10 ⁻³	10/10	10	0	18	0	18/10	100
10 ⁻⁴	6/10	6	4	8	4	8/12	67
10 ⁻⁵	2/10	2	8	2	12	2/14	14
10 ⁻⁶	0/10	0	10	0	22	0/22	0

10

$$\% \text{ mortality at dilution above 50\%-50\%} = \frac{67-50}{67-14} = \frac{17}{53} = .32 \text{ (Proportionate distance)}$$

% mortality at dilution next above 50%-

% mortality at dilution next below 50%

Negative log of lower dilution (next above 50% mortality)

$$= -4.0$$

Proportionate distance (0.32) x dilution factor (10g 10)

$$= -0.32$$

Log LD₅₀ titer

$$= -4.32$$

LD₅₀ titer

$$= 10^{-4.32} / 0.2 \text{ ml}$$

Production of Leukemia in CR Mice. A total of sixty conventional mice were arranged into six groups of 10 mice each, designated groups 1 through 6. Groups 1, 2, and 3 were given one combination of drug and were designated Experiment I. Groups 4, 5, and 6 were given another combination of drugs and were designated Experiment II. In each cage the mice were numbered from 1-10 by clipping their toes. Table III summarizes the grouping of mice with respect to their treatment.

A vial of stock virus was thawed out quickly at 37°C. The contents were centrifuged in an international High-Speed Refrigerated Centrifuge Model HR-1, at 5000 rpm and 4°C for 5 minutes. The supernatant containing the virus was used to make a 10^{-3} dilution in 1x sucrose stabilizer. Forty mice were injected IP with a dose of 0.2 ml of the virus dilution. These mice were left for 14 days to develop the disease.

Fourteen days after injection of the mice with virus, palpation of the mice showed 100% splenomegaly. At this time drug treatment was started and for one month thereafter, all the mice, except the virus control, received one regimen of drugs consisting of a combination of MTX + 6-MP + Vin-SO₄. The injections were given every other day, and the mice first received a dose of 10 mg/Kg given for 7 days in 0.25 ml quantities, but then maintained on 2.5 mg/Kg given

TABLE III. Grouping of Mice.

	Virus + Drugs (V+D) Mouse No.	Virus Control (V) Mouse No.	Drug Control (D) Mouse No.
Group 1	1-5	6-10	
Group 2	1-5	6-10	
Group 3			1-10
Group 4	1-5	6-10	
Group 5	1-5	6-10	
Group 6			1-10

in 0.06 ml quantities for the duration of the experiment.

The schedule of drug treatment was variable in that treatment was stopped whenever the mice exhibited the severe signs of wasting, i.e. ruffled fur, diarrhea, and excessive loss of weight. At one time drug treatment was discontinued for 20 days due to the condition of the mice. When it was resumed the mice in Experiment I were given the same regimen of drugs, but for the mice in Experiment II, the regimen was changed to a combination of MTX + 6-MP + cortisone; the mice were again injected every other day with the maintenance dose of 0.06 ml.

Production of Leukemia in GF Mice. A total of 30 GF mice were divided into 3 groups of 10 mice per group. One group was the virus control, another group the drug control while the third group served as the treated group. The virus was prepared for introduction into GF units by first diluting in 1x filter sterilized sucrose stabilizer (.45 millipore filter). For the GF mice a 10^{-2} dilution was used since it was presumed that some activity would be lost due to filtration, hence a 10^{-2} dilution would compensate for any loss of activity. This dilution was filtered with 0.8 μ millipore filter using a Sweeny adapter. An aliquot of this filtrate was taken out and the remainder was sprayed into the GF unit. Thirty minutes after bringing the virus into the unit the GF mice were injected IP

with a dose of 0.2 ml each mouse, and left for 14 days to develop the disease.

Part of the aliquot left outside the unit was used to inject IP 5 conventional BALB/c females with a 0.2 ml per mouse to determine whether the suspension was still active after filtration. The remainder of the aliquot was inoculated into thioglycollate broth and BHL semi-solid and also streaked on blood agar (BA) plates to determine whether the suspension was sterile. The BA plates were incubated aerobically and anaerobically at 37°C. After 3 days, the BA plates, thioglycollate broth, and BHL semi-solid were examined for any growth.

Parameters of Experiment

Organ and Body Weights of Mice. All the CR mice were weighed and their weights recorded. Upon the death of a mouse, the spleen and liver weights were taken.

Upon the death of an animal, tissue samples of the liver, spleen, kidney, intestines, and lungs were taken and sectioned.

After the experiment had proceeded for 6 months, hematologic studies were curtailed because the hematocrits were becoming rather low when other parameters were not changing noticeably. Hence it was assumed that the low values of the hematocrits for test and

control mice were probably due to the frequent bleeding of the mice, rather than to the leukemic process.

For the GF mice white blood cell counts and blood smears were made weekly to check the progress of the disease.

RESULTS

Experiment I

Experimental Approach - White blood cell counts, hematocrits, and differential counts were obtained from adult BALB/c mice, before they were challenged IP with 0.2 ml of a 10^{-3} dilution of Friend virus (20-LD₅₀). Ten readings for each parameter were taken from which the average for each parameter was established and considered to be the normal value. Twenty four days after challenge with the virus, the mice were treated with a combination of Methotrexate+6-Mercaptopurine+Vinblastin Sulfate. The drugs were given on alternate days for a period of 88 days.

The Leukemic Process

Hematology of BALB/c Mice - The data shown in Table IV indicate that the mean white blood cell count for normal mice was $10,270 \pm 18 \times 10^2$ and 54.6 ± 2.5 for the hematocrit. The lymphocyte constituted the major cell type, 81.8% in the white blood cell population.

The progress of the disease can be described from the tables and graphs given below. The data in Table V describe the change in the white blood cell (WBC) count and the hematocrit values during the experiment.

The results in Table V further show that there is an increase in the white count 29 days after introduction of the virus into the mice.

TABLE IV. Hematocrit total and differential white blood cell counts of BALB/c mice prior to challenge with virus and drugs.

Mouse Number	WBC Count	Hematocrit%	Differential Count %			
			Lymphs	Polys	Monos	Eos
1	8,500	54	74	24	1	2
2	14,000	56	82	15	2	2
3	10,750	58	84	14	1	1
4	12,450	59	86	12	0	2
5	9,450	53	81	16	2	0
6	8,450	52	78	21	0	1
7	8,850	52	85	13	1	0
8	10,300	52	79	20	1	2
9	9,150	54	86	14	0	1
10	10,750	56	83	15	0	2
Mean	$10,270 \pm 18 \times 10^2$	$54.6\% \pm 2.5$	81.8 ± 3.6	16.4	0.8	1.3

^a SE = Standard Error or deviation.

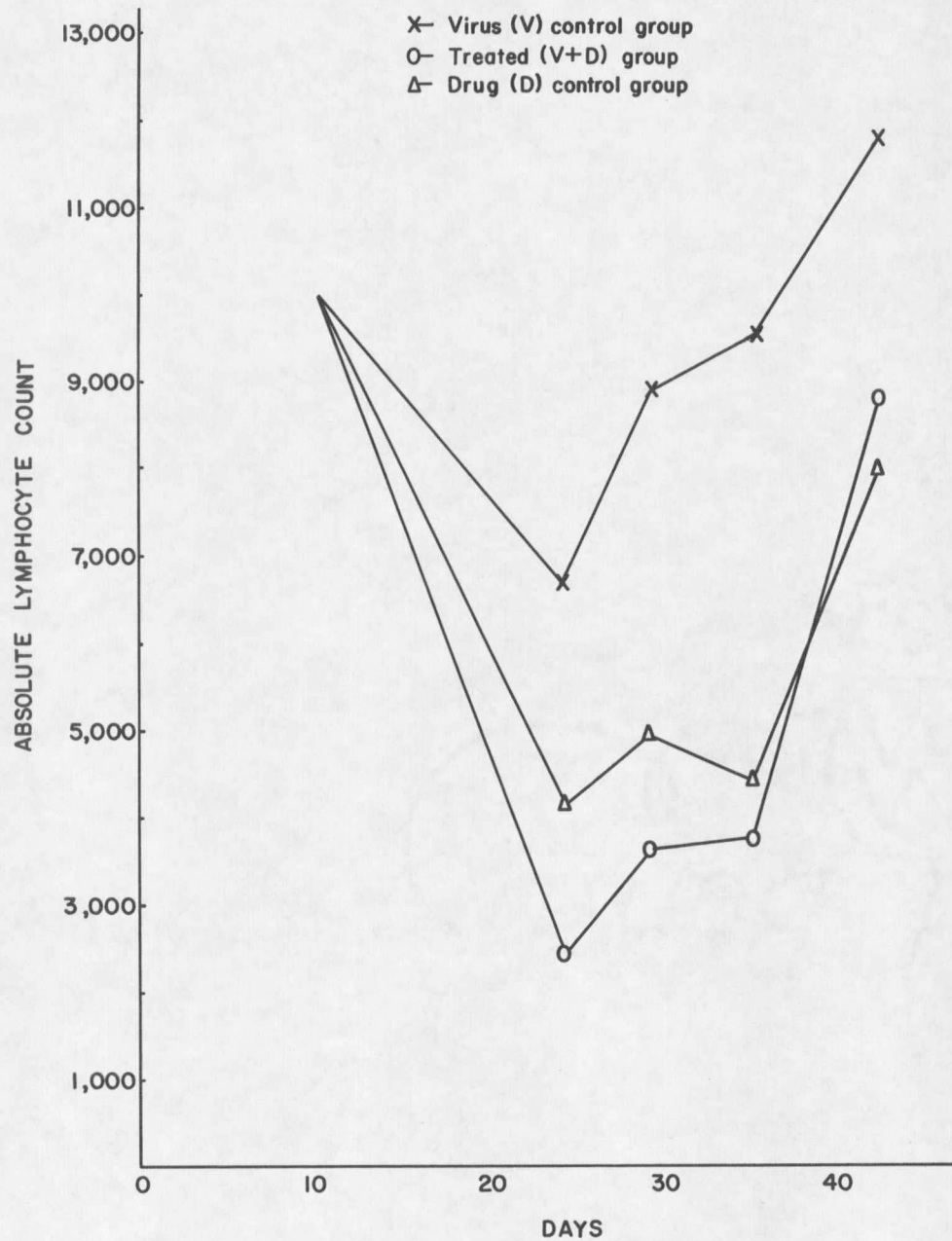


Fig.1 Absolute lymphocyte counts of BALB/c mice treated with Friend virus and/or antileukemic drugs (Exp. I)

TABLE V. White blood cell counts and hematocrit values of BALB/c mice treated with Friend virus and a drug Regimen. (Experiment I*)

No. of days since start of experiment	Ave. WBC Count			Ave. Hematocrit %		
	Group			Group		
	V ^a	V + D ^b	D ^c	V	V + D	D
24	9,300	5,390	5,120	52.8	51.8	54.0
29	12,010	5,890	6,570	55.2	45.2	55.2
35	13,730	5,720	6,067	53.3	48.5	50.3
42	15,360	11,790	11,130	52.8	44.0	49.2
Treatment stopped for 20 days						
69	167,880	58,016	14,520	42.8	50.3	58.4

^a V = Friend virus in a dose of 10^{-3}

^b V + D = Friend virus in a dose of 10^{-3} + Regimen

^c D = Drug control with Regimen

* Normal WBC count was 10,270 cells/cmm

For the virus control group, this increase becomes marked at the termination of the experiment. In contrast, the white counts of the treated and the drug control groups compare very well, but fall below the normal levels. This indicates that the treatment was successful in controlling the leukemic process. The increase in WBC count is presented graphically in Figure 1. The fact that the white count rose dramatically when treatment was stopped gives further evidence for the effect of treatment.

The hematocrit values presented in Table V show that anemia is a consequence of the leukemic process and was evident at the terminal stages of the disease. The data suggest that the treatment did not appear to alter the hematocrit value and at 35-42 days the low hematocrit values in the drug control group is perhaps more a consequence of frequent bleeding rather than due to treatment. This is suggested by the hematocrit values of the virus control group and the treated group which continued to be low, while the values in the drug control rose to above normal after treatment and bleeding of the mice had stopped.

The differential count (Table VI) shows some difference among the three groups; also, considering the groups individually, there were many changes from the normal. In the virus control group, the cells, particularly the lymphocytes showed abnormalities compatible

TABLE VI. Average differential count for BALB/c mice treated with MTX + 6-MP + Vin SO₄.* (Experiment I)

Days post virus in- jection	<u>Lymphs</u> Group			<u>Polys</u> Group			<u>Monos</u> Group			<u>Eos</u> Group		
	V	V+D	D	V	V+D	D	V	V+D	D	V	V+D	D
24	72.0	45.2	81.0	25.0	51.2	16.0	1.6	2.6	1.0	1.4	0.6	2.0
29	74.6	62.0	76.0	27.0	36.0	21.0	1.0	2.0	1.2	1.4	1.0	0.8
35	69.2	66.0	73.0	29.2	30.2	23.0	1.4	2.0	2.8	1.4	1.8	1.2
42	76.8	70.4	71.5	20.4	25.8	26.0	1.8	2.6	1.5	1.2	1.2	1.0
69	87.2	81.0	84.4	11.0	17.0	13.4	1.0	0.8	0.6	1.0	1.2	1.6

* Each value is expressed as % of white blood cell populations on Wright stained smears.

V = Virus control group

V+D = Treated group

D = Drug control group

with leukemia. Since the cellular changes in Experiment I and II are essentially the same. These changes will be described in detail with the results of Experiment II. In all three groups, the increase in neutrophils indicates that the mice may have become infected, most probably with their normal flora, soon after the initiation of treatment.

Pathological Studies - A compilation of data indicating the change in body weight, the difference in liver and spleen weights among the three groups, and the survival time for the mice in each group is presented in Table VII.

These data show that the average body weights at death of the mice in the virus control group and the treated group decreased markedly from the initial body weight averages. In contrast, there is an increase in average body weight in the drug control group. This increase is due to the apparent recuperation of the drug control group when treatment was stopped. Recuperation was accompanied by an improvement in the external appearance of the mice; continued treatment resulted in diarrhea and ruffled fur. The mice that died due to toxicity of the drugs showed congestion of the intestinal tract and other organs. These signs and others will be described in more detail in Experiment II.

TABLE VII. Comparison of body weights, spleen and liver weights, and survival time for Experiment I of BALB/c mice treated with Friend virus and/or drugs.

Mouse No.	Body Wt. (gms) ^a			Spl. & Liver Wt. (gms) ^c			Survival Time (days)		
	Group			Group			Group		
	V	V+D	D ^b	V	V+D	D	V	V+D	D
1	<u>15.26</u> 15.76	<u>20.90</u> 16.05	<u>17.23</u> 18.79	<u>1.25</u> 2.94	<u>0.80</u> 1.35		105	70	
2	<u>19.25</u> 17.80	<u>19.16</u> 16.33	<u>16.95</u> 16.68	<u>2.50</u> 1.85	<u>1.20</u> 1.40		68	74	
3	<u>16.76</u> 16.20	<u>20.77</u> 16.30	<u>19.95</u> 22.52	<u>2.05</u> 3.20	<u>0.70</u> 1.20		85	72	
4	<u>21.37</u> 20.69	<u>22.09</u> 18.52	<u>18.38</u> 16.32	<u>1.79</u> 5.52	<u>0.65</u> 1.50	<u>0.32</u> 0.69	90	70	
5	<u>20.01</u> 20.54	<u>20.06</u> 16.27	<u>16.89</u> 17.97	<u>2.01</u> 1.73	<u>0.94</u> 1.44		64	70	
6	<u>23.50</u> 15.47	<u>19.60</u> 14.41	<u>18.15</u> 17.77	<u>2.85</u> 2.05	<u>0.72</u> 1.54		71	70	
7	<u>19.95</u> 18.47	<u>20.30</u> 16.15	<u>19.92</u> 19.87	<u>0.54</u> 3.02	<u>0.18</u> 0.79		91	104	
8	<u>23.01</u> 15.87	<u>24.32</u> 20.05	<u>19.34</u> 22.94	<u>0.38</u> 0.52	<u>0.85</u> 1.40		92	91	

TABLE VII. Continued

Mouse No.	Body Wt. (gms) ^a			Spl. & Liver Wt. (gms) ^c			Survival Time (days)		
	Group			Group			Group		
	V	V+D	D ^b	V	V+D	D	V	V+D	D
9	$\frac{24.60}{20.10}$	$\frac{20.42}{16.21}$	$\frac{16.85}{15.92}$	$\frac{0.75}{3.29}$	$\frac{0.65}{1.50}$	$\frac{0.25}{0.64}$	95	70	64
10	$\frac{20.40}{21.43}$	$\frac{21.01}{15.76}$	$\frac{19.16}{20.69}$	$\frac{2.09}{1.29}$	$\frac{0.94}{1.44}$		91	70	
Ave.	$\frac{20.41}{16.86}$	$\frac{20.86}{16.60}$	$\frac{18.28}{19.03}$	$\frac{1.62}{2.54}$	$\frac{0.76}{1.35}$		83.2	77.1	

^a The numerator is body wt. at start, the denominator is body wt. at death.

^b The body wt. here is taken at the start, and the termination of the experiment.

^c The numerator is spleen wt., the denominator is liver wt.

V = Virus control group

V+D = Treated group

D = Drug control group

There is a distinct difference in spleen and liver average weights between the virus control group and the treated group. In the latter group the weights were not much above normal while they were more than twice the normal values in the virus control group. The two mice that died because of toxicity of the drugs displayed small spleens and livers. It is interesting to note that in the virus control group several of the mice that experienced prolonged survival showed greater involvement of the liver.

The mean survival time of treated group was unexpectedly lower than that of the virus control group. This however, is not an indication of the failure of the drugs to arrest the leukemic process. White blood cell counts, and liver and spleen weights clearly established the success of these drugs in controlling the disease. However, the combined effect of the drugs (immunosuppressive effects) and the proliferation of leukemia cells when treatment was stopped, apparently caused death of the mice. This is further suggested by the longer survival time of the virus control group, and few deaths in the drug control group.

Experiment II

Experimental Approach - Adult BALB/c mice were injected IP with 0.2 ml of a 10^{-3} dilution of Friend virus (20-LD₅₀) and then

treated for one month with drug regimen 1. Thereafter, the regimen was changed to include methotrexate+6-mercaptopurine+cortisone. The drugs were given on alternate days for a period of 242 days.

The Leukemic Process

Hematology and Survival Time - The normal WBC counts, hematocrits and differential counts for this experiment are represented by those taken for Experiment 1, since the mice used for the normal values were picked at random from those used for both experiments. The normal values for this experiment were then taken as $10270 \pm 18 \times 10^2$ for the WBC count, $54.6\% \pm 2.5$ for the hematocrit, 81.8% for lymphocyte count, and 16.4% for neutrophil count.

The survival time of the mice is given in Table VIII. Unlike the previous experiment, the mean survival time of the various groups in this experiment is very significant. The data show that the average survival time of the treated (V+D) group is three times that of the virus (V) control group. The longest survival in the latter group was 92 days, while in the former group, one mouse survived 262 days. The normal life span of the mice used is about 2 years, therefore about 30% of the treated group survived for about half their normal life span. The mean survival time of the drug (D) control group was about twice as high as the virus control group, but

TABLE VIII. Survival time of CR BALB/c mice challenged with Friend virus and/or treated with drugs. (Experiment II)

Group ^a	% Mortality	Death day	MST ^b (days)
V	10/10	(1) 78 ^c , (2) 92, (3) 65, (4) 68, (5) 89, (6) 68, (7) 71, (8) 42, (9) 67, (10) 61	71.1
V+D	10/10	(1) 248, (2) 262, (3) 160, (4) 216, (5) 234, (6) 160, (7) 184, (8) 140, (9) 95, (10) 168	183.1
D	9/10	(1) 107, (2) 207, (3) 23, (4) 185, (5) 233, (6) 25, (7) ---, (8) 151, (9) 177, (10) 225	133.7

^a V = Virus control group
V+D = Treated group
D = Drug control group

^b MST = Mean survival time

^c e.g., (1) = No. of mouse, 78 = survival time

significantly lower than the treated group. It is very likely that the mice in the drug control group died as a result of infection. Some of the mice that were dissected very soon after death showed congestion of the intestinal tract, liver, and kidneys. The lungs contained fluid, and the spleen was atrophied.

Table IX presents the average values obtained for WBC counts and hematocrits for the three groups of mice in Experiment II. The difference in WBC count among the three groups is striking. In all groups of mice the white count was at first depressed but then gradually rose to about normal levels in the interval between days 24-35. Thereafter, the white count in the virus control group increased at an extremely rapid rate to counts as high as 241,000/cm, 88 days post-challenge with virus.

In the treated (V+D) group, the white count was slightly above normal for the large part, but never approached the values found in leukemic (V) mice. This suggested that the drugs were responsible for keeping the white count far below the high levels reached in the virus control group. The treated mice appeared healthy, and there was no marked fluctuation in the white count except for two occasions. The two prominent rises in the white count seen in the treated group were at day 102 and day 137 (Table IX) where the counts were about 57,000 cells/cmm and 49,000 cells/cmm, respectively. Since there

TABLE IX. Average white blood cell count and hematocrit of female CR mice of the BALB/c strain treated with Friend virus and/or anti-leukemic drugs. (Experiment II)

Days post infection with virus	Ave. WBC count			Ave. Hematocrit %		
	Group			Group		
	V	V+D	D	V	V+D	D
24	8,452	6,780	6,240	54.8	52.8	54.0
31	10,502	6,290	6,870	53.2	40.2	56.4
38	14,310	8,405	7,856	53.3	41.5	49.5
45	16,634	13,975	14,457	53.5	49.0	44.3
52	25,283	15,450	13,846	50.0	50.2	49.3
69	110,205	18,350	9,950	40.6	53.0	55.0
81	203,830	13,730	5,230	40.4	48.6	54.4
88	241,562	14,780	8,434	39.0	42.8	58.6
95		19,260	10,470		43.0	54.0
102		56,502	14,808		47.6	48.8
109		28,705	7,583		42.8	45.8
116		25,844	12,550		40.6	46.6
123		21,980	7,377		39.4	43.6

TABLE IX. Continued

Days post infection with virus	Ave. WBC count			Ave. Hematocrit %		
	Group			Group		
	V	V+D	D	V	V+D	D
130		25,367	6,365		43.6	46.2
137		49,155	15,829		40.6	44.6
144		10,494	8,287		33.0	47.8
151		10,991	5,749		36.4	43.2
158		28,697	13,705		34.8	41.2
172		11,781	10,645		45.4	46.2
186		12,804	9,265		43.2	45.4
200		15,041	9,725		41.5	46.8
214		19,238	12,458		39.6	45.5
228		13,590	8,357		40.5	52.2
242		11,658	5,928		42.8	48.2

V = Virus control group

V+D = Treated group

D = Drug control group

was a corresponding rise of the white count in the drug control group (D), it was presumed that the rise in the treated group was due to the breakdown of one or more components of the drugs as a result of ageing. Thus, when a fresh combination of drugs was used, the white count was lowered dramatically. Other factors may contribute to the rise in white cell count, such as resistance to the drugs.

The rise and fall of the WBC population is illustrated by Figure 2 which gives the absolute value of lymphocytes over a 242 day period, and by Figure 3 which gives the absolute value of neutrophils over a 200 day period.

As mentioned earlier, there is initially a decrease in the white count in all three groups. In the drug control group this decrease can be attributed to the drugs which destroy cells of the lymphocytic series. However, in the treated group and in the virus control group, the probable explanation is that this is due to the virus which causes depression in the bone marrow. This depression is seen primarily in the granulocytes, while the lymphocytes are not affected.

The hematocrit values shown graphically (Figure 4) indicate that anemia was a consequence of the leukemic process and is especially prominent at the terminal stages of the disease. Great variation in hematocrit values is evident in the treated group where at times the values dropped to 33% such as on day 139. This however, as has been

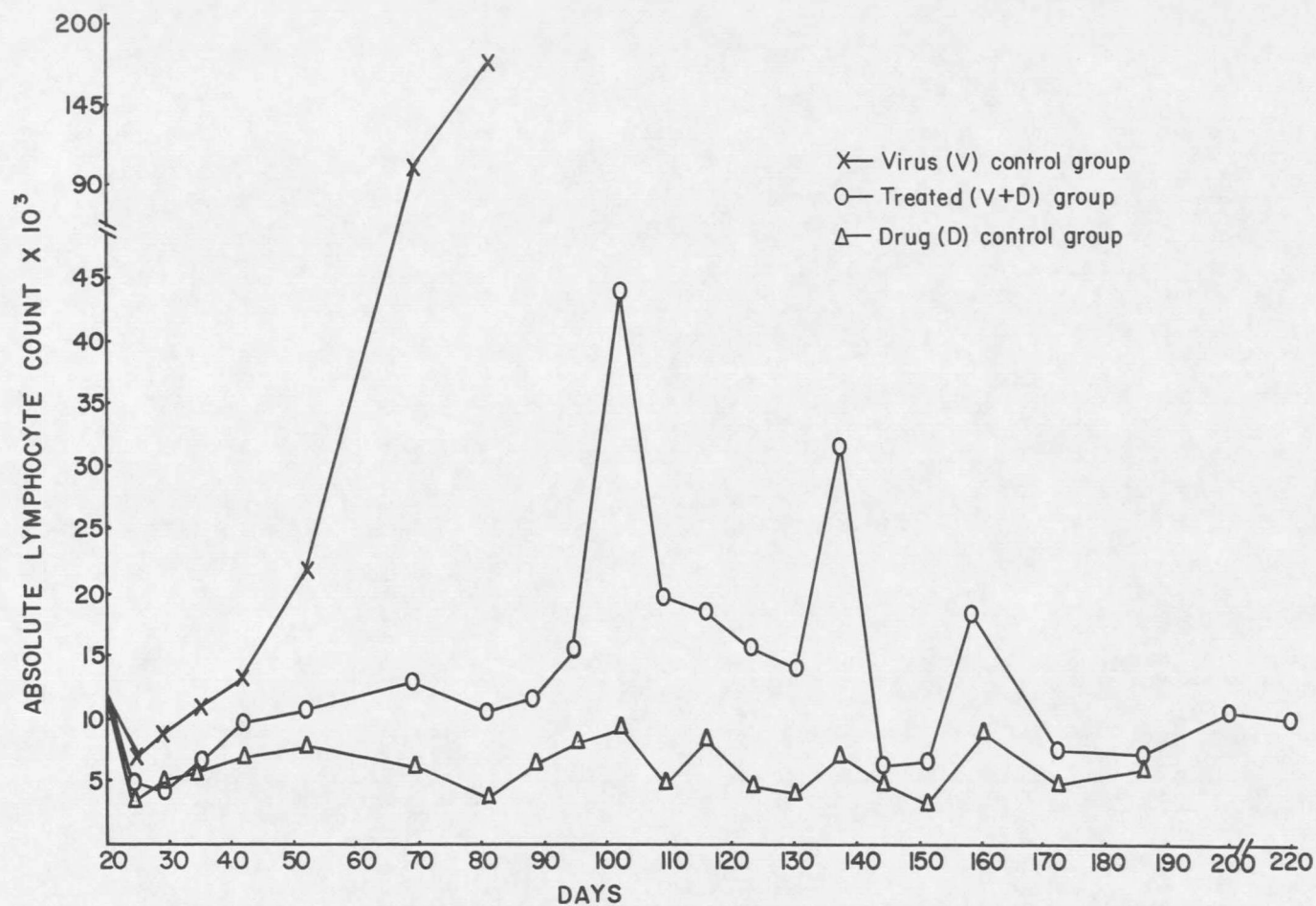


Fig.2 Absolute lymphocyte counts of CR BALB/c mice treated with Friend virus and/or antileukemic drugs (Exp. II)

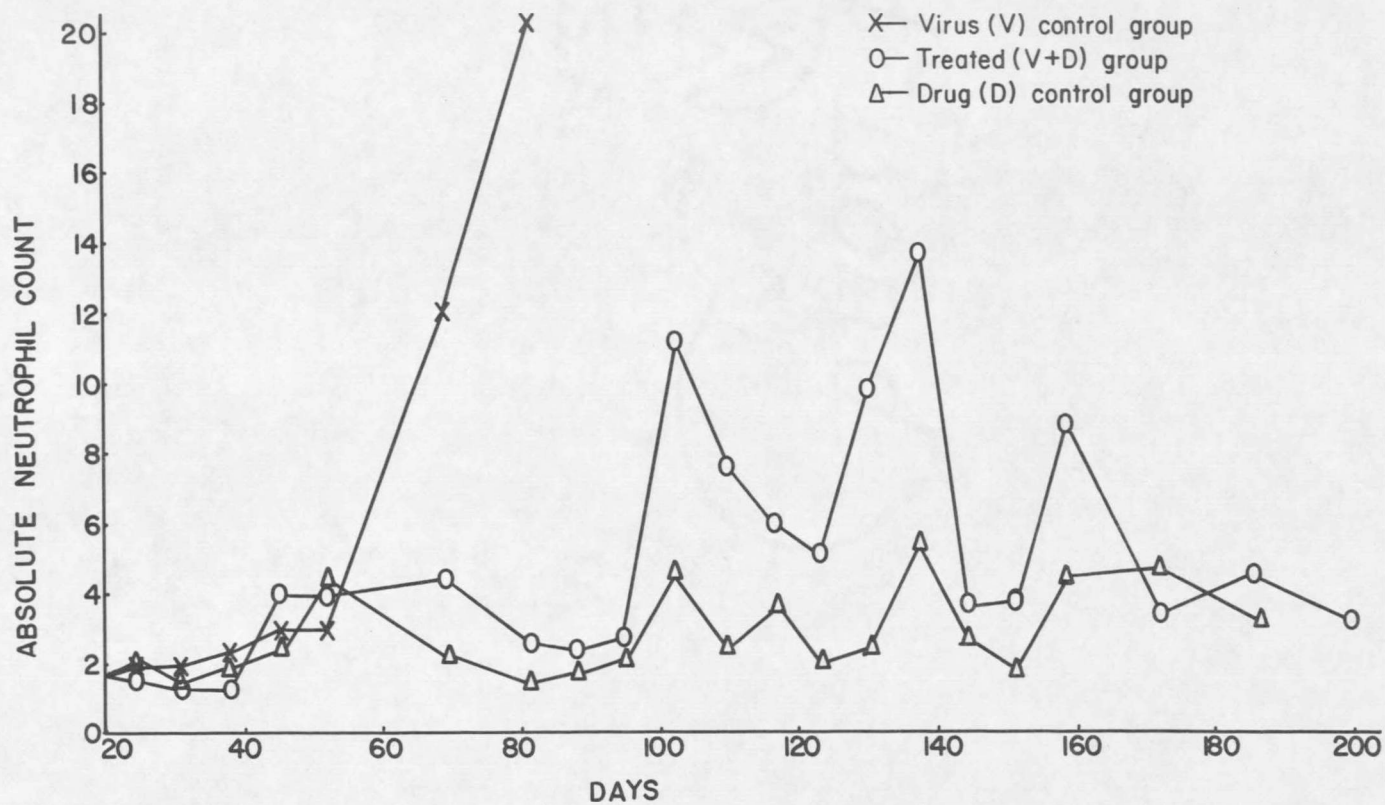


Fig. 3 Absolute neutrophil count of CR BALB/c mice treated with Friend virus and/or antileukemic drugs (Exp. II)

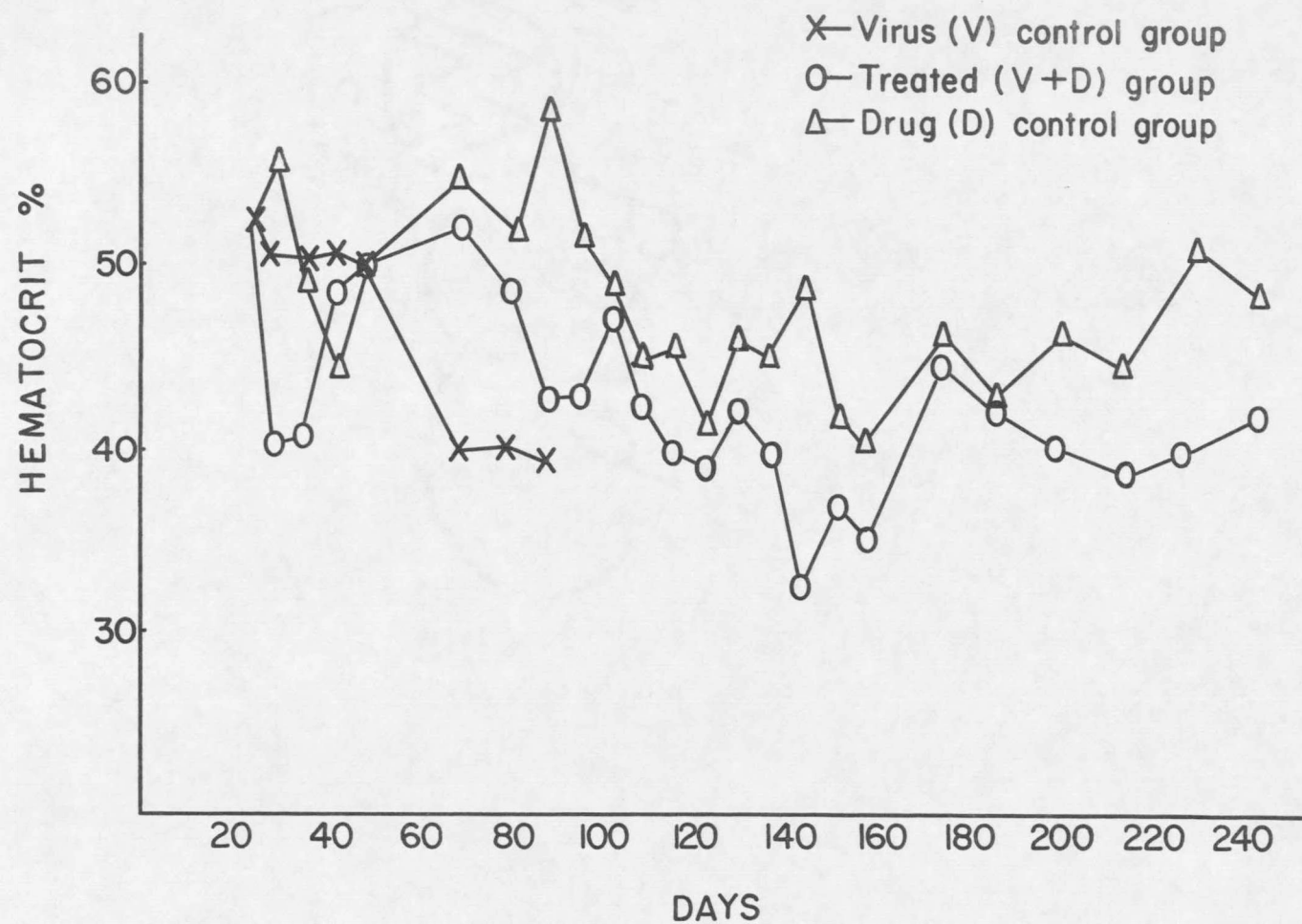


Fig. 4 Average hematocrit values for CR BALB/c mice treated with Friend virus and/or antileukemic drugs (Exp. II)

explained for Experiment 1, is mainly due to the frequent bleeding. Thus, when bleeding was done at two week intervals, the hematocrit values increased. Variation in hematocrit values in the drug control group were not as prominent as in the treated group.

Generally, the eosinophils appeared normal, and there was no change in the number of these cells in any of the three groups as compared to the normal values. The platelets did not seem to have been affected in any of the preparations examined.

The neutrophils presented an interesting case. The graph of absolute neutrophil count against time (Figure 3) is very similar to that of absolute lymphocyte count against time (Figure 2), indicating that there also was a significant increase of neutrophils in the virus control group. If the above two graphs are superimposed on one another, it becomes clear that the main increase in neutrophils in the virus control group comes after but corresponds with the large increase in absolute lymphocyte count. This and the fact that microscopic examination did not show abnormal neutrophils suggested that the increase in these cells is a result of factor or factors other than the virus.

Cell Morphology - Figures 5 and 6 show two blood smears, one from a normal mouse and the other from a leukemic mouse two months

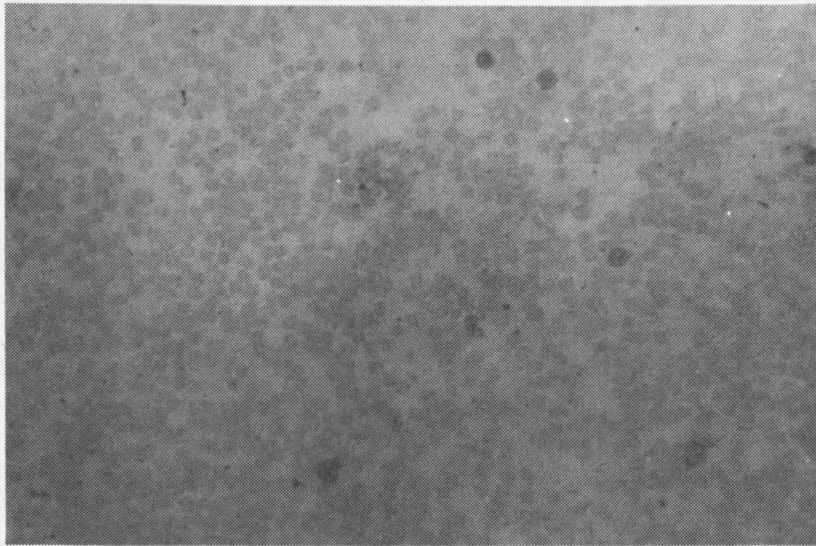


Figure 5. x100, Blood smear from a normal mouse showing a normal number of white cells.

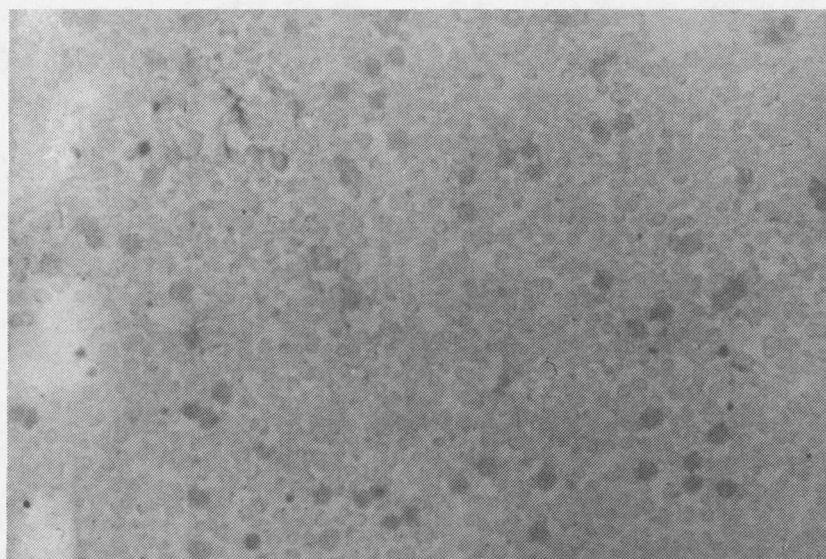


Figure 6. x100, Blood smear from a leukemic mouse, 2 months after challenge with virus. Note the large increase in the number of white cells.

after infection with virus. There are large numbers of white cells in the smear from leukemic mice in comparison to a few white cells in smears from normal mice. The main abnormalities seen in the blood smears were in the lymphocytes. The following description typifies the cellular morphology of the WBC population infected with Friend virus. The segmented neutrophils appeared normal (Figure 7), and a few of the lymphocytes appeared normal (Figure 8). There was a large number of degenerated cells (Figures 9 and 10) which were usually referred to as basket cells or smudge cells. Some of these cells appear to be streaked out, and it is possible that the only structure seen was the nucleus. Many abnormal lymphocytes looked doughnut-shaped, while others appeared like monocytes but could be distinguished by having a blue cytoplasm and were smaller in size than monocytes. Some of the lymphocytes had nucleoli typical of the very immature cell commonly found in leukemia. Many cells which were considered abnormal lymphocytes contained nuclei. The nucleus was lobulated as in the segmented neutrophil or large and indented resembling the nucleus of the monocytic cell. Therefore, the nucleus seemed to be the most abnormal structure in the leukemic cell. Many preparations contained large cells seen in Figure 11, and could not be positively identified. None of the abnormalities could be seen in the drug control group. Most of these abnormalities were seen in

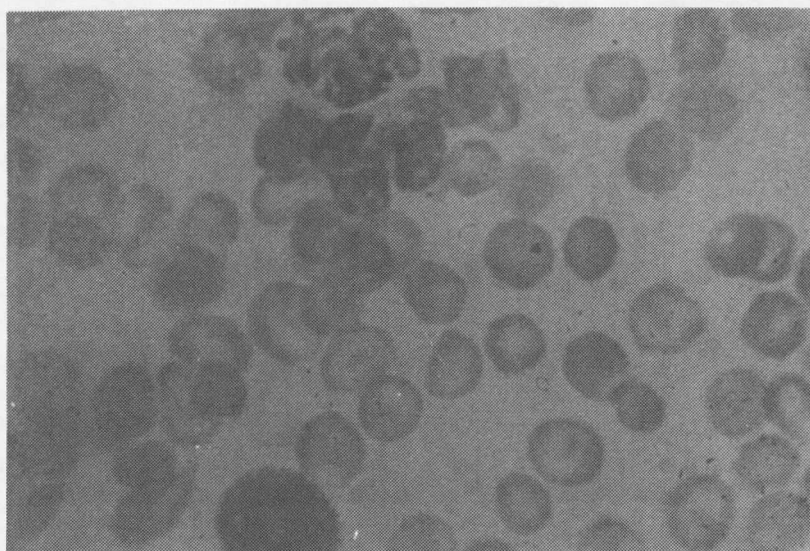


Figure 7. x1000, Normal appearing neutrophils obtained from a leukemic mouse 2 months after challenge with virus.

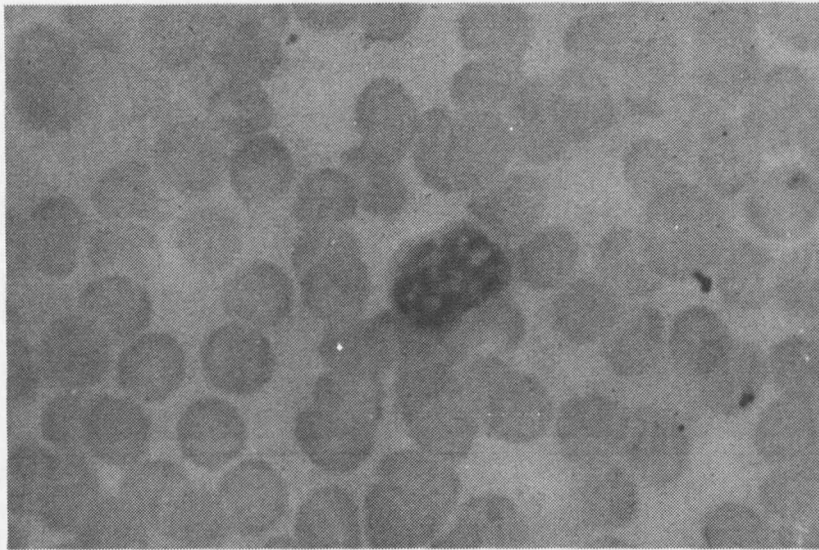


Figure 8. x1000, Normal appearing lymphocyte obtained from a leukemic mouse, 2 months post challenge with virus.

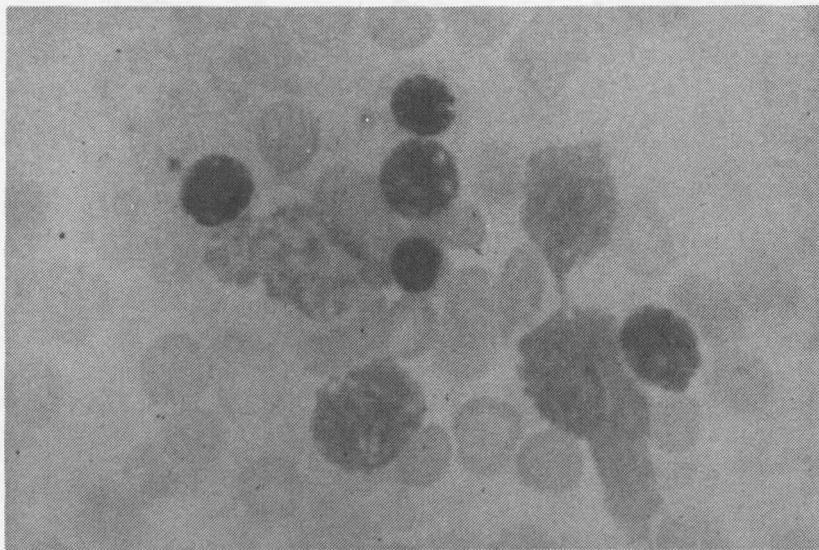


Figure 9. x1000, A blood smear obtained from a leukemic mouse, 2 months post challenge with virus. Note the presence of basket cells or smudge cells. Nucleated red blood cells are also present, and a Howell-Jolly body can be seen on one of the mature cells.

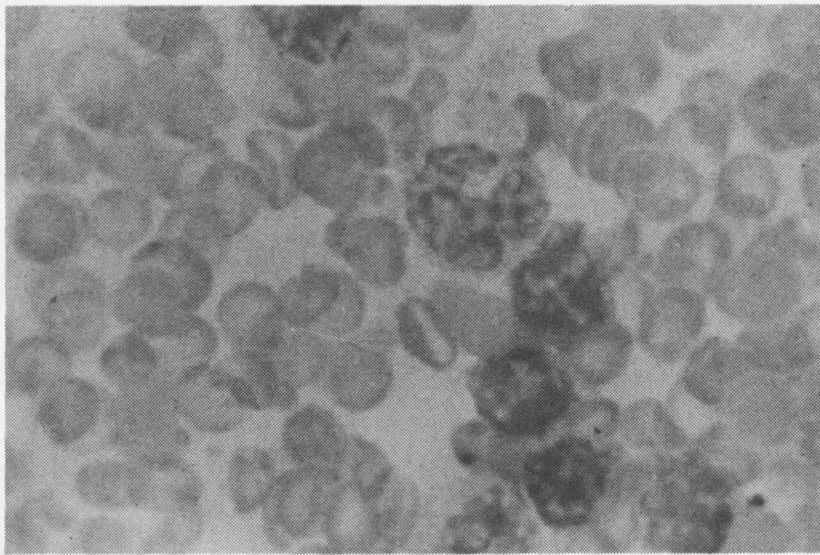


Figure 10. x1000, Blood smear from a leukemic mouse, 2 months post challenge with virus. There are several degenerated cells, and polychromatophilia is evident.

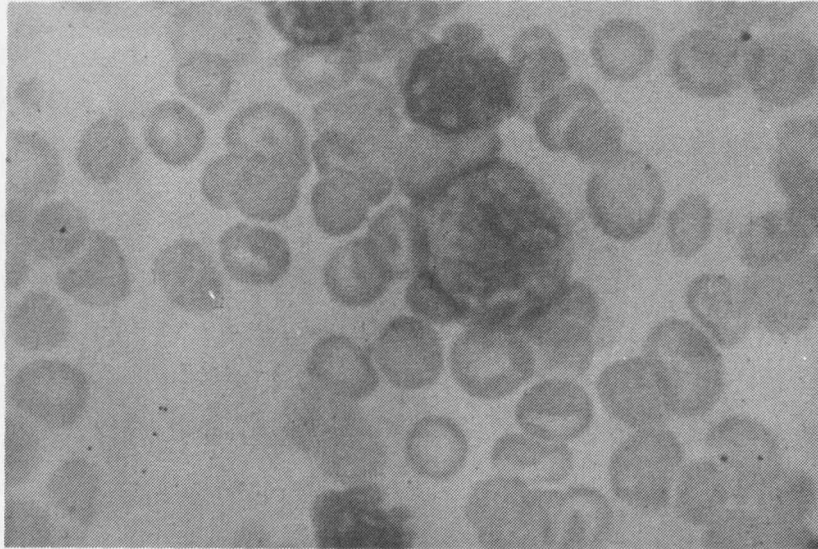


Figure 11. x1000, Blood smear from a leukemic mouse, 2 months post challenge with virus. Many preparations contained cells as the large cell present; these cells could not be positively identified.

cells from mice in the treated group but they were not as prominent or as frequent as in the virus control group, except during the period shortly prior to the death of the mice. In the latter group, some of the abnormalities were seen as early as 29 days after infection with virus, and they were extreme before the death of the animals.

The red blood cells in general appeared normal, however, there were some abnormalities which were seen at the late stages of the disease. Two of the most prominent abnormalities were the presence of many nucleated red blood cells (Figure 9) and polychromatophilia (Figure 10). The latter finding suggested that active hemopoiesis was occurring in the bone marrow, which is in agreement with the observation that at the terminal stages of the disease anemia was a constant feature. Howell-Jolly bodies were also present in some red blood cells (Figure 9).

Pathological Studies - Gross Findings

The data shown in Table X, summarizes the results of body, spleen and liver weights of the three experimental groups. In the virus control group, three of the mice (3, 4, 5) showed a decrease in body weight at death, however, the average body weight for the group was higher than at the beginning of the experiment. This is not true for the other two groups which showed a decrease in average body

TABLE X. Total body, spleen and liver weights of CR BALB/c female mice treated with Friend virus and/or anti-leukemic drugs. (Experiment II)

Mouse #	Body wt. at start and at death (gms) ^a			Spl. wt. & Liver wt. (gms) ^b		
	Group			Group		
	V	V+D	D	V	V+D	D
1	19.45	22.97	18.12	2.90	1.83	0.12
	20.06	19.92	13.04	2.15	2.25	0.40
2	15.71	20.70	19.25	1.01	1.54	0.32
	19.81	19.60	13.62	6.01	2.08	0.65
3	24.81	24.85	17.80	1.36	1.98	0.45
	21.64	16.65	15.72	4.94	2.70	1.05
4	16.25	24.92	17.90	2.60	0.55	0.34
	14.76	16.81	14.70	2.05	1.15	0.80
5	23.07	21.70	17.58	1.69	0.93	0.20
	22.38	17.38	13.15	1.48	1.08	0.62
6	20.94	23.81	16.10	3.40	0.41	0.41
	25.92	19.22	15.07	2.95	0.98	1.12
7	23.36	24.70	18.38	3.02	0.84	
	24.71	21.02	17.10	2.83	1.35	
8	20.65	22.37	16.85	2.90	0.52	0.35
	24.62	23.21	10.97	2.55	1.10	1.20

TABLE X. Continued

Mouse #	Body wt. at start and at death (gms) ^a			Spl. wt. & Liver wt. (gms) ^b		
	Group			Group		
	V	V+D	D	V	V+D	D
9	23.28	22.71	18.57	2.95	3.20	0.37
	27.08	22.85	13.51	2.50	2.02	0.95
10	21.44	21.16	18.69	3.50	0.59	0.28
	25.45	18.16	16.50	2.20	1.08	0.87
Ave.	20.90	22.97	17.92	2.53	1.24	0.284
	22.65	19.50	14.34	2.97	1.58	0.766

^a The numerator shows body wt. at start, the denominator shows body wt. at death.

^b The numerator shows spleen wt., the denominator shows liver wt.

V = Virus control group

V+D = Treated group

D = Drug control group

weight although two of the mice (8, 9) in the treated group had gained weight slightly over the experimental period. None gained weight in the drug control group.

In both the virus control group and the treated group, the average spleen and liver weights were above normal. The spleen weights for mice untreated in any way ranged from 0.4 gm to 0.7 gm, and the liver weights for similar mice ranged from 0.90 gm to 1.25 gm. Values for spleen and liver weights from the virus control group were twice those from the treated group. About half the mice in the drug control group exhibited normal or very slightly above normal values of spleen and liver weights, once again indicating the effectiveness of the drugs. In some of the mice in the virus control group increases in spleen and liver weights were dramatic. For example, the liver weights of mice 2 and 3 were 6.01 gm, and 4.91 gm, respectively, and the spleen weights of mice 6 and 10 were 3.40 gm and 3.50 gm, respectively. It is perhaps possible, at least in some mice in this group, to attribute the increase in body weight to the enlargement of these organs.

In the drug control group, mouse number 7 was still alive after the study had been stopped and therefore it was not included in the study. For the remaining mice in this group, the average spleen and liver weights were only slightly below normal values; however,

the drugs do cause some atrophy of these organs.

The spleen is of prime importance for all three groups, but mainly in the virus control group and therefore it deserves special mention as the key organ involved in the disease. It is the primary site for the replication of Friend virus, and when compared to other organs it contains the highest titer of virus. Furthermore, recoverable virus from the plasma was reduced by 75% when the mice were splenectomized (Chirigos and March, 1966). Examination of the leukemic mice soon after death showed the spleen to be very large, soft, and hemorrhagic. In BALB/c mice, the disease is progressive, and the spleens continue enlarging until they eventually rupture causing death of the animals. This is unlike Friend disease in other strains of mice such as BDF1 where, in many, the initial splenomegaly regresses (Dawson and Fieldsteel, 1964). The spleens upon examination contained small foci. These foci contain neoplastic cells, and were first demonstrated by Metcalf (Metcalf et al, 1959). In this present study, it was not determined how soon after injection these foci developed, but Chirigos has demonstrated that they were present in spleens of BALB/c mice by the third day after inoculation with a 10^{-1} to 10^{-4} dilution of Friend virus (Chirigos, 1967).

Pathological Studies - Microscopic Examination

Tissue sections from all three groups were examined

microscopically after death or sacrifice. In the virus control group, the main changes were present in the spleen, Figure 12, and liver, Figure 13. There is loss of normal architecture of the spleen and many tumor cells can be seen in this organ. The liver shows extensive destruction, and the lobules are separated by tumor cells. There is also evidence of infiltration of tumor cells in the lung, Figure 14. The alveolar septae are irregularly thickened due to infiltration by leukemia cells. Autolysis is also evident in the lung. The kidneys did not show any tumor, nor was there tumor involvement in the small bowel. However, there was apparent acute inflammation in the small intestine, as there was infiltration of leukocytes and neutrophils.

In the treated group, there were no, or very few tumor cells present in the liver, Figure 15. The leukemia cells (darkly staining) could be seen in the sinusoids. The overall architecture of the liver was still good, and trabeculae were still present. The lung, Figure 16, was mildly affected; leukemia cells could be seen in the septae and capillaries, however, they were less in number than in the virus control group. No real thickening of the alveolar septae could be seen. The small bowel and the kidneys looked normal with no signs of inflammation. The spleen, Figure 17, was well involved. There was no lymphoid architecture, and leukemia cells could be seen throughout the field. Megakaryocytes were also present in the spleen.

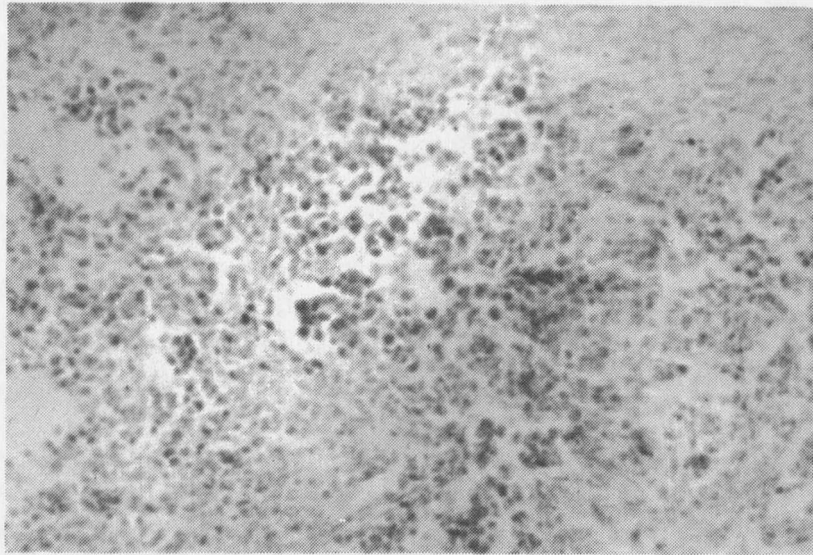


Figure 12. x100, Spleen of a leukemic mouse 2 months after challenge with virus, showing loss of architecture of the spleen, and the presence of tumor cells.

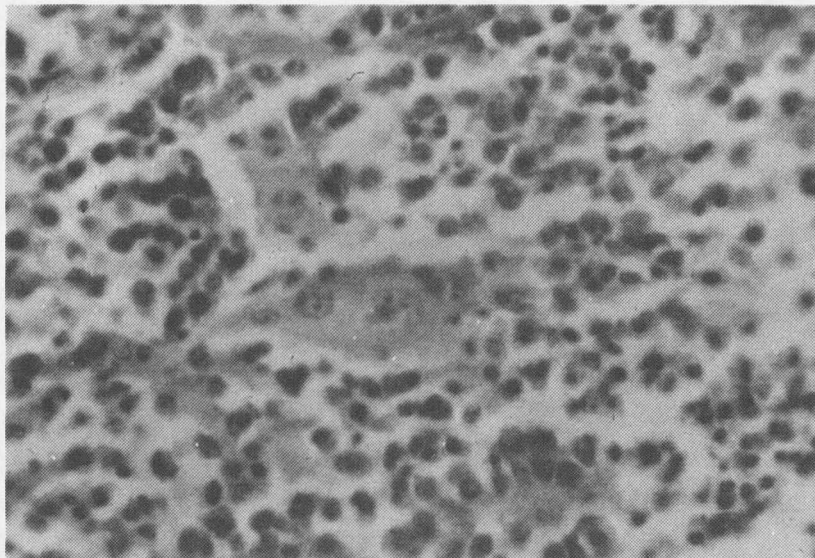


Figure 13. x400, Liver from a leukemic mouse 2 months after challenge with virus, showing extensive destruction, and separation of lobules.

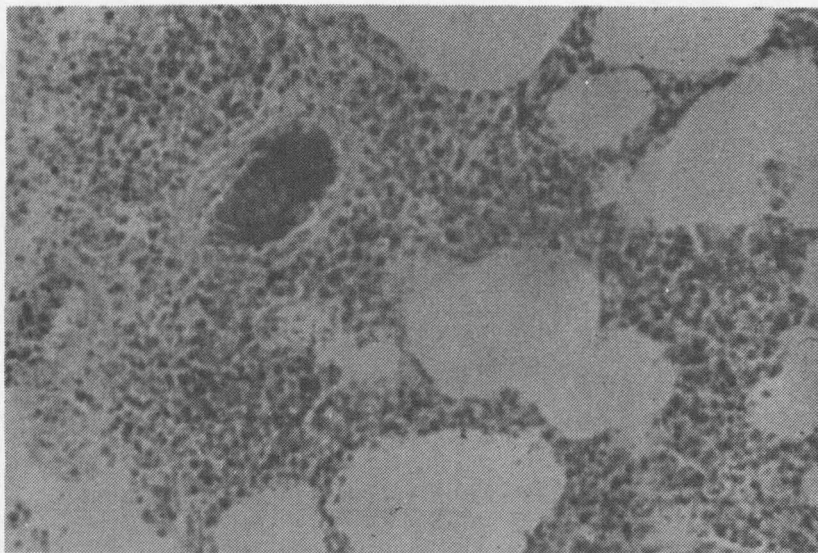


Figure 14. x100, Lung tissue from a leukemic mouse, 2 months after challenge with virus, showing infiltration of tumor cells, and thickening of the alveolar septae.

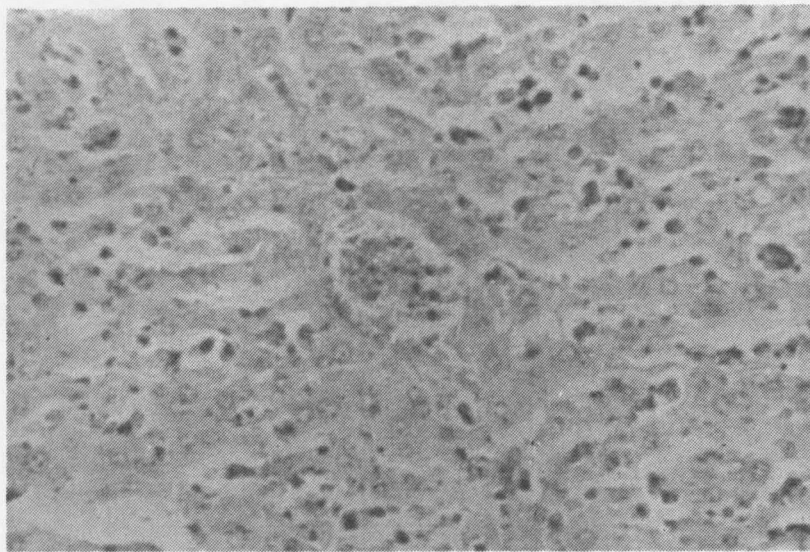


Figure 15. x100, Liver tissue from a mouse in the treated (V+D) group, treated for 160 days. There is slight involvement, and few tumor cells are present.

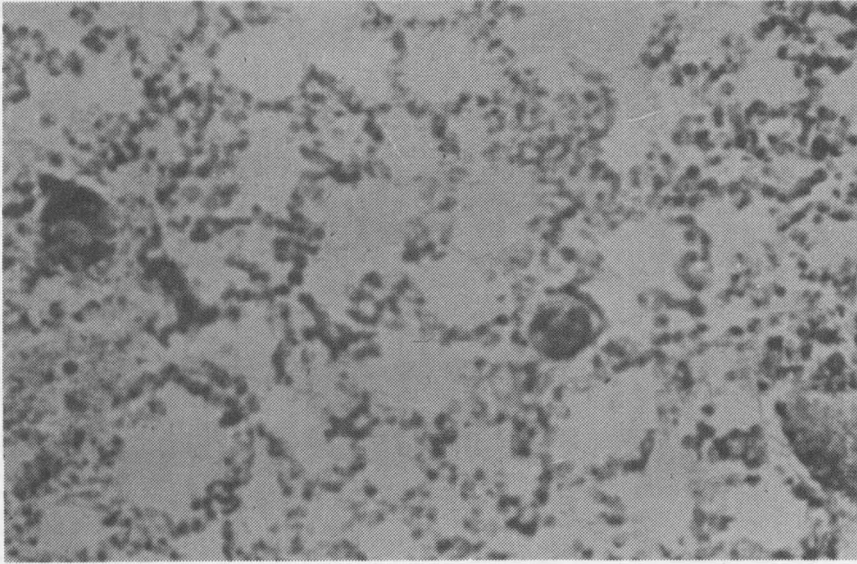


Figure 16. x100, Lung tissue from a mouse in the treated (V+D) group, treated for 160 days. Leukemia cells can be seen in the septae, however, there was only mild involvement.

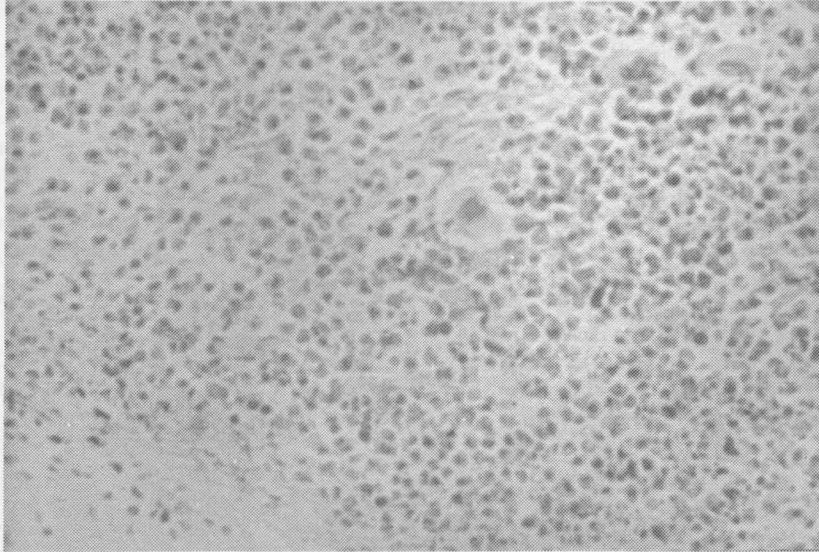


Figure 17. x100, Spleen tissue from a mouse in the treated (V+D) group, treated 160 days. Leukemia cells can be seen throughout and megakaryocytes are also present. Histologically spleens from the treated group could not be distinguished from those of the virus control (V) group.

Histologically spleens from the treated group looked very similar to those from the virus control group.

In the drug control group, inflammation was apparent in the small intestine. Most of the changes were observed in the spleen, Figure 18, however, they were not as extensive or marked as in the other two groups since no virus was injected in this group and the abnormalities are mainly due to the drugs. These changes included inflammation as suggested by the large numbers of neutrophils; many megakaryocytes were also seen in the spleen which is suggestive of extra medullary hematopoiesis. The liver, Figure 19, appeared normal.

Germ Free Experiment

Experimental Approach

Adult germ free BALB/c mice were injected IP with 0.2 ml of a 10^{-2} dilution of Friend virus (200-LD₅₀ doses) and then treated with a drug regimen consisting of a combination of MTX+6-MP+cortisone. The drugs were given on alternate days for a period of 60 days.

The Leukemic Process

Hematology and Survival Time

The normal WBC count for the germ free group was about 4224 $\pm$

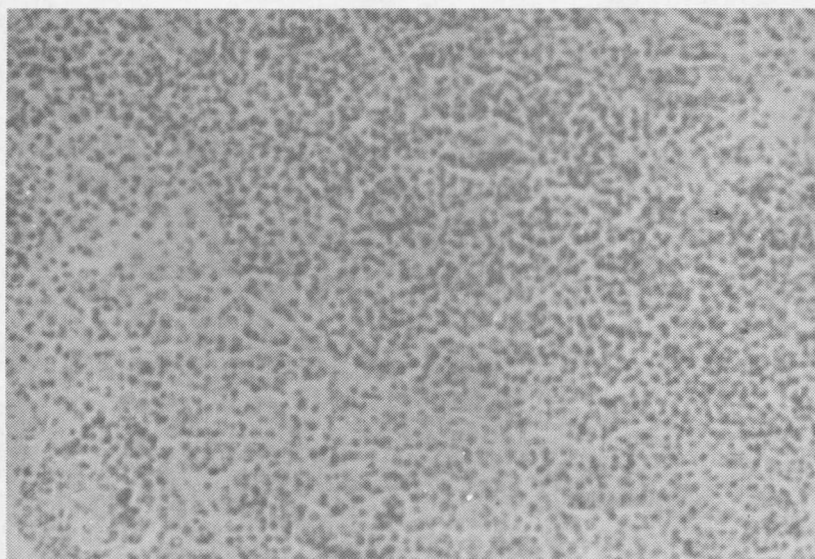


Figure 18. x100, Spleen tissue of a mouse from the drug control (D) group, treated for 107 days. Inflammation is suggested by the presence of large numbers of neutrophils. Megakaryocytes are also present.

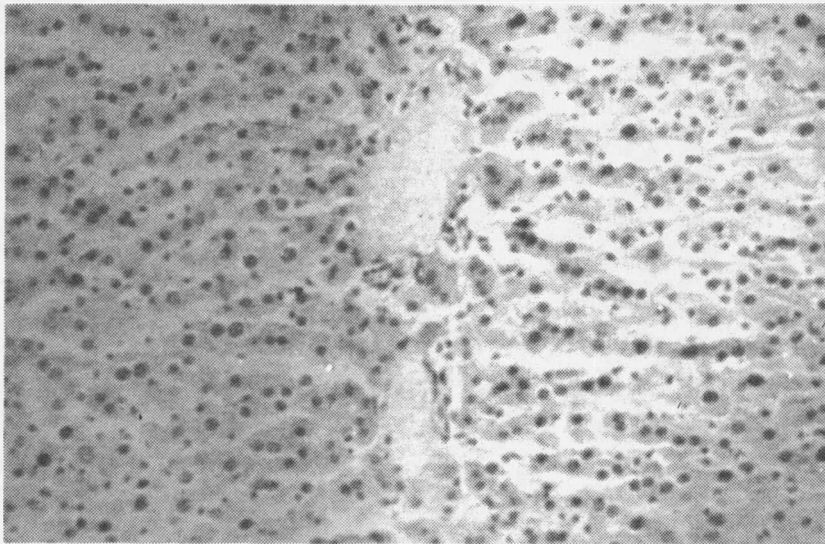


Figure 19. x100, Liver tissue of a mouse from the drug control (D) group, treated for 107 days. The tissue appears normal.

7.55×10^2 cells/cmm. The normal differential count showed that over 90% of the cells were lymphocytes, with few neutrophils, and occasional eosinophils and monocytes. These observations are explained by the fact that the GF mice are protected from microbial or antigenic challenge, hence their lymphoid tissue is underdeveloped.

The data in Table XI, in comparing the leukemic process in CR and GF animals, show that all the CR mice died, whereas only 30% of the GF mice died. Although the causes of death of the GF mice could not be accurately determined, it was presumed to be due to the leukemic process. The table shows that the mean survival time of those GF mice that died was 81 days as compared to 71 days for the whole of the CR virus control group.

Table XII, presents the average values obtained for WBC counts in the three experimental groups. In the virus control group, the WBC population rises, but very gradually. There is great variation in the WBC count in both the treated and drug control groups. The variation observed in the treated group indicates that the drugs may not have been too effective in controlling the disease.

The differential count is given in Table XIII. Generally, the eosinophils appeared normal, and there was no changes in the number of these cells in any of the three groups as compared to the normal values. The platelets did not seem to have been affected in any of

TABLE XI. Mortality among germfree (GF) and conventionally-reared (CR) BALB/c mice injected with Friend virus.

Challenge Dose		No. survived/ No. died	Mean Survival time
CR	20-LD ₅₀	0/10	71.1 days
GF	200-LD ₅₀	7/10	81.3 days

TABLE XII. White cell count for GF BALB/c mice treated with Friend virus and/or anti-leukemic drugs.*

Days Post Injection of Virus*	V	V+D	D
24	5,729	10,208	4,347
31	6,163	8,594	4,605
38	7,641	6,583	2,539
45	7,911	8,660	3,044
52	9,231	10,872	4,112
59	11,496	9,067	4,781

* The normal white count was 4224 cells/cmm.

** Mice were challenged with 0.2 ml of a 10^{-2} dilution (200-LD₅₀)

V = Virus control group

V+D = Treated group

D = Drug control group

TABLE XIII. Differential count for GF BALB/c mice treated with Friend virus and/or anti-leukemic drugs. *

Days	V				V+D				D			
	Lymphs	Polys	Monos	Eos	Lymphs	Polys	Monos	Eos	Lymphs	Polys	Monos	Eos
24	80.8	15.6	3.8	0	88.0	10.8	1.3	0	79.2	20.6	0.6	0
31	87.4	11.6	0.8	0	90.3	9.5	0.3	0	81.0	17.5	1.0	0.3
38	92.8	6.3	0.8	0.5	94.5	4.5	0	1.0	87.5	12.0	0	0.5
45	91.8	7.9	0.3	0	90.0	9.0	1.0	0	89.0	9.8	1.0	0.3
52	94.0	8.5	0	0.3	86.0	13.5	0.5	0	89.5	10.0	0.5	0
59	91.3	8.7	0.7	0	92.5	7.0	0.5	0	86.5	13.3	0.3	0

* The normal differential count showed that over 90% of the cells were lymphocytes, and only occasional monocytes and neutrophils.

** Days post injection of virus. Mice were challenged with 0.2 ml of a 10^{-2} dilution of virus (200-LD₅₀).

Lymphs = lymphocytes

Polys = neutrophils

Monos = monocytes

Eos = eosinophils

the preparations examined. A slight increase has been observed in the number of both the monocytes and neutrophils, however, no morphologic abnormalities could be seen in these cell types.

The lymphocytes showed most, if not all, the abnormalities seen in CR mice. Many smudge cells and immature cells were seen, and were as severe in the treated group as in the virus control group; at times they were more frequent.

The disease process in the GF mice is different than that observed in the CR mice. First, GF mice challenged with virus died rapidly when treated with drugs. Indeed the symptoms in this group were more severe than in either control group. This is illustrated by the data in Figure 20, which shows the absolute lymphocyte count for the three GF groups over the experimental period of 60 days. Note, that in the treated group, the white count is often higher than in the virus control mice. Second, the lymphocyte count in the virus control mice receiving 200-LD₅₀ doses of leukemia virus displayed no, or only mild symptoms of leukemia, and exhibited lymphocyte counts slightly higher than normal (Figure 21). Hence, at day 70 post infection the lymphocyte count in GF mice was about 11,000 cells/cmm, whereas the lymphocyte count in the CR mice was above 90,000 cells/cmm.

The red cells in the GF animals showed abnormalities similar to those present in CR mice. Many nucleated red blood cells, extreme

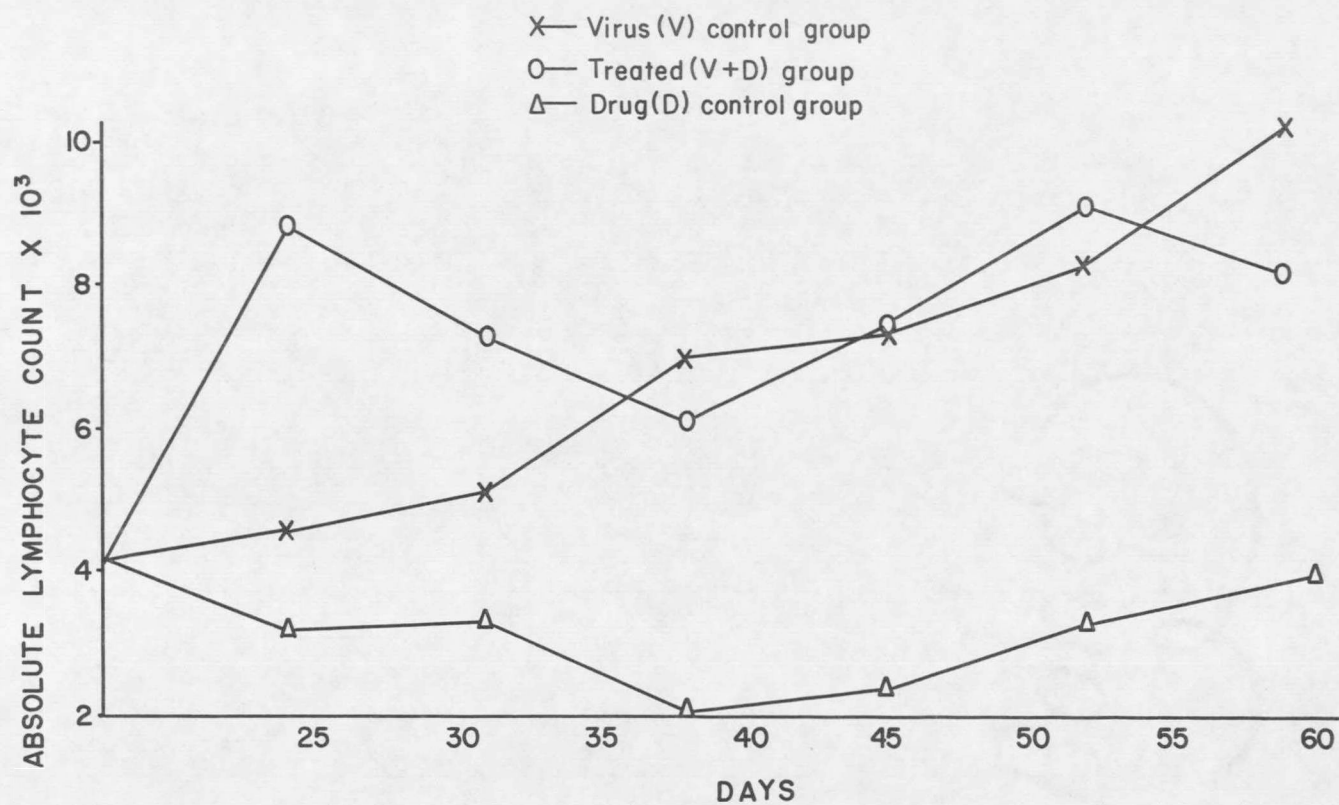


Fig. 20 Absolute lymphocyte counts of GF BALB/c mice treated with Friend virus and/or antileukemic drugs (Exp. II)

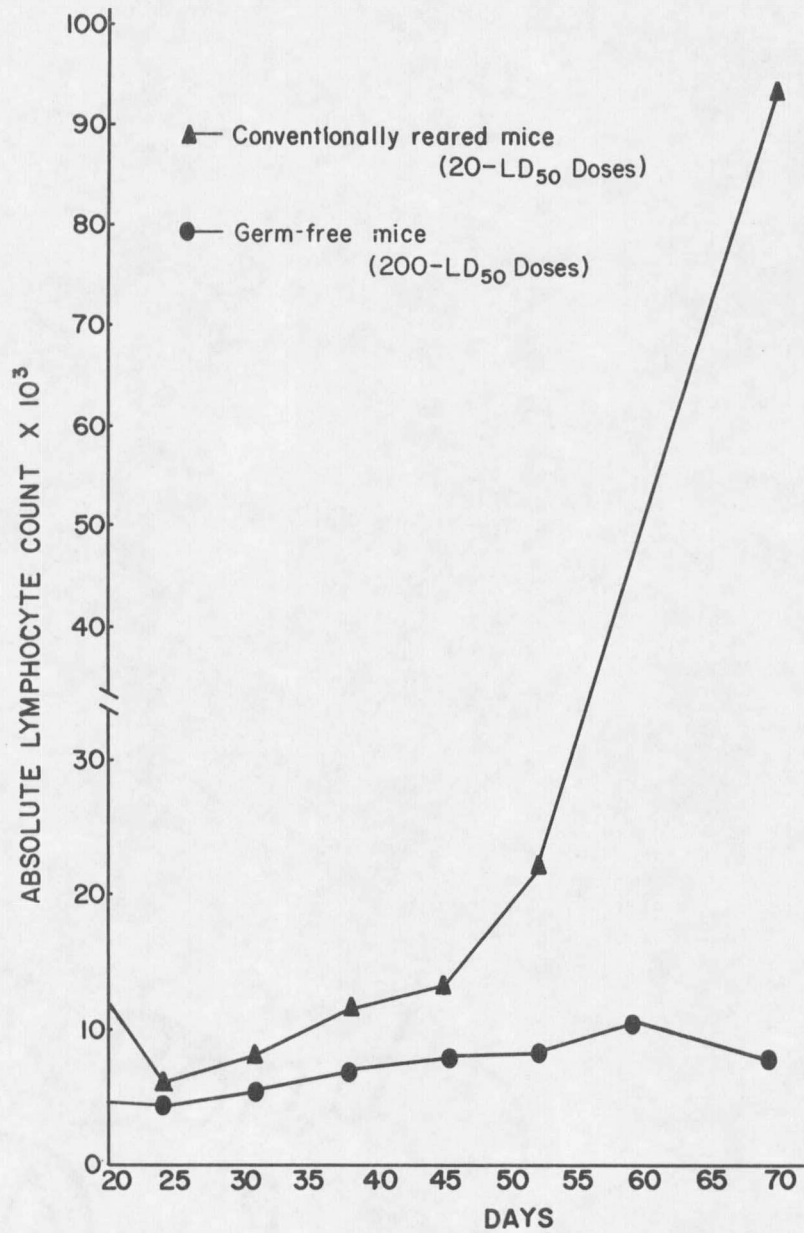


Fig. 21 Absolute lymphocyte counts for GF and CR BALB/c mice treated with Friend virus

polychromatophilia, and Howell-Jolly bodies were seen in both the treated and virus control groups.

DISCUSSION

In this study, combined chemotherapy has been used to evaluate the effects of drug combinations on CR and GF BALB/c mice challenged with Friend Leukemia Virus. The leukemic process established by the virus was also described and compared in the mice.

Combined chemotherapy has been found to be very effective in the treatment of various types of leukemias in both humans and experimental animals (Acute leukemia Group B, 1965, George et al., 1968, Hardisty et al., 1969). Any investigator using combination chemotherapy has to cope with several problems; among these are, the selection of a schedule, dose and a route that will be the most effective in treatment, and the effectiveness of drugs in combination with others. It is not uncommon to find that the best dose and schedule for one drug is not desirable, or even feasible when that drug is combined with others. One should also be aware of the fact that certain leukemias are more responsive to treatment than others, and that the age of the host is of importance in response to treatment. For example, the response of children between the ages of 2 and 19 is better than that of infants and adults (Henderson, 1969). In addition, there are other basic problems to consider when using any anti-leukemic drug whether singly or in combination, such as, the eventual resistance of the host to the drug, and the subsequent effect of the drug or drugs on normal cells. We do not, as yet,

have available anti-leukemic drugs that selectively destroy leukemic cells, hence we have to rely on other methods for evaluation of the treatment. According to Henderson, evaluation must depend upon incidence and duration of remission, and frequency of morbidity and mortality during treatment (Henderson, 1969). There is one final consideration, that is, most of the anti-leukemic drugs presently in use are anti-metabolites, replacing the natural metabolites during proliferation of the cells, which are thought to impair the multiplication of leukemic cells. Since the question whether leukemic cells proliferate or not has never been satisfactorily resolved, the efficacy of such treatment is in question.

Since the cure for leukemia is still in the future, the usual result of chemotherapy has been to extend the life of the host for a finite period. These studies ~~whoped~~ to extend the survival time to the point that could be regarded as a cure. To accomplish this, two regimens of drugs were used; the first consisted of a combination of MTX+6-MP+cortisone. Mercaptopurine is a purine analogue exerting its effect in 3 ways; first by producing 6-MP ribonucleotide which is the active tumor-inhibitory compound (Brockman, 1965); second, by preventing the first step in purine biosynthesis by inhibiting the formation of phosphoribosylamine (Brockman and Chumley, 1965); third, by preventing the enzymatic

conversion of inosinic acid to adenylic acid (Salser and Balis, 1965). Although 6-MP does produce toxic side effects which mainly affect the hematopoietic system, it is considered as one of the mildest anti-leukemic drugs. Methotrexate functions by inhibiting folic acid or dihydrofolic reductase which is responsible for the conversion of folic acid to tetrahydrofolic acid (Werkheiser, 1963, Bertino, 1963). Methotrexate is a rather toxic drug and can cause anemia and thrombocytopenia among other effects. Hydrocortisone acetate, a steroid hormone, destroys lymphocytes, regresses lymph nodes, and inhibits growth of certain mesenchymal cells (Meyers et al, 1968). The mechanisms involved are lysis of cells and inhibition of mitosis (Dougherty and White, 1945), and inhibition of DNA synthesis (Stevens et al., 1965). Recently it was found that cortisone reduces the hematopoietic activity, antibody-forming and proliferative responses of spleen cells but not bone marrow cells (Levine and Claman, 1970). Toxic effects of this drug include hypertension, diabetes, and increased susceptibility to infection. Vinblastin sulfate is the sulfate of a dimeric alkaloid obtained from the periwinkle plant, Vinca rosea. Its precise mode of action is still unknown, however, it is probably an antimetabolite which interferes with the utilization of glutamic acid, and in humans and animal cells it stops mitosis in metaphase (Krantz and Carr, 1969). Its

toxic effects include stomatitis, muscular pain, gastrointestinal distress, and weight loss.

In this study, for the best results, the drug dosages of 4 mg/kg body weight for MTX and Vin SO₄, and 10 mg/kg body weight for 6-MP and cortisone were injected IP in 3 initial doses of 0.25 ml each, and then in a maintenance dose of 0.06 ml given on alternate days. The combinations were effective for inhibiting the proliferation of leukemic cells, and also by destruction of the lymphocytes by cortisone thus keeping the white count at a low level.

Although the first regimen was very effective in controlling the disease, it was too toxic and its adverse effects on the mice surpassed the beneficial effects. The mice exhibited weight loss and severe weakness, and the hematocrit fell to low levels. Treatment had to be stopped several times to allow the mice to recuperate, and many of them died during treatment. The second regimen was found to be the more effective in terms of being less toxic, and therefore could be maintained for long periods. Hematologic and pathological studies showed that the drugs in regimen 2 were effective in keeping the leukemic process in check. This was characterized by lower white cell counts than in mice, low level of abnormalities in the lymphocytes, and healthy appearance of the mice. It is of interest to note that there was a significant increase in neutrophils

in the virus control group, however, no abnormalities were seen in these cells. A study conducted by Nieburgs and Goldberg on malignant neoplasia including various leukemias showed that malignancy-associated changes were seen in polymorphonuclear leukocytes when the cells were examined by a light microscope using a X100 oil immersion objective and X16 wide field oculars, and by closed circuit television microscopy at X16,000 (Nieburgs and Goldberg, 1968). Nevertheless, these changes were seen in humans, and it does not follow that they would be present in animals. Also, if they were found in animal cells, it would still have to be shown whether they are caused directly by the virus or by some other process associated with the disease.

The response of GF mice to treatment was appreciably different from that observed in the CR mice. The drugs were not nearly as effective for controlling the disease; it was found that the treated GF mice had more marked symptoms of disease, and died sooner than mice in either the virus control or the drug control groups. The mice in the GF virus control group did not show a marked increase in white cells as their CR counterparts. The causes of death of the three mice in this group can be attributed to the leukemic process since the mean survival time approached that for the CR animals.

Why is there a difference in the development of the leukemic process between the CR and GF mice? The reasons for the severity of the disease in the GF treated group are not clear, however, a remote possibility is that the poorly developed reticuloendothelial system is unable to cope with leukemic cell debris that accumulates following treatment with drugs. Based on the data, the following concept is proposed to describe the leukemic process. It is suggested that leukemia cells do not proliferate and that their rate of growth is much slower than corresponding normal lymphoid cells. Also it is speculated that the blast population of acute leukemia may be continually primed from a lymphoid stem cell compartment (Gavosto et al., 1968, Mauer et al., 1969). Hence, in the CR animals, where there is constant stimulation by antigens and commitment of stem cells to antibody production, the stem cell pool is continuously being replenished. When leukemia virus enters the environment of the stem cell it parasitizes those cells that enter into a differentiation phase characterized by DNA and, to some extent, RNA synthesis but little or no protein synthesis. The stimulatory effect of a cell commitment induced by antigen and virus on the stem cell gives rise to more susceptible cells and increased numbers of leukemia cells. In the GF animals, there is no large stem cell pool and when the virus parasitizes the available cells there are no

other cells, since the lack of antigenic stimulation does not replenish the stem cell pool. Nevertheless, we cannot rule out the possibility that some of the leukemic cells could reenter a phase of division (Gabutti et al., 1969). Also, it was shown by Baserga and Gold, that when nonproliferating Ehrlich ascites tumor cells were introduced into a new host, these cells synthesized DNA and entered a phase of division (Baserga and Gold, 1963). Mauer comments that if this is true in leukemias, then this cell population may be the most important in chemotherapy. That is, we could perhaps cause these nonproliferating cells to reenter a phase of cell division and thus make them extremely susceptible to intensive therapy (Mauer et al., 1969).

Certainly, the ultimate victory would be to eradicate leukemia; but we cannot deny that great advances have already been made. In the past, a child with acute leukemia had a maximum survival rate of one year, and most died within two to six months after diagnosis. Now, however, the use of chemotherapeutic agents has extended the survival time to several years, and for the most part the child leads a normal life. In the words of Dr. Lane, "Emphasis is now placed on total eradication of the leukemic cell population. New drugs and new forms of treatment are being investigated in hope that a cure might be found" (Lane, 1970).

SUMMARY

The effects of combined drug therapy on leukemia in conventionally-reared and germ free mice was studied. Leukemia was produced in conventionally-reared mice by the IP injection of 0.2 ml (20-LD₅₀ doses) of Friend Leukemia Virus, and in germ free animal by injecting 200-LD₅₀ doses of the same virus.

After allowing 24 days for the development of the disease, two regimens of drugs were used for treatment in conventional mice. The first consisting of a combination of methotrexate, 6-mercaptopurine, and vinblastin sulfate; in the second regimen, hydrocortisone acetate was substituted for vinblastin sulfate. Methotrexate and vinblastin sulfate were used in concentrations of 4 mg/kg body weight, and 6-mercaptopurine and cortisone were used in concentrations of 10 mg/kg body weight. Only the second regimen was used in germ free animals.

The leukemic process in conventional animals was monitored by hematological and pathological techniques, and in germ free animals by hematological techniques only.

The results indicated that although the first regimen was very effective in controlling the leukemic process, it was too toxic, and treatment had to be stopped prematurely. The second regimen however, was more effective in terms of being less toxic, and therefore treatment could be maintained for long periods. It was possible with the second regimen to alleviate the symptoms and prolong the survival

time of the mice to about 3 times the expected survival with this disease.

In the germ free animals the disease process was different than that seen in the conventional animals.. In general, the disease was much milder in germ free than in conventional mice. The germ free leukemic mice treated with drugs exhibited more severe symptoms than either of the other two germ free control mice. A thesis has been offered in an attempt to explain the observed difference.

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