Studies on chemotherapy of leukemia in conventionally-reared and germfree mice
by Nicola Mitri Kouttab

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements of the degree of MASTER OF SCIENCE in Microbiology
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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 consistring of a combination of methotrexate, 6-mercgptopurine, 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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Date May 27, 1970

STUDIES ON CHEMOTHERAPY OF LEUKEMIA IN CONVENTIONALLY-REARED AND GERMFREE MICE

by

NICOLA MITRI KOUTTAB

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements of the degree

of

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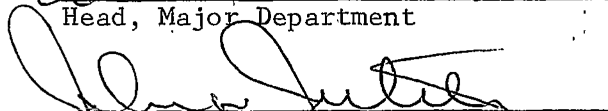
Microbiology

Approved:

Head, Major Department



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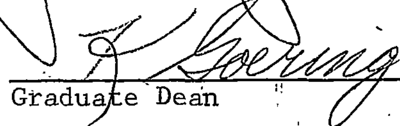

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ABSTRACT

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 hemopoeitic 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, vinblastine 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 antileukemic 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 CF 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 $4^{\circ}C$, 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 $-70^{\circ}C$.

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_2HPO_4	2.508 gms
KH_2PO_4	1.034 gms
Sucrose	150.000 gms
H_2O	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 $121^{\circ}C$) was added to give a 2% final concentration of gelatin in the stabilizer virus mixture.

Determination of LD_{50} for Virus. Virus titration was carried out to estimate the LD_{50} 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 $4^{\circ}C$ 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 LD_{50} 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°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 LD_{50} 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

% mortality at dilution above 50%-50% $= \frac{67-50}{67-14} = \frac{17}{53} = .32$ (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 LD_{50} titer = -4.32
LD_{50} titer = $10^{-4.32}$ /0.2 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-SO_4. 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

<u>Experimental Approach</u> - 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-LD_{50}). 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

<u>Hematology of BALB/c Mice</u> - The data shown in Table IV indicate that the mean white blood cell count for normal mice was 10,270 $\pm$ 18 x 10^2 and 54.6 $\pm$ 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.