THE EFFECT OF LEUCOGENENOL AND CHEMOTHERAPY ON LEUKEMIA L1210

An abstract of a Thesis by
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The problem. This study determined the effect of leucogenenol, an untried immunostimulant, in conjunction with known effective chemotherapy on mouse leukemia L1210.

Procedure. L1210 infected animals in various treatment groups were monitored for changes in several parameters of the disease. Among these parameters were differential smears, hematocrits, total nucleated cell counts, spleen and liver weights to total body weight ratios and survival times.

Findings. Although some of the parameters of the leukemia were decreased after leucogenenol therapy, a minimal detrimental effect was observed in the survival times of the animals. The humoral antibody levels were elevated after leucogenenol and chemotherapeutic treatment, but these were obviously not effective in controlling the number of proliferating L1210 leukemic cells.

Conclusion. It was concluded that leucogenenol was able to increase the humoral antibody response but not the number of sensitized lymphocytes such that the residual leukemic cells could be destroyed immunologically. Hence, even in combination with chemotherapy, leucogenenol's action could not turn the race between the residual leukemic cells and sensitized lymphocytes in favor of the host.

Recommendations. The recent demonstration of immunological enhancement of a tumor by humoral antibodies makes it important to determine if leucogenenol's action is specific for the B lymphoid cells or does it affect both the B and T lymphoid cells? A method for determining the molecular action of leucogenenol might lead to a greater understanding of the control of hematopoiesis and leukemias.

THE EFFECT OF LEUCOGENENOL AND CHEMOTHERAPY ON LEUKEMIA L1210

A Thesis

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by

Mark Alan Barnhill

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INTRODUCTION AND REVIEW OF THE LITERATURE

Remission of a tumor involves the elimination or the control of growth of a tumor cell population and is subject to a balance of factors pertaining to the drug, tumor and the host. Three important variables which have an influence on the attainment of complete remission are the extent of drug action on the tumor cells, the drug toxicity for the host tissue, and the immunocompetence of the host. Recent research has dealt with a combination of chemotherapy and immunotherapy in which maximum destruction of the tumor cells is followed by either specific or non-specific immunological stimulation to kill the remainder of the tumor cells present and/or to prevent the growth of new tumor cells. In this study combined chemotherapy of Leukemia L1210 with known effective drugs will be accompanied by immunotherapy with an untried immunostimulant, leucogenenol.

Leukemia L1210

Leukemia L1210, a tumor originally found in an animal treated with methylcholanthracene, was first reported by Law et al. (1949). The initial tumor arose in a female DBA mouse, subline 212, at 8 months of age following skin paintings with 0.2% methylcholanthracene in ethyl ether. The tumor still only grows in DBA mice and in F_1 hybrids and backcrosses derived from it (Mihich and Kitano, 1971). The disease was characterized by a great enlargement of the axillary, inguinal

and cervical lymph nodes and moderate enlargement of the spleen and liver. Law also reported enlargement of the internal lymph nodes and the thymus as well as extensive infiltration of the kidneys with leukemic cells. Following the inoculation of leukemic cells, there is a rapid local growth with infiltration of the cells into the liver, spleen, subcutaneous lymph nodes and internal lymph nodes. Within 48 hours, immature lymphoid cells are found in the peripheral blood. This proliferation in the blood is followed by massive infiltration of the tissue resulting in the death of the animal approximately 8 days after challenge. The rapid death was attributed to infarctions occurring in the vital tissues and organs. This leukemia has since this time been carried in DBA mice by intraperitoneal or intramuscular passage of viable cells suspended in a balanced salt solution. Although $1x10^5$ viable cells are usually injected to transplant the tumor, Schabel et al. (1966) showed that a single viable L1210 cell isolated with a micromanipulator and transplanted into a BDF_1 mouse gives rise to a population of cells that quadruples daily resulting in about 1 billion widely disseminated cells and death of the mouse in 15 to 18 days.

Skipper et al. (1967) showed that the average doubling time and the average generation time of L1210 leukemic cell populations are approximately equal. In contrast, Yankee et al. (1967) determined from radio-autograph studies that the generation time was 11.7 hours with a doubling time of

14.5 hours. This apparent discrepancy was explained by the extensive migration of the cells from the peritoneum during the course of the study. The period of DNA synthesis during the cell cycle was 8.9 hours and occupied 75% of the entire cell cycle. An elongation of the cell cycle as the tumor enlarges has been shown to be consistent with the crowding effect of a rapidly growing tumor in which the potential to proliferate is maintained but delayed (Dombernowsky and Hartmann, 1972).

Gresser et al. (1970) demonstrated that various mouse interferon preparations inhibited the multiplication of L1210 cells in cell suspension, and the degree of inhibition was found to be directly related to the anti-viral titers of the preparation. This finding has led to a search for a viral etiology for Leukemia L1210, but at this time although A-type particles have been seen and isolated from L1210 infected animals, viruses have not been conclusively demonstrated as a causitive agent for the leukemia (Brandes et al., 1966 and Kuff et al., 1972).

Glynn et al. (1963) established that CDF_1 mice are competent to react immunologically against L1210 leukemic cells. However, this immunological response varies over the course of the tumor growth. When CDF_1 mice were immunized to sheep erythrocytes (SRBC) during the early stages of the L1210 growth, a definite increase in the number of anti-SRBC plaque forming cells per spleen was observed. If immunization

was delayed until an advanced stage of the disease, a decreased immunological response was found (Bonmasser et al., 1971). Thus, as the tumor growth progresses, the animal becomes progressively less immunoresponsive.

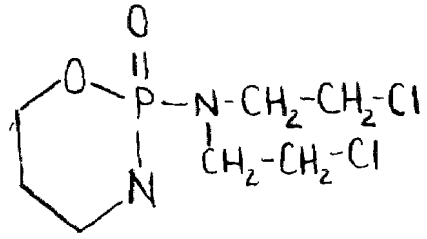
Goldin and Humphreys (1960) demonstrated that mice surviving systemic Leukemia L1210 after treatment with halogenated derivatives of amethopterin may be refractory to reinoculation of L1210 leukemic cells. Further evidence of an immune response to L1210 was demonstrated by Glynn, et al. (1963). They immunized CBBA mice against L1210 by multiple inoculations of irradiated leukemic splenic tissue from DBA/2 mice of one of the parental types. These mice were later challenged with L1210 tumor cells and exhibited no signs of the leukemia. Hence, it is possible to mount a successful immunological response to the L1210 leukemic cells; however, the response would vary with the stage of the disease and the kind, amount and success of chemotherapy and immunotherapy.

Chemotherapy

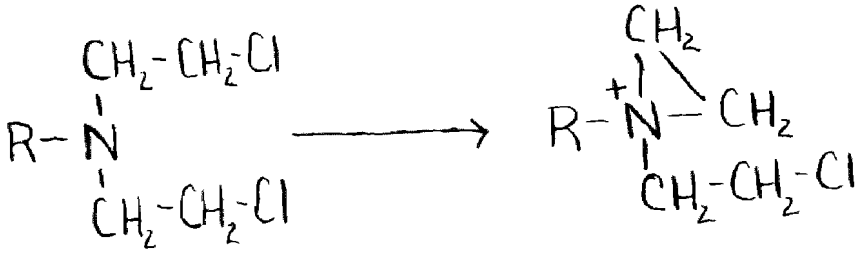
There has been much experimentation to test the effectiveness of various types of chemotherapy against lymphoid leukemias. Leukemia L1210 is now routinely used by many as the standard against which new drugs are tested.

One of the most effective of these drugs is cyclophosphamide, also known as Cytoxan (Mead Johnson Laboratories,

Evansville, Ind.).



Cytoxan is an alkylating agent and like other nitrogen mustards it reacts with many nucleophilic substances within the cell including a number of biologically active groups such as phosphate, amino, sulfhydryl, hydroxyl, imidazole and carboxyl groups (Livingston and Carter, 1970). The first attempt to explain the mechanism of action of nitrogen mustard drugs proposed the formation of an ethylenimine intermediate.



This three member ring can either hydrolyze or alkylate one of the biologically active groups previously mentioned (Furst, 1963). This internal cyclization is necessary for the biological activity of all these drugs.

In the case of Cytoxan, the drug cannot be ionized or activated until the ring is cleaved at the level of the phosphorous-nitrogen bond. Following this cleavage, internal cyclization to a three member ring probably occurs as in other nitrogen mustards. Thus, it is a metabolite of the

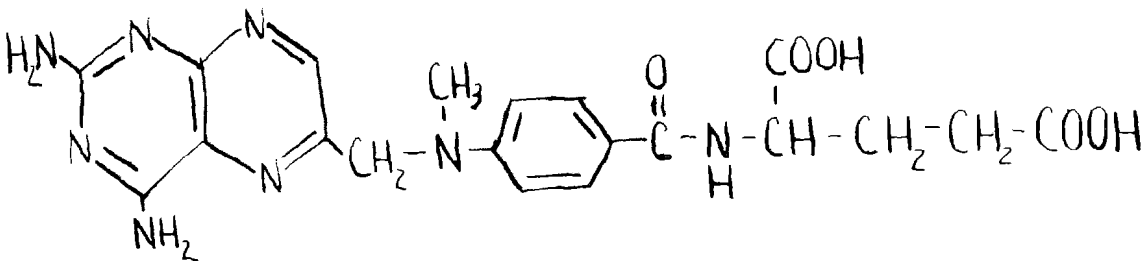
drug which gives the observed biological activity. This activation or cleavage is believed to occur in the microsomes of the liver rather than in the tumor cells themselves, even though it was originally believed that the high levels of phosphatase and phosphamidase in the tumor cells might activate Cytoxan in the tumor cells (Livingston and Carter, 1970). The active metabolite that produces the anti-tumor effect of the drug has not yet been isolated or identified (Bagley et al., 1973).

Cytoxan has been shown to have great activity against Leukemia L1210. Schabel et al. (1966) showed that a single maximum dose will reduce the number of L1210 cells in the peritoneum of BDF_1 mice by 99.9%. Lane (1959) has shown that a single treatment with cyclophosphamide produces a high percentage of tumor-free survivors if the drug is administered early in the course of the disease. However, if the treatment was deferred until later in the disease, maximum effectiveness of the drug was achieved only when the drug was given at weekly intervals (Hoffman et al., 1969).

With this evidence of the effectiveness of Cytoxan against L1210, research efforts were turned to dosage levels and treatment schedules in an attempt to determine the maximum efficiency of the drug. Venditti et al. (1959) observed that with a daily treatment schedule beginning early in the disease, none of the test animals exhibited total recovery from the leukemia. They also noted that using the daily

dose which yielded the greatest increase in survival time, Cytoxan toxicity for the host became extensive. When treatment was begun later in the disease and the drug administered either as a single dose or every two days or every four days, the effectiveness of Cytoxan against L1210 increased as the interval between treatments was increased.

Another drug which has shown a high degree of success against Leukemia L1210 is amethopterin or Methotrexate (Lederle Laboratories, Pearl River, N.Y.). Methotrexate



is a folic acid antagonist which competitively inhibits dihydrofolate reductase and this results in a restriction of the amount of tetrahydrofolate available to the cell (Livingston and Carter, 1970). Methotrexate unites with dihydrofolate reductase, having an affinity 100,000 times greater than that of the normal substrate for the enzyme (Borsa and Whitmore, 1969). Since the supply of tetrahydrofolic acid must be renewed to replace the amounts lost by oxidation, folic acid from the diet must be reduced to form tetrahydrofolic acid (Porter and Wiltshaw, 1962). Because Methotrexate competitively inhibits the action of dihydrofolate reductase, no tetrahydrofolic acid can be formed and

DNA synthesis and replication are altered. The synthesis of DNA is much more sensitive to the action of Methotrexate than RNA. This probably is the result of a selective effect of Methotrexate on thymidylate synthesis since the efficiency of the drug can be altered by the addition of pre-formed thymidine to the cell population in vitro (Borsa and Whitmore, 1969).

Aminopterin, another folic acid antagonist, was first used by Law et al. (1949) in the treatment of Leukemia L1210. They showed that the drug causes a significant increase in the survival time, a pronounced inhibition of the leukemic cell growth in the peritoneum, and an inhibition of the infiltration of the leukemic cells into the spleen, liver and axillary lymph nodes. Further work with aminopterin showed it to be severely toxic for the host, and Methotrexate, a methylated derivative of aminopterin, was found to reduce this problem. Friedkin and Goldin (1962) showed that treatment with Methotrexate increased the life span of mice inoculated with L1210 leukemia cells. Hofer et al. (1969) confirmed this finding by labeling leukemic cells with ^{125}I-iododeoxyuridine. They found that with daily treatments of Methotrexate (1 mg/kg) a similar fraction of the tumor cell population dies each day.

Venditti and Goldin (1964) showed that it is possible with the use of sequential treatment to improve the therapeutic response to advanced Leukemia L1210. This was achieved

by using a priming dose of cyclophosphamide, followed by daily or intermittent doses of Methotrexate. Cyclophosphamide when used alone exerted its maximum degree of effectiveness on a weekly treatment schedule and the combination of daily Methotrexate plus weekly cyclophosphamide provided a marked therapeutic synergism. This advantage is attributable to a decrease or attenuation of the systemic leukemic cell population from the initial treatment with cyclophosphamide followed by subsequent growth retardation by Methotrexate.

Straus et al. (1971) showed similar results when Methotrexate was used both as the priming dose and as the scheduled or intermittent dose. Here the priming dose effect was attributed to the possibility that the percentage of tumor cell kill of Methotrexate increases as the tumor cell population decreases. If this were so, the priming dose by lowering the tumor cell number to a low level, would result in subsequent intermittent doses causing a greater percentage of kill for a given dose of the drug.

Straus et al. (1972) later compared the effect of the priming dose with respect to the drug used and the dosage. When Cytoxan was used as the priming dose, the survival time was increased over that which was obtained when Methotrexate was used at a comparable dose. By using a maximal single dose of Cytoxan (120 mg/kg) at day two followed by Methotrexate (4 mg/kg) every two days thereafter, it was possible to obtain the best chemotherapeutic effect with the survival being

increased to about 27 days. This was about twice that achieved when either drug was used by itself and about four times longer than when untreated.

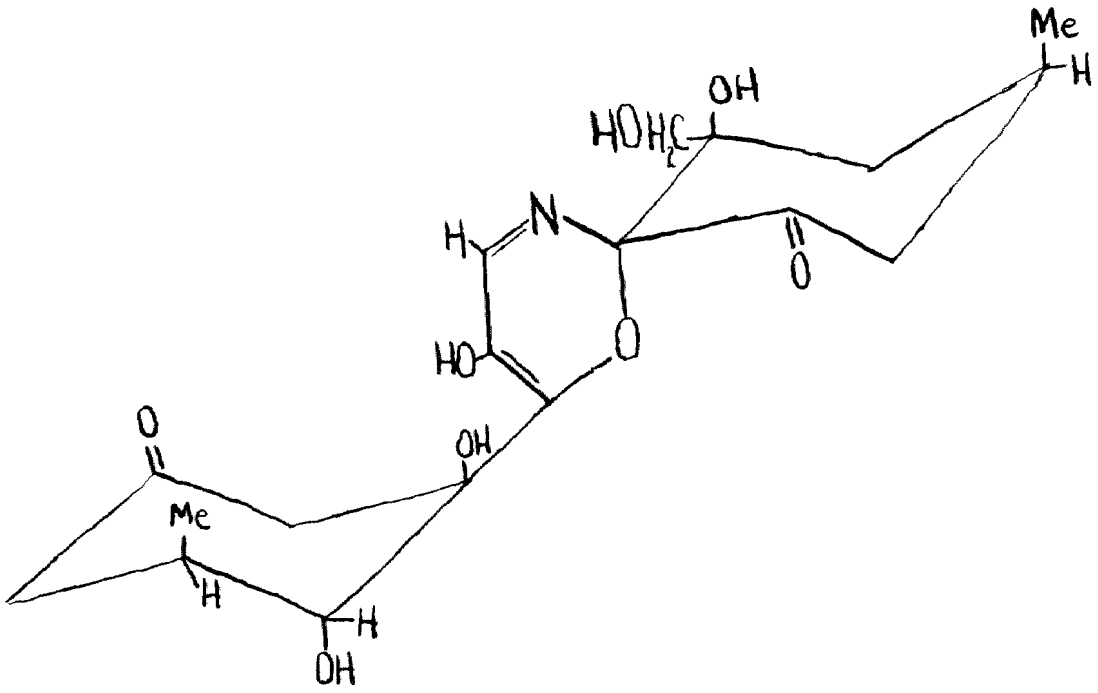
At the same time that these drugs are exerting their anti-leukemic effect, they are also causing a depression of the host's defense mechanisms. Cyclophosphamide, an alkylating agent, has several effects: (1) It prevents the differentiation of small lymphocytes into immunoblasts after antigenic stimulation. (2) It has a toxic effect on the immunoblasts that do develop. (3) It is an anti-mitotic drug. In contrast, Methotrexate appears to block the development of a population of small lymphocytes, and also slows down the division of immunoblasts (Mihich, 1967). When treatment is concluded, the drug effect stops and both residual leukemic cells and sensitized lymphocytes begin to proliferate again (Mihich, 1969). The race between these two proliferating cell lines will determine the eventual outcome for the host.

Immunotherapy

Leucogenenol, a leucocytosis inducing factor, has been isolated from the mold, Penicillium gilmanii, as well as bovine and human liver (Rice, 1966; Rice and Shaikh, 1970). It has the properties of an enol and has the molecular formula, $C_{18}H_{25}NO_8$ (Rice, 1971).

Extensive chemical analysis of leucogenenol showed a

single spot on thin layer chromatography of silica gel when the slides were developed with methanol-benzene (1:19, v/v), ethanol-benzene (1:19, v/v), dioxane-benzene (1:9, v/v). The R_F values in these systems were respectively 18, 28 and 66 (Rice and Barrow, 1967). The ultraviolet absorption of leucogenenol isolated by thin layer chromatography showed no sharp maxima. Therefore, it was necessary to determine the relationship between optical density and concentration at several wavelengths. The relationships were found to be: optical density/concentration (mg/ml) = 1.030, 1.054 and 1.285 at 264, 270 and 280 nm, respectively. Leucogenenol readily forms salts with sodium, potassium and calcium, and solutions of these salts in water retained their biological activity for over a year. An aqueous solution of leucogenenol itself, however, readily lost this activity at room temperature (Rice, 1971). When hydrolyzed it formed 1,2 dihydroxy-3-methyl-5-oxycyclohexanecarboxylic acid, 3-hydroxy-3-hydroxymethyl-5-methylcyclohexane-1,2-dione, glycoaldehyde, aminoacetaldehyde and ammonia. This evidence and spectroscopic data indicate that leucogenenol is 2-(1,2-dihydroxy-3-methyl-5-oxocyclohexyl)-3,11-dihydroxy-11-(hydroxymethyl)-9-methyl-1-oxa-5-azaspiro 5,5 undeca-2,4-dien-7-one.



Injection of leucogenenol, as little as 1 mg/kg, elicits a leucocytosis without a febrile response (Rice, 1968) and is not toxic when as much as 500 ug/g is injected into a mouse (Rice and Darden, 1968). Injection of leucogenenol, either intravenously or intraperitoneally, leads first to an increase in the concentration of neutrophils and later to an increase in the concentration of lymphocytes in the peripheral blood of rabbits and mice (Rice, Connolly, Aziz and McCurdy, 1971). This is accompanied by an increase in the number of myeloblasts in the bone marrow and lymphoblasts in the spleen (Rice, Lepick and Darden, 1968). In mice (strain unspecified) which were injected with a large dose (200 ug/kg) of leucogenenol, a decrease in neutrophils to less than one-half of the normal and a corresponding

greater than two-fold increase in lymphocytes was seen within 4 hours after injection. This change remained relatively constant for 12 days (Rice, 1968).

Studies of the biological activity of leucogenenol indicate that it stimulates DNA replication in the precursor cells and increases their rate of transformation (Rice, McCurdy and Aziz, 1971). Rice, Blum and Rene (1970) showed that the addition of leucogenenol to a tissue culture of lymphoblastoid cells increases the respiratory quotient as well as increasing the rate of replication, and concluded that leucogenenol's action in an animal is due to a direct effect of leucogenenol on the blood cells. Further evidence for this hypothesis came from studies with sublethally irradiated mice. Rice, Lepick and Hepner (1970) showed that these mice when injected with leucogenenol and SRBC exhibited a shorter latent period and a greater hemolysin titer than non-leucogenenol treated animals. These results indicate that leucogenenol increases the rate of transformation of precursor cells into cells capable of antibody synthesis. Rice and Ciavarra (1971) extended this idea by treating splenectomized rats with leucogenenol. Within 3 days after splenectomy, treated rats became capable of producing normal titers of hemolysin in response to injections of SRBC whereas untreated mice still showed reduced levels.

Rice, Ciavarra and Borsos (1972) showed that injections of leucogenenol into splenectomized rats along with SRBC

results in the formation of normal titers of 19S hemolysin. This is the result of a marked increase in the concentration of antibody forming cells in the lymphatic tissue and the liver (Rice, Das and Koo, 1972). Although normal 19S titers were formed, no increase in the peak titer was found. This is in contrast to the findings of Jacoby (1972) in which animals that were injected with several doses of leucogenenol and SRBC showed increased hemolysin titers over that of non-leucogenenol treated controls. From this evidence, it is concluded that leucogenenol acts by increasing the rate of transformation of immunocompetent cells, possibly a type of lymphocyte, into antigen-reactive lymphocytes (Rice, Ciavarra and Borsos, 1972).

Summary

It is the purpose of this study to determine if leucogenenol used in conjunction with conventional chemotherapy can help maintain the immunocompetence of the host throughout the course of Leukemia L1210 disease. The number of leukemic cells will be kept at a low level through sequential chemotherapy, using Cytoxan and Methotrexate. When chemotherapy is stopped, it has been stated that there will be a race between the residual leukemic cells and the remaining sensitized lymphocytes. By treating the animals through the course of the disease with leucogenenol, it is postulated that the number of sensitized lymphocytes will be increased

such that this race will be turned in the host's favor.

METHODS AND MATERIALS

Leukemia L1210

The Leukemia L1210 cell line, LE-29, used in these experiments was received from Mr. N. H. Greenberg of the National Cancer Institute. It was at generation B-10 when received. The leukemic cells were carried in DBA/2 mice by intraperitoneal injection of $1x10^5$ viable cells. Ascites tumor cells were counted in a hemocytometer and their viability determined by the Trypan Blue exclusion method. The cells were then diluted in Hank's balanced salt solution to $1x10^6$ viable cells per milliliter. The suspension was kept in an ice bath until ready for use and each test animal then received 0.1 ml ($1x10^5$) viable leukemic cells.

Mice

Mice used in this experiment were $C_3D_2F_1/J$ males, approximately 25 grams in weight. They were obtained from The Jackson Laboratories, Bar Harbor, Maine. The DBA/2J male mice in which the tumor line was carried were also obtained from this laboratory. All animals were kept in groups of 5 and 6 in plastic disposable cages with sterilized sawdust. Water and standard laboratory chow were given ad libitum.

Drugs

Cytoxan (cyclophosphamide; Mead Johnson Laboratories, Evansville, Ind.) was dissolved in sterile non-pyrogenic 0.85% NaCl and injected intraperitoneally at a dose of 120 mg per kilogram of body weight. Methotrexate (Nutritional Biochemical Corporation, Cleveland, Ohio) was freshly prepared at each injection time. It was dissolved in sterile non-pyrogenic 2% sodium bicarbonate and injected intraperitoneally at a dose of 4 mg per kilogram of body weight.

Antigenic Stimulation

The status of the humoral immune response was monitored by the injection of a 10% suspension of sterile SRBC. These cells were washed three times in sterile saline and then diluted to a 10% suspension with sterile saline. Each animal received 0.1 ml doses at days 0, 7 and 14. Serum for antibody titers was collected for each animal when sacrificed. The serum was separated from the whole blood after the cellular elements had clotted and were centrifuged out. The serum was decanted from the tube and frozen immediately. Both hemolysin and hemagglutination titers were done by standard microtiter techniques. For hemagglutination titers, .025 ml of serum, .050 ml of veronal buffered saline and .025 ml of 2% SRBC were used. For hemolysin titers, .025 ml of serum, .025 ml of veronal buffered saline, .025 ml of a 1:15 dilution of guinea pig complement and .025 ml of 2% SRBC were used. All titers

were read as the last dilution showing a positive result and reported as the Log_2.

Leucogenenol

Leucogenenol was isolated using the procedure of Rice (1966) with several minor modifications. Approximately 20 grams of lyophilized bovine liver were extracted with 80 or 90 ml of methanol in a Soxhlet apparatus. Equal volumes of liver from three different lots were used and were extracted for 24 hours. These extracts were pooled until approximately 1000 grams of liver powder had been extracted. The pool was then evaporated at 40-50°C to a thick syrup in a flash evaporator under 20-22 mm Hg pressure. Subsequent steps were similar to that of Rice (1966). Leucogenenol was dissolved in sterile non-pyrogenic water and used at a concentration of 8 ug per animal in 0.2 ml.

Experimental Procedure

Cytoxan was given two days following L1210 leukemic cell inoculation and Methotrexate was given every two days beginning on the fourth day after infection until day sixteen when the last injection was given. Leucogenenol was given two days prior to infection as well as day 3, 7, 14 and 21 after infection.

A total of 114 animals were used and they were divided into the following groups:

Group I: Normals - 3 sub-groups of 6 animals.

Group II : Leukemia L1210 controls - 2 sub-groups of 6 animals.

Group III: Leukemia L1210 + Cytoxan - 3 sub-groups of 5 animals.

Group IV : Leukemia L1210 + Methotrexate - 3 sub-groups of 5 animals.

Group V : Leukemia L1210 + Leucogenenol - 3 sub-groups of 6 animals.

Group VI : Leukemia L1210 + Cytoxan + Methotrexate 3 sub-groups of 6 animals.

Group VII: Leukemia L1210 + Cytoxan + Methotrexate + Leucogenenol - 3 sub-groups of 6 animals.

The first sub-group of each group was sacrificed on day 7; the second sub-group was sacrificed on day 14 except for Groups II and V in which no animals were still living; the third sub-group was kept for survival data.

Blood samples at day 7 and day 14 were obtained by decapitation and blood collected for differential smears, hematocrits, total nucleated cell counts and antibody studies. The nucleated cell counts were made with the Coulter Counter, Model B, in which 20 λ of blood was diluted in 10 ml of diluting fluid (Coulter Counter, Inc.; Hialeah, Florida).

Differentials were stained with Wright's stain and 100 cells were counted and classified. Multiple counts were made on questionable differentials and the average of these counts used.

Hematocrits were made using pre-calibrated Micro-Hematocrit Tubes (Clay Adams No. 1025; Parsippany, N.J.). The tubes were then sealed and centrifuged for five minutes in an Adams' Micro-Hematocrit Centrifuge. The values were then read directly from the pre-calibrated scale.

Upon termination, all animals were weighed and then the spleens and livers removed and weighed. Ratios of the spleen and liver weight to the total body weight were then determined. The relative enlargement of the inguinal and axillary lymph nodes was also observed at this time.

Statistical analyses were carried out by using a t-test for simple correlation (Chou, 1969). The p values were then determined from a standard t distribution table. The criteria for significance was $p < .1$.

RESULTS

In reporting the differential counts for the various animal groups in these experiments, the term lymphoid cell is used instead of lymphocyte to avoid confusion and controversy. Thus, small, large and immature lymphocytes were all classified in this one category. Although no significant change occurred in the number of monocytes and eosinophils, their results are shown in Table 1.

Normal CDF_1 control animals showed similar differential counts at each bleeding during the experiment with

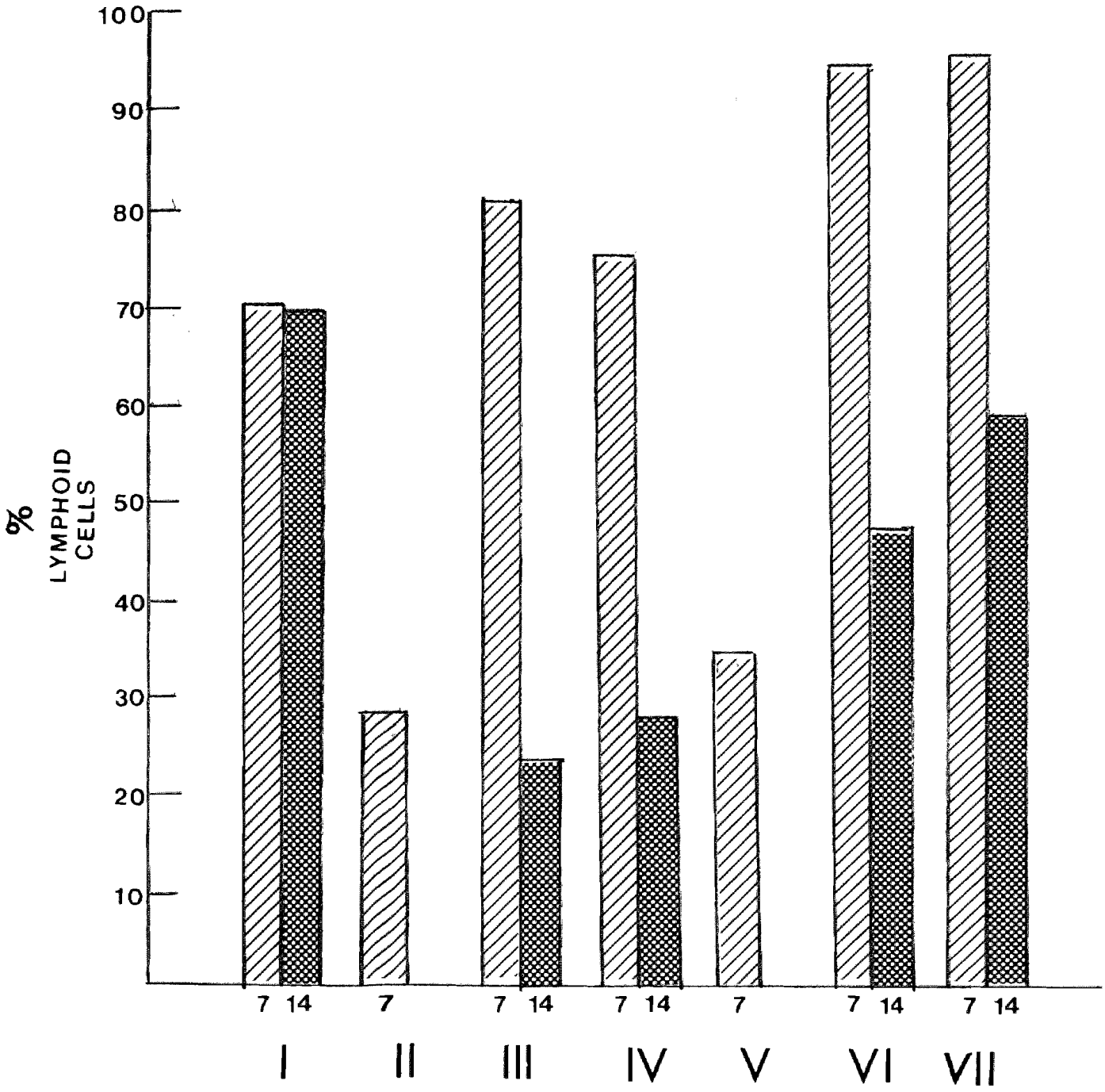
Table 1. Average Differential Counts.

Animal Group	Day	% Lymphoid	% PMN	% Monocytes	% Eosinophils
Group I Normal Control	7	70.0	26.6	1.2	2.2
	14	69.3	26.5	2.0	2.2
Group II L1210 Control	7	28.2	69.6	1.8	0.4
	14	----*	----	---	---
Group III L1210 + Cytoxan	7	81.4	18.0	0.6	0.0
	14	23.0	75.3	1.7	0.0
Group IV L1210 + MTX	7	75.8	21.6	1.8	0.8
	14	28.3	70.2	0.5	1.0
Group V L1210 + Leucogenenol	7	34.2	65.0	0.5	0.3
	14	----*	----	---	---
Group VI L1210 + Cytoxan + MTX	7	95.7	4.3	0.0	0.0
	14	48.5	50.1	0.7	0.7
Group VII L1210 + Cytoxan + MTX + Leucogenenol	7	96.3	3.7	0.0	0.0
	14	59.0	39.6	0.7	0.7

*No animals were living in these groups at day 14.

Figure 1. Average percent lymphoid cells from peripheral differential smears. No animals were living at day 14 in Groups II and V.

Group I : Normal Controls
Group II : L1210 Controls
Group III: L1210 + Cytoxan
Group IV : L1210 + Methotrexate
Group V : L1210 + Leucogenenol
Group VI : L1210 + Cytoxan + Methotrexate
Group VII: L1210 + Cytoxan + Methotrexate + Leucogenenol


100
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ANIMAL GROUPS AND DAYS

lymphoid cell counts in the range of 65%-77% with an average of 70.0% and polymorphonuclear cells (PMN's) in the range of 20%-32% with an average of 26.6%. These counts were greatly changed in the L1210 controls. Mice in this group had lymphoid cell counts that ranged from 24%-31% and PMN counts in the range of 66%-74%. When L1210 infected animals were treated with either drug, this reduction in the number of lymphoid cells was delayed. L1210 animals that were treated with Cytoxan exhibited 81.4% lymphoid cells at day 7 compared to 69.6% for L1210 controls ($p < .01$), while animals treated with only Methotrexate showed 75.8% lymphoid cells ($p < .1$). However, at the fourteenth day after infection, the counts for both groups were reversed. L1210 infected animals treated with Cytoxan showed 23.0% lymphoid cells and those treated with Methotrexate showed 28.3% lymphoid cells. These values approached the levels that the untreated controls showed at day 7. When these drugs were combined in the treatment of infected animals, there was a marked increase in the percentage of lymphoid cells at day 7 as compared to groups treated with either drug alone ($p < .001$). The percentage of lymphoid cells dropped from 95.7% at day 7 to 48.5% at day 14 and this value was again significantly higher than either group which received only one drug ($p < .001$).

When L1210 animals were treated with leucogenenol, the percentage of lymphoid cells was higher than that of the L1210 controls ($p < .05$), showing 34.2% lymphoid cells at

day 7. If leucogenenol treatment was given in conjunction with combined chemotherapy, there was no significant change (p=NS) in the differential counts at day 7 over that of animals receiving only drug therapy. At day 14, however, the leucogenenol treated animals showed 59.0% lymphoid cells while non-leucogenenol treated animals showed 48.5% lymphoid cells ($p < .1$).

The hematocrit values of all animal groups are shown in Table 2 and Figure 2. Uninfected CDF_1 control animals showed little difference in their hematocrits throughout the experiment, showing values of 49.0 and 49.2 at days 7 and 14 respectively, while L1210 infected animals showed a lower hematocrit of 30.0 ($p < .001$) at day 7. Drug treated animals showed similar hematocrits to that of L1210 controls at day 7, but at day 14, Cytoxan treated animals showed a lowered hematocrit of 28.2. When chemotherapy was combined, the hematocrit showed little change at day 7 but was reduced to 16.7 at day 14; this was the lowest hematocrit value recorded for any group. L1210 infected animals that were injected with leucogenenol showed a hematocrit of 27.5 at day 7 which was significantly lower than L1210 controls at the same time ($p < .05$), and when leucogenenol was given with combined chemotherapy, the hematocrit value was higher than in animals receiving only combined chemotherapy at day 7. However, this was not a significant difference. At day 14, the animals receiving leucogenenol with chemotherapy showed a hematocrit

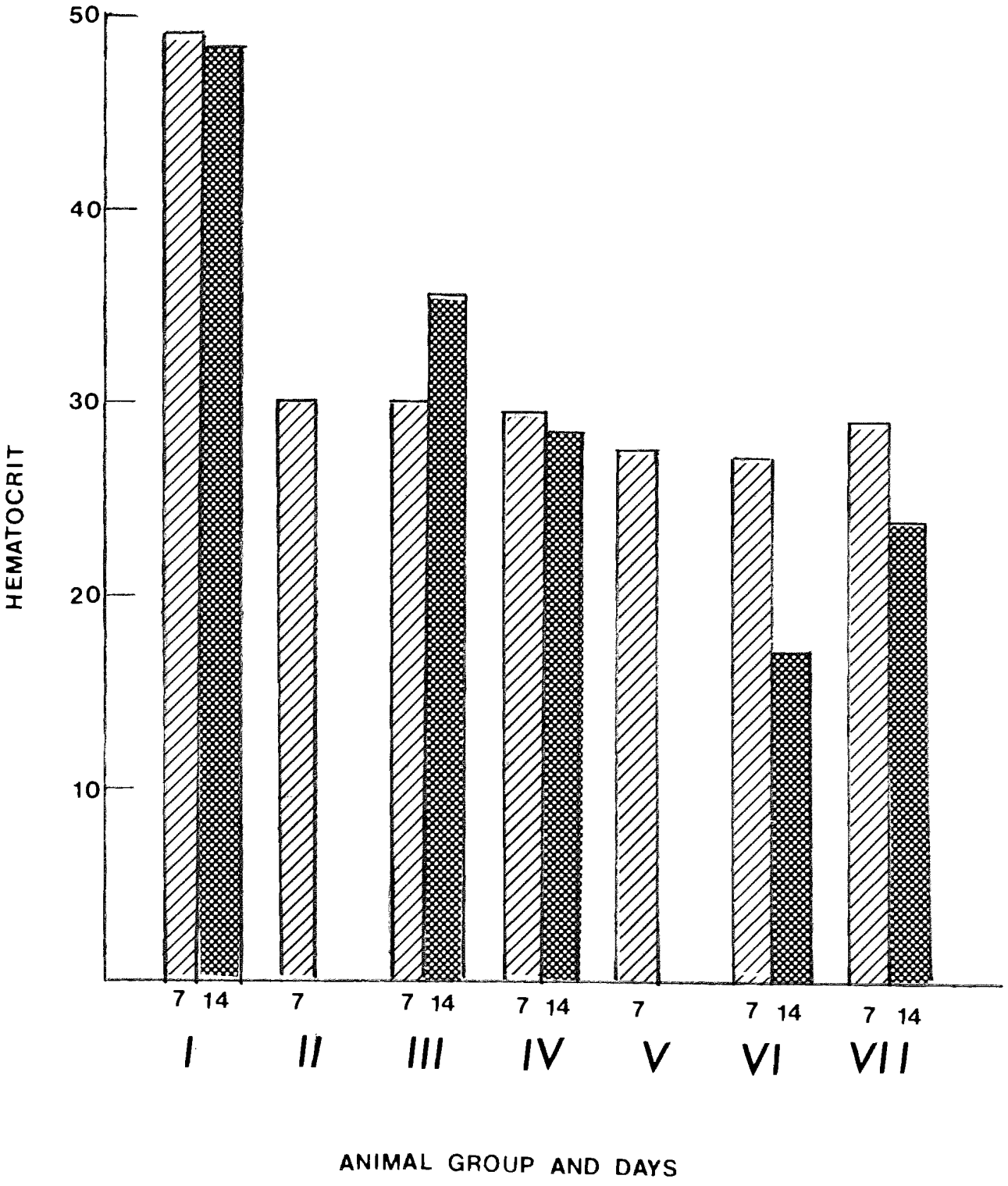
Table 2. Average Hematocrit Values.

Animal Group	Day	Hematocrit (Average)
Group I Normal Control	7	49.0
	14	49.2
Group II L1210 Control	7	30.0
	14	----*
Group III L1210 + Cytoxan	7	30.0
	14	35.7
Group IV L1210 + MTX	7	29.4
	14	28.2
Group V L1210 + Leucogenenol	7	27.5
	14	----*
Group VI L1210 + Cytoxan + MTX	7	27.1
	14	16.7
Group VII L1210 + Cytoxan + MTX + Leucogenenol	7	28.8
	14	23.5

*No animals were living in these groups at day 14.

Figure 2. Average hematocrit values. No animals were living at day 14 in Groups II and V.

Group I : Normal Controls
Group II : L1210 Controls
Group III: L1210 + Cytoxan
Group IV : L1210 + Methotrexate
Group V : L1210 + Leucogenenol
Group VI : L1210 + Cytoxan + Methotrexate
Group VII: L1210 + Cytoxan + Methotrexate + Leucogenenol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VII
ANIMAL GROUP AND DAYS

of 23.5 versus 16.7 for groups receiving only combined chemotherapy ($p < .05$).

The average nucleated cell counts for all animal groups can be seen in Table 3 and Figure 3. While uninfected control animals showed similar counts at days 7 and 14 of 6.06 and 6.16 x 10^3 cells/cu mm respectively, the L1210 controls showed an elevated count of 15.54 x 10^3 cells/cu mm at day 7. Drug treated groups usually showed decreased nucleated cell counts and the group given combined chemotherapy had the lowest value of 1.93 x 10^3 cells/cu mm at day 7. The group that received only Methotrexate was an exception and instead of having a leukopenia had relatively normal counts of 6.71 x 10^3 cells/cu mm at day 7 and 5.51 x 10^3 cells/cu mm at day 14. In contrast, the remaining groups showed elevated counts at day 14. Those animals that received only Cytoxan showed 91.18 x 10^3 cells/cu mm while those receiving both drugs in combination showed 6.8 x 10^3 cells/cu mm.

When L1210 infected animals were injected with leucogenenol, the total counts were significantly higher than that of the L1210 controls ($p > .1$) at day 7. When leucogenenol was administered along with chemotherapy, the nucleated cell count reached its lowest level at day 7 of 1.11 x 10^3 cells/cu mm. This was significantly lower than for animals receiving only combined chemotherapy ($p < .001$). However, by day 14, the count for leucogenenol and drug treated animals had increased to 20.09 x 10^3 cells/cu mm, while those animals

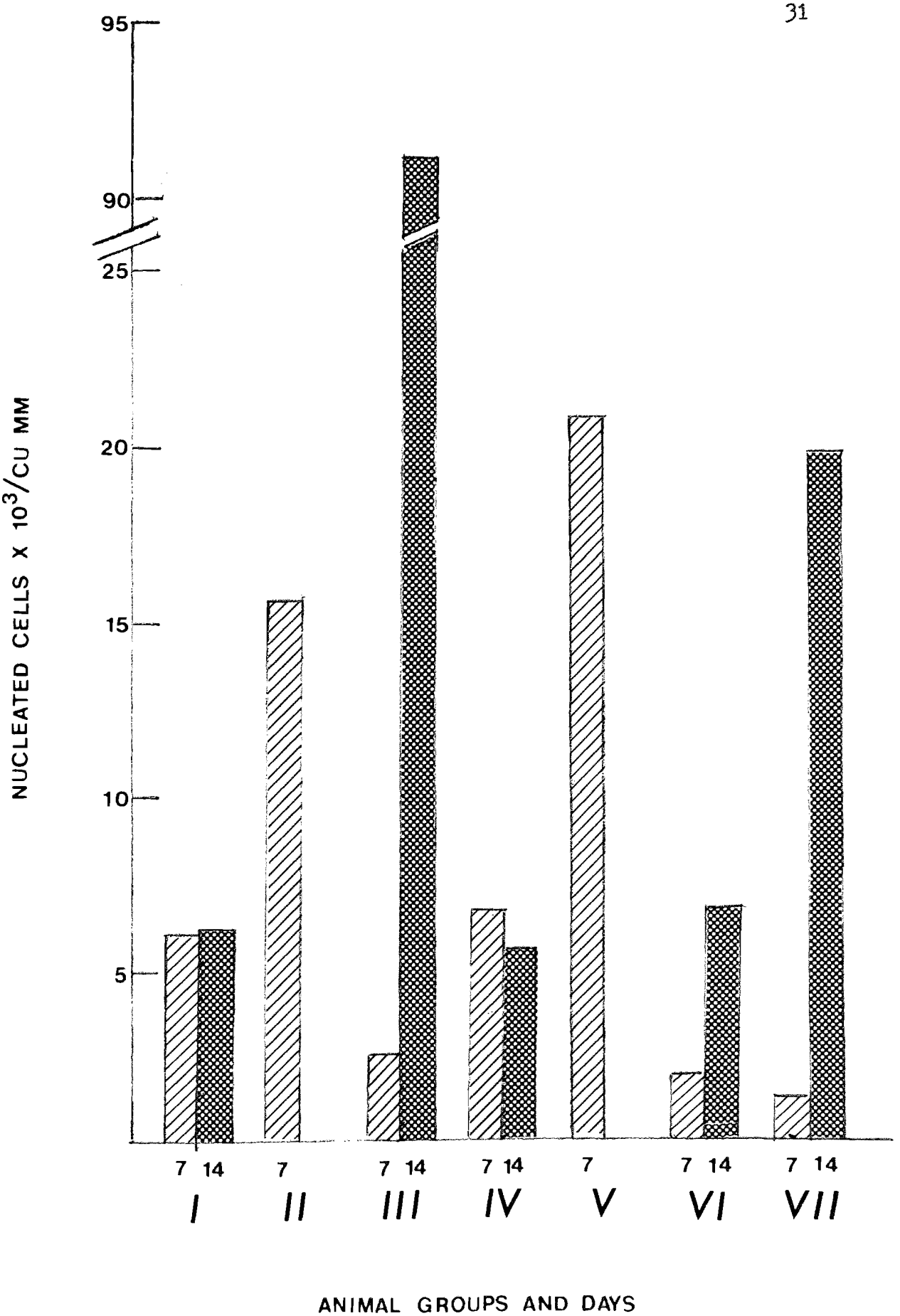
Table 3. Average Peripheral Nucleated Cell Counts X 10^3 Cells/cu mm.

Animal Group	Day 7	Day 14
Group I Normal Controls	6.06	6.16
Group II L1210 Controls	15.54	----*
Group III L1210 + Cytoxan	2.57	91.18
Group IV L1210 + MTX	6.71	5.51
Group V L1210 + Leucogenenol	20.86	----*
Group VI L1210 + Cytoxan + MTX	1.93	6.80
Group VII L1210 + Cytoxan + MTX + Leucogenenol	1.11	20.09

*No animals were living in these groups at day 14.

Figure 3. Average peripheral total nucleated cell count x 10^3 cells/cu mm. No animals were living at day 14 in Groups II and V.

Group I : Normal Controls
Group II : L1210 Controls
Group III: L1210 + Cytoxan
Group IV : L1210 + Methotrexate
Group V : L1210 + Leucogenenol
Group VI : L1210 + Cytoxan + Methotrexate
Group VII: L1210 + Cytoxan + Methotrexate + Leucogenenol


95
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NUCLEATED CELLS X 10^3/CU MM
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ANIMAL GROUPS AND DAYS

receiving combined chemotherapy showed only 6.80×10^3 cells/cu mm ($p < .001$).

The average survival times in days can be seen in Table 4 and Figure 4. L1210 controls showed an average survival time of 8.3 days. This was not significantly different from an average of 8.0 days for L1210 animals treated with leucogenenol. All remaining groups showed increased survival times. Those that received Cytoxan lived an average of 13.0 days while those that received Methotrexate survived an average of 15.0 days. If these drugs were combined, the survival time was increased to 26.0 days compared to 23.8 days when leucogenenol was given in conjunction with combined chemotherapy ($p > .1$).

The ratio of spleen and liver weight to total body weight can be seen in Table 5 and Figure 5. These ratios were calculated after the animals died or in the case of the normals when sacrificed. L1210 controls showed a ratio greater than that of the uninfected controls, .077 compared to .060 (p=NS). When infected animals were treated with leucogenenol, this ratio was increased to .089 (p=NS). Following the administration of combined chemotherapy, the ratio was further increased. Animals that received combined chemotherapy showed the highest ratio, .161, while those that received leucogenenol along with combined chemotherapy showed a slightly decreased ratio. It should be noted that the ratio for treated groups increased as the survival time

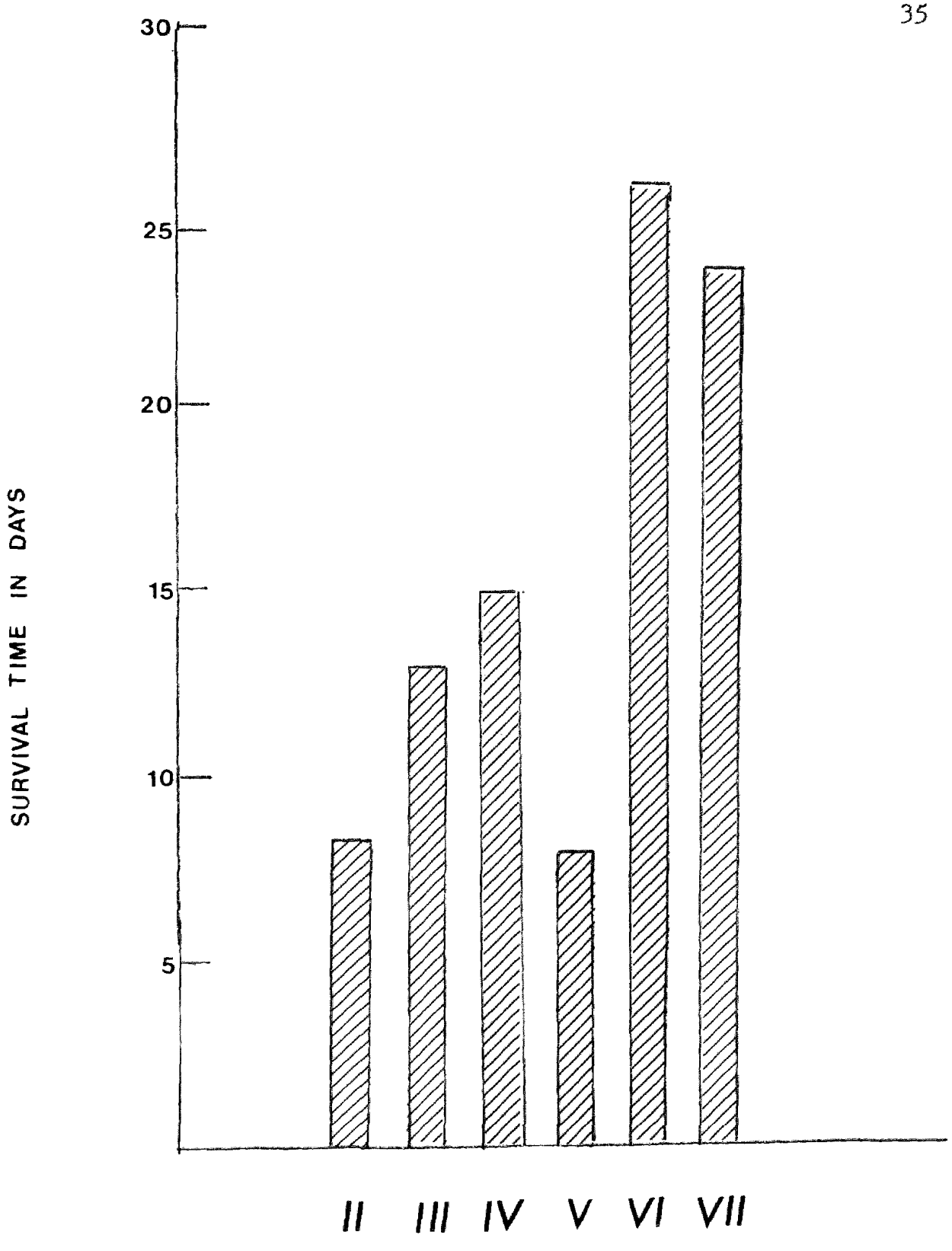
Table 4. Average Survival Times in Days.

Animal Group	Survival Time (Average in Days)	Range
Group I Normal Control	----*	-----*
Group II L1210 Controls	8.3	8- 9
Group III L1210 + Cytoxan	13.0	12-14
Group IV L1210 + MTX	15.0	14-16
Group V L1210 + Leucogenenol	8.0	7- 9
Group VI L1210 + Cytoxan + MTX	26.0	22-33
Group VII L1210 + Cytoxan + MTX + Leucogenenol	23.8	20-26

*No deaths occurred in the normal controls.

Figure 4. Average survival times in days. Normals not included.

Group II : L1210 Controls
Group III: L1210 + Cytoxan
Group IV : L1210 + Methotrexate
Group V : L1210 + Leucogenenol
Group VI : L1210 + Cytoxan + Methotrexate
Group VII: L1210 + Cytoxan + Methotrexate + Leucogenenol


30
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5
SURVIVAL TIME IN DAYS
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VI
VII
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Table 5. Average ratio of the spleen and liver weight to the total body weight.

Animal Group	Liver + Spleen Wt./Total Body Wt. (Average)	Range
Group I Normal Control	.060	.056-.061
Group II L1210 Control	.077	.064-.091
Group III L1210 + Cytoxan	.116 (3)*	.106-.128
Group IV L1210 + MTX	.117 (4)	.100-.136
Group V L1210 + Leucogenenol	.089	.080-.110
Group VI L1210 + Cytoxan + MTX	.161	.149-.170
Group VII L1210 + Cytoxan + MTX + Leucogenenol	.153	.137-.184

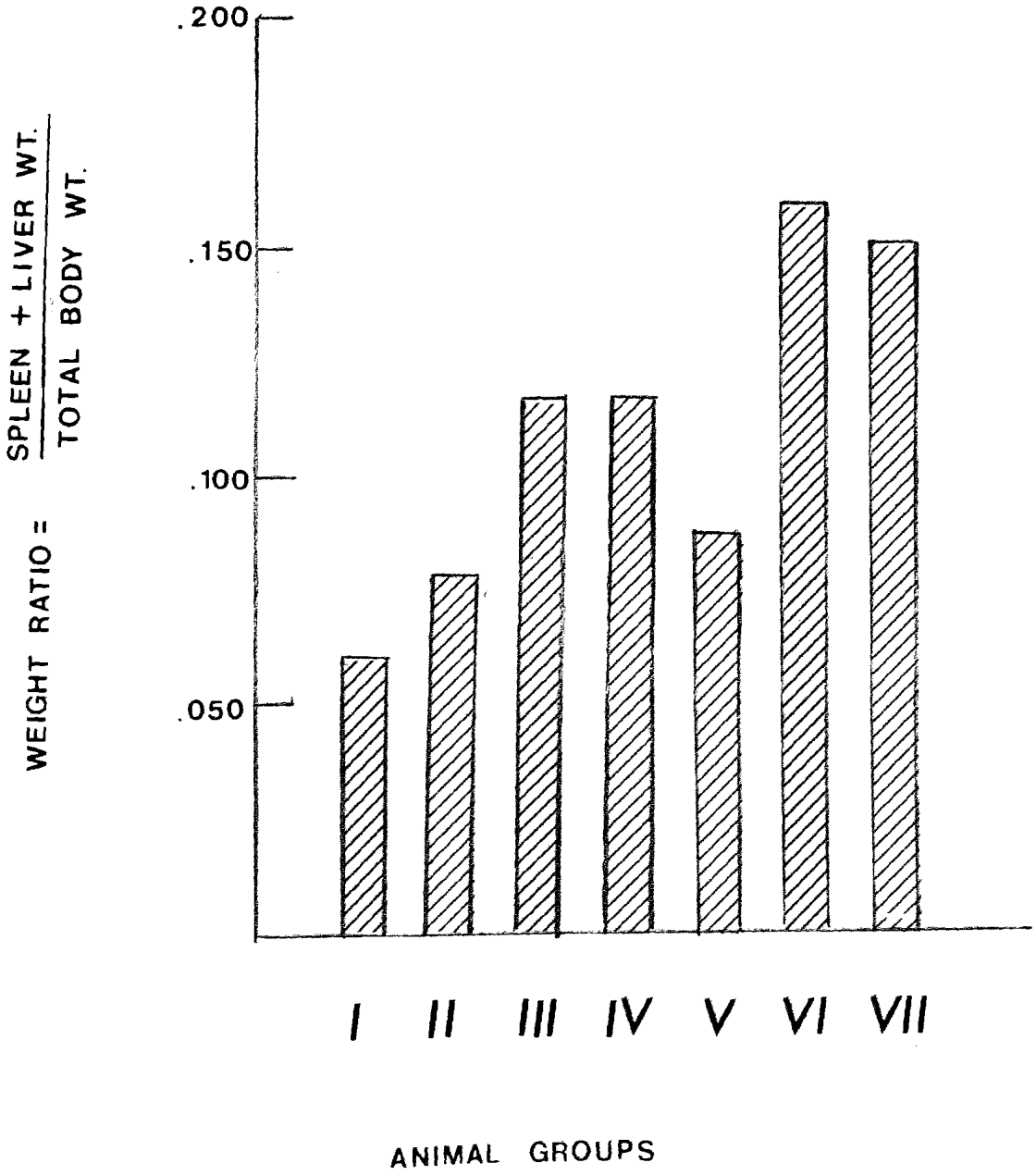
*Six animals were used per group unless specified, in which case, the number in parenthesis represents the number of animals used to calculate the average.

Figure 5. Average weight ratio of the liver and spleen compared to the total body weight.

Group I : Normal Controls
Group II : L1210 Controls
Group III: L1210 + Cytoxan
Group IV : L1210 + Methotrexate
Group V : L1210 + Leucogenenol
Group VI : L1210 + Cytoxan + Methotrexate
Group VII: L1210 + Cytoxan + Methotrexate + Leucogenenol

liver

exate
exate



increased.

Antibody studies are shown in Table 6 and Figures 6 and 7. L1210 controls showed no significant difference in either hemagglutination (HA) or hemolysin titers from that of normal controls at day 7 (p=NS). Infected animals which were injected with leucogenenol showed slightly decreased titers compared to L1210 controls but this was not a significant difference. All animals that received drug treatment showed significantly reduced titers. Those animals receiving Cytoxan showed HA titers of 1.00 and 2.67 at days 7 and 14 respectively. These animals exhibited the same values for hemolysin titers at days 7 and 14. Animals receiving Methotrexate showed reduced HA titers of 4.60 and 2.50 and hemolysin titers of 4.60 and 3.50 at days 7 and 14 respectively. In animals with combined therapy, HA titers of 1.67 and 1.83 and hemolysin titers of 1.17 and 2.17 were found at days 7 and 14 respectively. If leucogenenol was administered along with combined chemotherapy, there was no significant difference in either HA or hemolysin titers as compared to animals receiving only combined chemotherapy. However, by day 14, both titers were increased over that of groups receiving only chemotherapy. They showed an average HA titer of 3.17 and an average hemolysin titer of 3.00 compared to 1.83 and 2.17 respectively for animals receiving combined chemotherapy ($p < .05$ for HA and p=NS for hemolysin).

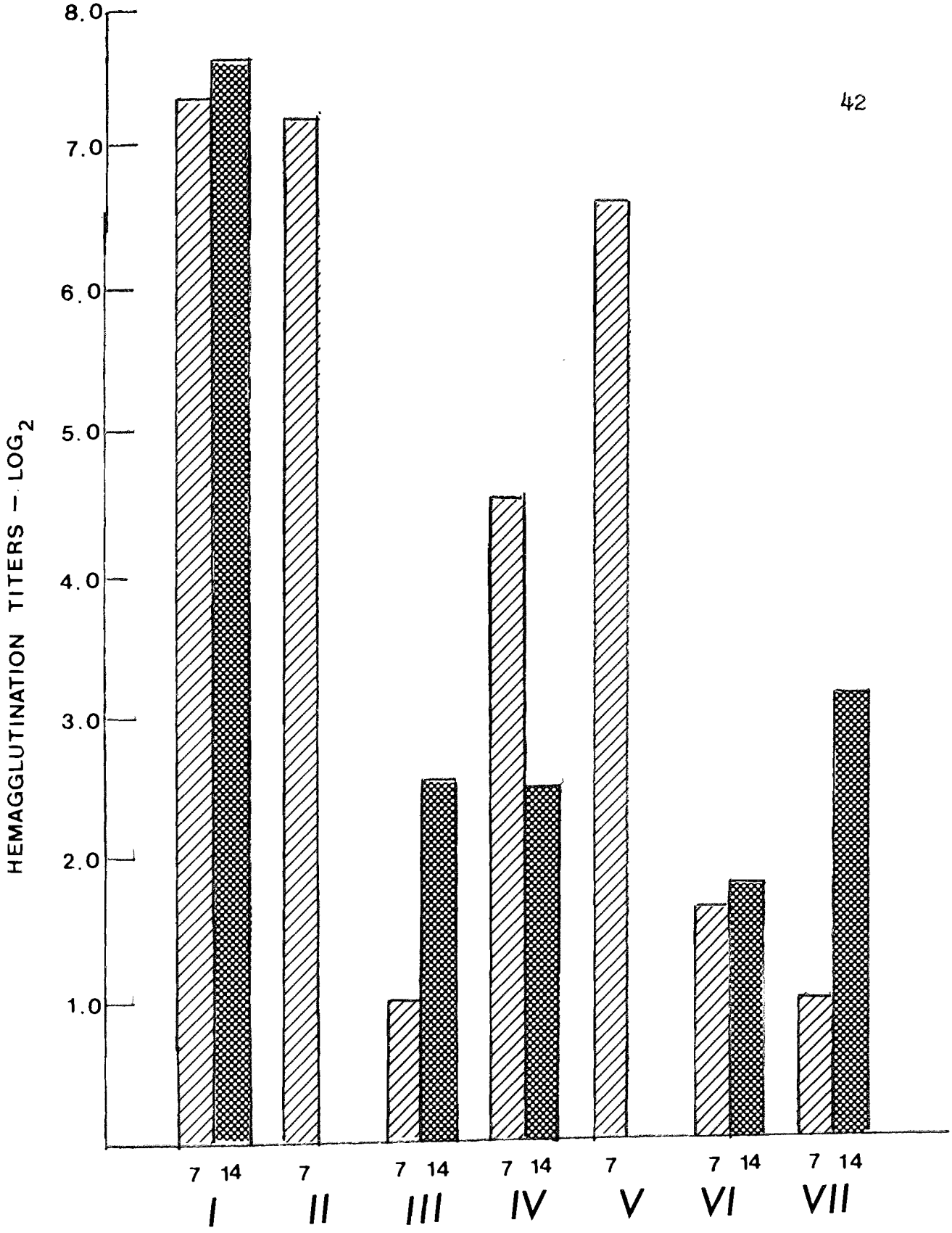
Table 6. Average hemagglutination and complement-fixation titers to SRBC at day 7 and day 14 (expressed as Log_2.

Animal Group	Day	Hemagglutination Titers (Average)	Complement-fixation Titers (Average)
Group I Normal Controls	7	7.33	6.67
	14	7.67	7.50
Group II L1210 Controls	7	7.20	6.80
	14	----*	----
Group III L1210 + Cytoxan	7	1.00	1.00
	14	2.67 (3)**	2.67 (3)**
Group IV L1210 + MTX	7	4.60	4.60
	14	2.50 (4)	3.50 (4)
Group V L1210 + Leucogenenol	7	6.67	6.50
	14	----*	----*
Group VI L1210 + Cytoxan + MTX	7	1.67	1.17
	14	1.83	2.17
Group VII L1210 + Cytoxan + MTX + Leucogenenol	7	1.00	1.00
	14	3.17	3.00

*No animals were living in these groups at day 14.
**Six animals were used per group unless specified, in which case, the number in parenthesis represents the number of animals used to calculate the average.

Figure 6. Average SRBC hemagglutination titers expressed as Log_2. No animals were living at day 14 in Groups II and V.

Group I : Normal Controls
Group II : L1210 Controls
Group III: L1210 + Cytoxan
Group IV : L1210 + Methotrexate
Group V : L1210 + Leucogenenol
Group VI : L1210 + Cytoxan + Methotrexate
Group VII: L1210 + Cytoxan + Methotrexate + Leucogenenol


8.0
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HEMAGGLUTINATION TITERS – LOG_2
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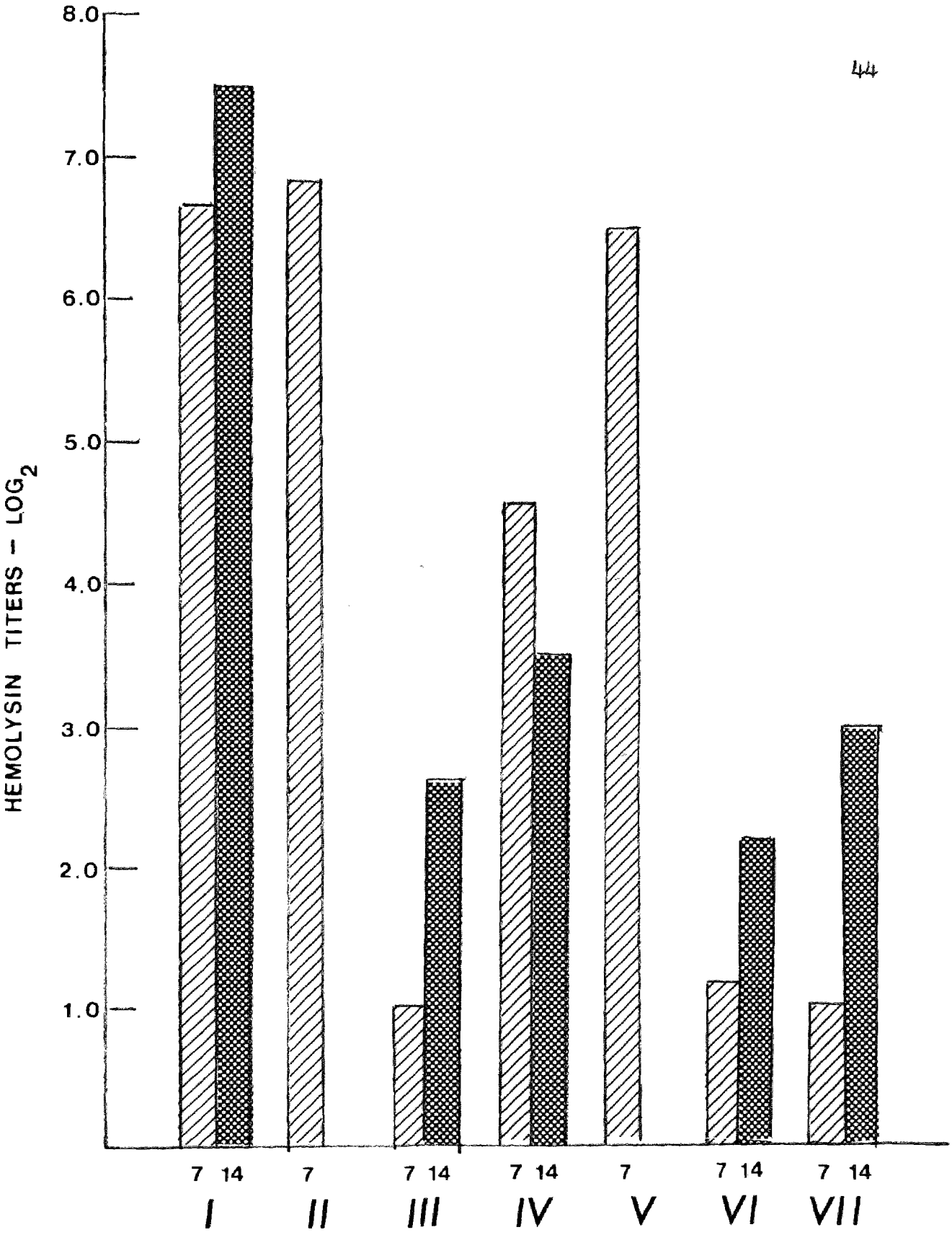
Figure 7. Average SRBC hemolysin titers expressed as Log_2. No animals were living at day 14 in Groups II and V.

Group I : Normal Controls
Group II : L1210 Controls
Group III: L1210 + Cytoxan
Group IV : L1210 + Methotrexate
Group V : L1210 + Leucogenenol
Group VI : L1210 + Cytoxan + Methotrexate
Group VII: L1210 + Cytoxan + Methotrexate + Leucogenenol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HEMOLYSIN TITERS - LOG2
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ANIMAL GROUPS AND DAYS

DISCUSSION

The isolation and the biological activity of the lot of leucogenenol used in this experiment was previously reported by Jacoby (1972). He indicated that leucogenenol might be more effective in the treatment of leukemias if it were given after an initial course of anti-tumor drugs and in a system in which a virus is not known to be actively proliferating within the precursor cell stimulated by leucogenenol. The findings of this study do not support this hypothesis. Although there was a significant beneficial change in some of the parameters of the disease when leucogenenol was administered after chemotherapy, a minimal detrimental effect was observed in the survival times of the animals.

Leucogenenol treated animals showed increased total nucleated cell counts, hematocrits and percent lymphoid cells in the peripheral blood compared to non-leucogenenol treated animals by day 14. The total nucleated cell count was the only parameter of the disease which indicated an enhancement of the leukemia, while both the increased hematocrit and the increased percentage of lymphoid cells would seem to be beneficial to the host. However, even though leucogenenol was successful in bringing about beneficial changes in parameters of the disease, it showed little effect on the survival times of the animal groups involved. Little can be said of the

spleen and liver weights since the only correlation that can be drawn is that the longer the animal survives after being infected with Leukemia L1210, the greater will be the spleen and liver to total body weight ratios. Thus, leucogenenol, while influencing in a beneficial fashion some parameters of the disease, had only a minimal enhancing effect on the survival times of the mice.

Rice, Das and Koo (1972) have reported that splenectomized rats injected with leucogenenol showed normal antibody titers, but no increase in the peak titer. The results of this experiment confirm their finding. L1210 infected animals did not show a significant suppression of the immune response and when treated with leucogenenol showed little change in either HA or hemolysin titers. These titers in either case never went above the level of normal animals. This seems to suggest that there is a cellular control or feedback mechanism which controls the amount of antibody produced in response to an antigenic stimulation. The lack of immunosuppression in L1210 infected animals can best be explained by the time of antigenic stimulation in relation to the state of the disease. As previously stated, the humoral immunological response varies over the course of the disease. Bonmasser, et al. (1971) showed that with immunizations early in the course of the disease, there is no decrease in the number of anti-SRBC plaque forming cells per spleen. Thus, by early antigenic stimulation the cells have already been

committed and are not affected by the leukemia throughout the course of the disease. The administration of leucogenenol did not raise either the HA or hemolysin titer past its peak value. The near normal humoral antibody levels obtained in these experiments was obviously not effective in controlling the number of proliferating L1210 leukemic cells. Fass and Fefer (1972) have suggested that the presence of immune lymphocytes as the sole basis for anti-tumor therapy is ineffective after the tumor becomes detectable. This is believed to be due to the unfavorable ratio of immune cells to tumor cells. Hence, the effect of leucogenenol was not great enough to change this unfavorable ratio, and the number of tumor cells was too great for the host to immunologically destroy them.

The depression of the immune response during combined chemotherapy as previously stated (Mihich, 1967) was confirmed by the greatly reduced HA and hemolysin titers found in these experiments. Although by day 14, the humoral titers were increased by leucogenenol, they were never close to normal levels. These reduced levels of humoral antibody, even after the number of leukemic cells had been reduced, were not able to affect the disease to any great extent. This can best be explained in light of Mihich's (1969) theory which has previously been mentioned. When treatment was concluded, the race between the residual leukemic cells and the sensitized lymphocytes begins. In this case, leucogenenol

was not able to raise the antibody levels to a high enough level to overcome the residual leukemic cells and therefore the rapidly growing residual leukemic cells proliferated faster than the remaining immune lymphocytes following discontinuance of chemotherapy.

Since L1210 cells have an associated mammary tumor virus (MTV) or related antigen (ML), it would be expected that leucogenenol through its action would stimulate an enhanced specific immune response to these new antigens. This immunological response of the host would lead to the death of the leukemic cell population. The results of this experiment, however, indicate that the immune response even though stimulated by leucogenenol was still not strong enough to overcome the leukemic cell population.

While humoral antibody levels were increased by treatment with leucogenenol, recent evidence has shown that this may not necessarily be beneficial to the host. Hellstrom and Hellstrom (1970) showed that humoral antibodies may cause a phenomenon known as immunological enhancement of a tumor. It is now known that one of the main purposes of T-lymphocytes is the inhibition or destruction of tumor cells (Hellstrom et al., 1968). However, the presence of humoral antibodies directed to the same tumor antigens as the sensitized lymphocytes can combine with the antigenic determinates on the tumor cells and block the cytotoxic action of the T-lymphocytes. If these sites are blocked by the high titers of humoral

antibody, then the tumor cell population will continue to proliferate since the cells are not accessible to the cytotoxic action of the sensitized lymphocytes (Sinkovics, Reeves and Cabiness, 1972).

Hence, since leucogenenol stimulated increased levels of humoral antibodies while drug treatment was given, these antibodies may in fact have been coating the tumor cell antigens. This would have the effect of rendering them inaccessible to the cytotoxic action of any sensitized lymphocyte produced. If this blocking effect did occur it had a minimal but significant effect on the survival times of the animals tested.

This study leaves many questions to be answered. All studies of leucogenenol at this point indicate that it stimulates the humoral immune response in immunosuppressed animals (Rice, Lepick and Darden, 1968; Rice and Ciavarra, 1971; Jacoby, 1972). This would suggest that leucogenenol is definitely stimulating B cells. Is leucogenenol's action specific for the B cells or does it affect both B and T cell lines? This is especially important for two reasons. Hellstrom and Hellstrom (1970) showed that humoral antibodies can cause immunological enhancement of a tumor while T cells are largely responsible for the cytotoxic effect on tumor cells. It is also important from the standpoint of the cell line affected by the leukemia. A recent study by Thomas (1972) showed that the L1210 cell is derived from the B cell

precursor while studies are still underway to determine which cell line is affected by various other experimental and human leukemias. Does leucogenenol play a role in oncogenesis and is it present in increased levels in oncogenic tissue where cells are rapidly dividing? If this is the case, would an antibody directed to leucogenenol be effective in slowing down the tumor growth? Answers to these questions may provide a better understanding of the cellular control of abnormal tumor cell division.

SUMMARY

The purpose of this experiment was to determine if leucogenenol in conjunction with conventional chemotherapy can enhance the survival of L1210 infected animals. The initial findings indicated that some of the parameters of the leukemia were reduced. An increased hematocrit, an increase in the percent of lymphoid cells in the peripheral blood and increased antibody titers indicated that the infected animals might be successfully fighting off the leukemia. However, these changes in the parameters of the disease did not significantly increase the final survival time of the test groups. It was therefore concluded that leucogenenol was able to increase the humoral antibody response but not the number of sensitized lymphocytes such that the residual leukemic cells could be destroyed immunologically. Hence, even in combination

with chemotherapy, leucogenenol's action could not turn the race between the residual leukemic cells and sensitized lymphocytes in favor of the host under the conditions of these experiments.

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