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SUPPRESSION BY DRUGS AND X-RAY OF THE IMMUNE RESPONSE OF MICE TO MALIGNANT LYMPHOCYTES

by

Douglas Martinez

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Microbiology)
in The University of Michigan
1974

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LIST OF ABBREVIATIONS

CY	Cyclophosphamide
MTX	Methotrexate
PRD	Prednisolone
XR	X-Ray

INTRODUCTION

I. Chemotherapy of Acute Leukemia in Children

The leukemias are a complex group of diseases (4, 24, 92) characterized by uncontrolled proliferation of one or more cell types derived from the hematopoietic tissue. Thus, leukemias are classified as lymphocytic, monocytic, granulocytic, etc. Traditionally, they are also classified in terms of duration from the time of recognition of illness to death of the untreated patient: acute is less than 6 months, subacute ranges 6 to 12 months, and chronic lasts for more than a year.

Acute leukemia occurs mainly in children below the age of five or in adolescents between 15 and 20 (24). It is typified by (i) infiltration of the bone marrow by immature leukocytes, chiefly blasts, (ii) reduction of normal myeloid precursors with secondary anemia, granulocytopenia and thrombocytopenia, and (iii) a rapidly fatal course if treatment is unsuccessful or withheld. Leukemia in children is primarily the acute lymphocytic type (22, 56, 92). The reported incidence of leukemia in children has steadily increased over the last five decades (53, 59, 142). Because the diagnosis of leukemia has greatly improved, it is impossible to determine whether the rise in leukemia incidence is more apparent than real (24).

Before the latter part of the 1940's there was no treatment available which would reduce the leukemic cell burden and/or induce a return of normal marrow function with any degree of constancy in any form of the disease. Irradiation with X or gamma emissions reduced the number of blast cells in the peripheral blood and the infiltration of

malignant cells into organs. However, total eradication of disease with radiation was impossible (72,111,143). Similarly, in mouse model systems it was found that advanced transplanted leukemia could not be totally eradicated even with supralethal doses of x-ray (14). Beginning with the positive results obtained by Farber and his associates in clinical trials with the folic acid antagonist aminopterin (34), and augmented by the concordant development of new drugs during the succeeding years, systemic chemotherapy has become the mainstay of therapy of acute leukemias.

The design of clinical trials has substantially benefitted by basic knowledge (61, 66,155) gained in quantitative and kinetic experiments with mouse leukemia and lymphoma. The major contributions to this basic information (mostly the work of Skipper and his co-workers and of Bruce and his associates) have been reviewed (128,129) and can be summarized as follows:

(i) A single viable leukemic cell will proliferate relentlessly to a number ultimately lethal to the host (40,130). Thus, to "cure" leukemia it is necessary to eradicate every viable leukemic cell in the host (130).

(ii) Drug-induced increase in the life span of leukemic animals is largely the result of leukemic cell kill (12, 44,130,131).

(iii) Under certain conditions, antileukemic drugs do possess rather marked selective toxicity to leukemia or lymphoma cells in vivo (12, 49,132).

(iv) A given dose of a given antileukemic drug usually kills about the same percentage of leukemic (or hematopoietic) cell populations (130,131,132,144).

(v) Some antileukemic drugs are cell-cycle stage specific; others do not appear to be (12,119,132).

(vi) The dose-response of leukemic (or hematopoietic) cell populations in vivo is often steep and,depending on the mechanism of action of the drug, may be linear or asymptotic on semi-log graph paper (12,131,132). Cell-cycle stage specific drugs yield asymptotic dose-responses.

(vii) The rate of recovery of host hematopoietic tissue from sublethal doses of antileukemic agents is dose-related and seems to be longer for equitoxic doses (LD10) of alkylating agents than for certain antimetabolites (128).

(viii) In order to achieve "cell cure" of leukemias, it is necessary to kill leukemic cells faster than they are being replaced by proliferation of surviving cells (130,132).

Chemotherapeutic agents are traditionally classified according to their source or their biochemical activity (21, 71 , 78), i.e., ionizing radiation, alkylating drugs, adrenal steroids, antibiotics, purine analogues, pyrimidine analogues, folic acid antagonists, and vinca alkaloids. Bruce and his co-workers found that the grouping of drugs, based on dose effects, does not correspond to groupings based on chemical classification (12). Using the spleen-colony method of Till and McCullogh (144) they were able to calculate the fraction of normal hematopoietic colony-forming cells and of lymphoma cells surviving after a given dose of a chemotherapeutic agent. According to the type of curve obtained, they identified 3 classes of agents. The agents of the first class (γ-radiation, nitrogen mustard) kill cells in all phases of the cell growth cycle; thus, the sensitivity of the cells is not a

function of the proliferative state of the cells. The agents of the second class (tritiated thymidine, methrotrexate, azaserine, vinblastin, cytosine arabinoside) kill cells in only one phase of the growth cycle (the mitotic or the S phase). The agents of the third class (5-fluorouracil , cylcophosphamide, actinomycin D) kill cells in all or most phases of the cell growth cycle. Drugs of the second and third classes are much less active on cells in the non-proliferative state. Consequently, the sensitivity of a given cell population to these drugs depends on the fraction of cells in the proliferative state. A relatively large proportion of lymphoma cells are in the proliferative state, while only a relatively small proportion of the bone marrow cells are proliferating. As a result, drugs of the second and third classes have a more profound effect on lymphoma cells. Nitrogen mustard and cyclophosphamide are both alkylating agents; however, nitrogen mustard is in the first class and cyclophosphamide in the third class. Similarly, methotrexate, azaserine, cytosine arabinoside, and 5-fluorouracil are all antimetabolites; yet, 5-fluorouracil is in the third class while the others are in the second.

The therapy of acute leukemia has yielded its most promising results in children (56, 156). With the development of therapeutic drugs (21 , 71 , 78 , 153) during the last 2 decades, the life expectancy of a child with acute lymphocytic leukemia has increased greatly (13, 155). By using drugs in combination (5 , 39 , 62) some treatment centers have been able to obtain a 3 year period of survival free of leukemia in over 50% of children with acute lymphocytic leukemia. In some cases, eradication of all the leukemic cells appears to have been achieved. However, because the toxicity of the drugs is not sufficiently selective, total destruction of malignant cells is improbable in most

instances. Thus, it is very desirable to develop other modalities of treatment to be used in conjunction with chemotherapy.

II. Immunotherapy as an Adjunct to Chemotherapy

Interest in using immunotherapy for treating cancer developed when it was observed by Erlich (32), and later by others, that a malignant tumor arising in one laboratory animal frequently could not be transplanted to other animals of the same species (151). However, the experiments demonstrating resistance to tumor transplantation were carried out in outbred animals. Thus, when the basis of allograft rejection was established, the existance of tumor-specific immunity became suspect (32), i.e., the rejection of tumors in the early experiments was attributable to histocompatability differences between the malignant cells and the host.

Although immunity to syngeneic tumors in inbred mice was demonstrated in the 1930's (79), the current recrudescence of interest in immunotherapy of cancer is based primarily on the studies done by Gross in 1943 (50), by Foley in 1953 (37), and by Prehn and Main in 1958 (109), viz., methylcholanthrene-induced mouse sarcomas were immunologically rejected in syngeneic hosts. Furthermore, Kline et al. (70) determined that an immune reaction was mounted against these tumors in the autochthonous host. Similarly, it was found that both DNA (51 , 126) and RNA (69, 127) virus-induced tumors were rejected in syngeneic hosts which were immunized by homologous virus or tumor inoculation (27 , 104).

Cancer, especially leukemia, is more prevalent in individuals who are immunologically deficient either congenitally (45), or as a result of the aging process (16), immunosuppressive therapy (33 ,

105), or exposure to irradiation (42, 58, 60). The importance of the immune response in preventing leukemia (65) is also brought out by the studies done by Haran-Ghera and by Law. Haran-Ghera (54) presented evidence indicating that the immunosuppressive effect of irradiation may play a significant role in its leukemogenic activity in strain C57BL mice. She postulated that immune surveillance mechanisms normally are capable of detecting antigens induced on the surface of malignant lymphoid cells by "radiation leukemia virus" resulting in the destruction of such cells before they give rise to disease. Transient immunosuppression by x-ray, or various leukemogenic agents, provided an opportunity for clones of malignant lymphoid cells to gain reproductive momentum and to outgrow the immune response after it recovered. Most of the known chemical carcinogens (28), including the hydrocarbons (8 , 110), urethane (110), and nitroquinoline oxide (67), are also immunosuppressive and induce lymphomas from which leukemogenic viruses can be extracted. Law (73 , 74) found that the frequency of leukemia in mice injected with mouse leukemia virus was increased in mice rendered immunologically defective by neonatal thymectomy, treatment with anti-lymphocyte serum, or x-irradiation.

Approaches to immunotherapy of leukemia can be divided into two categories: specific and nonspecific (134). Specific immunotherapy is directed at increasing the host reactivity specifically to the antigens of the malignant cell line. Included in this category is active immunization with killed leukemic cells or their subcellular fractions, passive immunization, and adoptive immunization. Nonspecific immunotherapy is aimed at obtaining a generalized increase in host immune reactivity. Adjuvants commonly used in experimental tumor systems for this

purpose are microorganisms, subcellular fractions of microorganisms, and nonbacterial materials such as synthetic polynucleotides (154). In experiments with transplantable mouse leukemias it has been found that specific and nonspecific immunotherapy administered to mice with established disease significantly increases their survival time and, if the leukemic cell load is sufficiently small, these measures increase survival rate in treated mice. The degree of success obtained with any method appears to be dependent on the experimental system employed. Positive results have been obtained with x-ray inactivated leukemic cells (90), membrane fractions of leukemic cells (77), immune serum (94), immune lymphocytes from syngeneic (35) or allogeneic (10,36) donors. Adjuvants such as Bacillus Calmette-Gûerin (BCG) (19 ,89 ,90), Bordetella pertussis (89), Corynebacterium parvum (89), and poly I:C (11) appear to significantly increase immunity through nonspecific mechanisms.

Baker and Taub (7) have reviewed the literature on clinical trials of immunotherapy in human leukemia. (For a review of immunological methods used for treatment of human cancer in general, see references 23 and 98 .) The studies of Mathé and his associates have enormously stimulated interest. Based on the results obtained with immunotherapy of transplantable leukemia in mice, they conducted over 100 clinical trials (starting in 1964) in patients who had their leukemic cell load reduced by aggressive chemotherapy (91). They administered irradiated allogeneic leukemic cells and nonspecific stimulators of the immune response such as BCG, C. parvum, C. granulosum, and poly I:C in various combinations. Because the procedures used to treat patients varied greatly, results were difficult to interpret. However, their re-

sults were sufficiently encouraging that they recommend continuation of clinical trials of immunotherapy as an adjunct to conventional therapy. They have also established a program designed to screen adjuvants in mice for their effects on antibody production, graft versus host reaction, and resistance to transplantable L1210 leukemia and various other tumors (88).

III. Immunosuppressive Effects of Chemotherapeutic Agents: An Obstacle to Immunotherapy

Drugs used to treat leukemic patients suppress immune processes and, therefore, may interfere with immunotherapy (121). Extensive reviews on the immunosuppressive effects of x-irradiation (93 , 140) and drugs (41 , 84 , 120) have been published. The existing data on drug-induced immunosuppression are somewhat confusing, mostly because of the lack of uniformity in the approaches used to study immunosuppression. As a result, reports concerning a given agent often appear to be discordant. Nonetheless, there is a concensus that, under the proper conditions, the commonly used chemotherapeutic agents suppress antibody production and cell-mediated immune reactions. Moreover, some of the agents have been found to effect the complement system, phagocytic mechanisms, and inflammation (41).

Makinodin et al. (84) classified the immunosuppressive agents according to the stage at which the immune response is most sensitive to their action. Three categories were obtained but they do not correspond with the categories by Bruce and his associates as noted above.

Class I agents are most effective in suppressing an immune

response when given just before antigenic stimulation. The very early processes of the immune response on which these agents are assumed to act include "antigen processing" and early "cell cooperation". The class I agents used in the present study were x-irradiation (XR) and prednisolone (PRD).

The immunosuppressive effects of XR are mainly due to its lympholytic activity (93), i.e., lymphocytes are among the most radiosensitive cells. Even the small nondividing lymphocytes are rapidly destroyed. Their precursors, like all proliferating cell populations, are subject to the lethal effects of XR. The effects of various doses of XR on lymphoid cells of rabbits, rats and mice were studied by Murray (100,101) and de Bruyn (26). De Bruyn (26) showed that the germinal centers were almost completely destroyed by 600 r and reformed 3 weeks later. In contrast, 400 r only partially damaged such germinal centers. They returned to a fairly normal state in less than 1 week. The lowest dose of XR which caused histologically detectable damage was 25 r. This dose also significantly depressed the number of circulating lymphocytes in the blood.

XR produces effects such as chromosome breaks by direct action on key molecules in the chromosomes. However, a large part of the damage is due to an indirect mechanism, i.e., as the result of ionization of the intracellular water, with the production of very numerous active H ions and HO radicals (125). How much of the final radiation effect is due to direct or indirect mechanisms is not clear (6). Extremely little is known about why nondividing lymphocytes are so vulnerable to radiation injury.

The effects of PRD and the other adrenal steroids on the im-

mune response has been reviewed extensively (41, 86). However, the mode of action of corticosteroids on the various types of immune responses is poorly understood. These agents produce lympholytic effects which do not appear to depend on cell proliferation (30). In vitro studies indicate that the steroids can inhibit RNA and protein synthesis of lymphocytes (85,152). This inhibitory action might ultimately result in the destruction of the cells (149). The corticosteroids also inhibit phagocytosis (102) and inflammation (43). All of these effects may be important in explaining their immunosuppressive effects.

Class II agents are most effective as immunosuppressants when given a day or two after antigenic stimulation. The period of maximal sensitivity may last only a day or two. In general, immunocytes undergoing differentiation and proliferation are more sensitive to the action of these compounds. The major action of most of the drugs appears to be cell killing. The class II agent used in the present study was methotrexate (MTX). MTX and aminopterin are the most commonly used folic acid antagonists. The biochemical basis for their immunosuppressive effects is well characterized (9). These drugs inhibit dihydrofolate reductase, thus preventing the conversion of folic acid to tetrahydrofolic acid. This step is necessary for the synthesis of many biochemical compounds, including DNA, RNA, and several coenzymes. Turk and Stone (145) studied the dynamics of the large pyroninophilic cells and small lymphocytes involved in the hypersensitivity response. Histologic and autoradiographic studies were made of regional lymph nodes during sensitization. MTX did not block the formation of the large pyronophilic cells seen during sensitization, but inhibited the development of small lymphocytes that appeared to be derived from the large pyroni-

nophilic cells.

Class III agents are immunosuppressive whether applied before or after antigenic stimulation. Cyclophosphamide (CY), used in this study, is representative of this class. It is the alkylating agent most widely used for immunosuppression and has proved to be one of the most effective immunosuppressive agents studied (117). CY is converted to its active form in the liver (38). It is most immunosuppressive when given after the antigen (116). It is able to partially depress antibody synthesis even when given after antibody appears in the serum (146). Nevertheless, as stressed by Santos and Owens (118), the drug is very immunosuppressive when given before the antigenic stimulus in rodents. Turk and Stone (145) concluded, on the basis of histologic studies, that the major effect of CY was to block the appearance of the large pyroninophilic cells that in turn gave rise to the small effector lymphocytes. Alkylating drugs, in general, interact with the nucleophilic centers of other molecules. The most favorable sites of reaction are molecules possessing $-NH_2$, -COOH, -SH, and $-PO_3H_2$ groups and those possessing tertiary nitrogen compounds in heterocyclic systems (113). The most common biological components that possess such reactive groups are DNA, RNA, enzymes, and structural proteins.

Because the drugs in class II and class III have preferential toxicity for lymphoid cells in the proliferative phase, they are able to induce specific immunological tolerance to a given antigen, presumably by inactivating the immunocompetent cells responding to that antigen (84,122,123). This phenomenon was first observed by Schwartz and Dameshek (123) while studying the effects of 6-mercaptopurine on the antibody response of rabbits to human serum albumen. Subsequently, MTX

was found to induce immunological tolerance to lymphocytic choriomeningitis virus and sheep red blood cells (55,116). CY also has been used successfully for the induction of tolerance. Salvin and Smith (115) found that immunological tolerance induced with CY in guinea pigs to a given hapten-carrier conjugate was directed at the whole molecule. When either the hapten or protein portion of the conjugate was altered, an immune response to the newly substituted portion developed. Nevertheless, tolerance to the original conjugate persisted. Aisenberg and Davis (1) noted that if thymectomy was performed after initiation of tolerance toward heterologous erythrocytes, the tolerant state was significantly prolonged. Other studies, employing cell transfer techniques, also indicate that the antigen-activated thymus-dependent lymphocyte is the main target for CY in the induction of tolerance (2 ,87 ,96).

Under certain conditions, animals treated with an immunosuppressive agent respond to an antigen more vigorously than normal. This paradoxical phenomenon was first observed in the 1920's and since then has been confirmed by others (29,139). For example, 500 r XR given to rabbits 24 hr after immunization with sheep red blood cells resulted in a 10-fold rise in peak antibody titer (139). Most studies on this subject are consistant with the hypothesis that XR-enhanced antibody production is due to an increase in "space" in which the activated immunocytes can proliferate (84). Similarly, rabbits treated with 6-mercaptopurine, a class II immunosuppressant, developed hyperplastic lymphoid tissues 5 to 7 days after a 1-week course of the drug, and antibody formation was enhanced when bovine gamma globulin was injected at the time of maximum hyperplasia (17). Similar studies in the rat also showed that 6-mercaptopurine-enhanced antibody production coincided with the

period of increased lymphopoiesis (150). This adjuvant effect has been produced with other immunosuppressive agents including CY (15) and MTX (138).

The effects of immunosuppressive agents on tumor-specific immunity have not been studied in detail. Most of the work has been done with tumor allografts and xenografts. Thus, the phenomena observed in these studies were mostly, if not completely, due to the effects of the agents on the immune response to the strong allogeneic or xenogeneic antigens. Radiation (93) and adrenal steroids (41) very commonly are used to facilitate transplantation of allogeneic and xenogeneic tumors. The newer chemotherapeutic agents also have been used (95). In allogeneic and xenogeneic systems, it is easier to suppress antibody production or graft rejection when weaker antigens are employed (31 , 81 , 93). Thus, one might expect that the immune response to the relatively weak tumor-specific antigens to be more easily suppressed than the immune response to allogeneic or xenogeneic tumors. Therefore, for the purpose of predicting and avoiding suppressive effects on immunotherapy, it would be useful to know the effects of the therapeutic drugs on the immune response to weak tumor-specific antigens. Some qualitative studies have been done on the effects of therapeutic agents on the immune response to syngeneic methylcholanthrene-induced sarcomas (97 , 112) and to syngeneic leukemias (94). However, to rationally develop a chemo-immunotherapy of leukemia, basic quantitative and kinetic information is needed from appropriate animal model systems concerning the effects of chemotherapeutic drugs on the immune response to malignant lymphoid cells.

This study records experimental results dealing with the ef-

fects of selected therapeutic drugs and x-ray on the immune response of C58 mice to syngeneic transplantable line I_b leukemic cells. The line I_b-C58 mouse model system was selected because it provides a very sensitive assay for I_b cells. Moreover, the basic requirements for establishing immunity have been thoroughly investigated. In C58 mice injected with viable I_b cells, the time for death is inversely proportional to the log of the number of leukemic cells injected (99). Thus it is possible to bioassay tissues and fluids for viable I_b cells (77). A first-set immune reaction to I_b cells can be induced by injecting 10^7 formalinized I_b cells (containing ca. 10^3 viable cells) into normal C58 mice. Survival of this treatment is close to 100% (76).Two weeks after primary injection, mice mount a very strong second-set immune reaction, viz., they withstand a subsequent injection of 10^7 viable I_b cells. The immunity developed in the mice is transferable with lymphoid cells but not with serum (99). Furthermore, immune serum is not cytotoxic *in vitro* (75). Thus, the immunity to I_b cells is primarily, if not exclusively, cell-mediated.

A detailed study was made of the immunosuppressive effects of CY, MTX, PRD, and XR on both the first- and second-set immune reaction of C58 mice to line I_b cells. Attention was focused on time and dose effects of the immunosuppressive agents, the growth kinetics of I_b cells in normal and immunosuppressed mice, the relative immunosuppressive activity of the test agents, and the effects of the agents on immunocyte number and their immunologic competence.

MATERIALS AND METHODS

I. Experimental Mice

Breeding stocks of C58 mice were obtained from the University of Minnesota and inbred by approved methods to establish the Michigan subline C58/wm (48 ,135). Within a given experiment only male or female C58/wm were used. Mice given drugs were 6 to 8 weeks old at the beginning of experiments; those given x-ray were 9 to 11 weeks old.

II. Preparation of Leukemic Cell Suspensions

A. Frozen Line I_b Cell Stocks

The test strain of line I_b cells originated from passage 40 obtained from the University of Minnesota. Stock preparations of I_b cells (passage 78 and 79) were used for all experiments. The stock was prepared from the spleens of C58 mice morihund from transplanted I_b cells (passage 77 and 78). Freshly prepared I_b cells were suspended at a concentration of 10^7 viable cells/ml in cold (4°C) Hanks balanced salt solution (52) containing 10% glycerol (v/v), transferred to ampoules and stored at -70°C until used. It was not necessary to control the rate of freezing to obtain good viability. A half ml of stock I_b cell preparation killed mice 6 or 7 days after i.p. injection.

B. Fresh Preparations of Line I_b Cells

Spleens removed aseptically from C58 mice moribund from transplanted leukemia were gently dispersed into suspension in cold

saline (0.85 gm NaCl/100 ml) by use of a glass tissue homogenizer. Gross particles were allowed to settle for 5 min and the cells in the supernatant fluid transferred to a centrifuge tube. I_b cells were sedimented at 15 g for 5 min and the supernatant fluid (erythrocytes) aspirated. The cells were washed twice more in cold saline. Total and viable cell counts (trypan-blue exclusion) were done as described previously (99, 141). Line I_b cells were distinguished by their characteristic morphology (108, 136). They are round or slightly oval, and about twice the diameter of a small lymphocyte. The nucleus is coarsely lobulated, occupies an eccentric position, and fills most of the cell, leaving a small amount of nongranular cytoplasm. Appropriate dilutions of viable I_b cells were made so that the number of I_b cells to be injected i.p. was contained in 1 ml.

C. Formalin Treated Line I_b Cells

A 37.4% (gm/100 ml) analytical grade formaldehyde solution was diluted 1/10 in saline and stored at room temperature. Dilute formalin was prepared fresh by diluting the 1/10 formalin solution 1/250 in cold saline. I_b cells, prepared as described above, were suspended at a concentration 2×10^7 viable cells/ml in cold saline. I_b cells were mixed 1:1 with dilute formalin (giving final concentrations of 10^7 I_b cells/ml and 0.0075% formaldehyde) and kept at 4°C for 18 hr (76). Mice were injected i.p. with 1 ml of the formalinized cell suspension. This preparation contained approximately 10^3 viable I_b cells/ml at the time of injection. It thus served as both an immunizing and challenge preparation.

III. Bioassay for Viable Leukemic Cells

To assay for viable leukemic cells (99) in C58 mice that received an injection of formalinized I_b cells, spleens from 5 recipient mice were gently dispersed into a single cell suspension in cold saline. A series of 10-fold dilutions of pooled spleen cells were injected i.p. into groups of 4 or more normal C58 mice. Mice injected with I_b cell preparations were observed twice daily for 15 days (maximum time for leukemic death to result from 1 transplanted I_b cell). The gross pathology of dead mice was noted to assure that deaths were from leukemia. The mean time of death gave an estimate of the number of viable I_b cells contained in each preparation. Time of death for mice injected with viable I_b cells was a linear function of the log of the number of I_b cells injected, and accurate to 0.5 day (Figure 1).

IV. Immunization of Mice

An i.p. injection of 10^7 formalinized I_b cells elicited a first-set immune reaction in essentially 100% of recipients. Two weeks after injection with formalinized I_b cells, mice survived a dose of 10^7 viable I_b cells (second-set immune reaction). Thus, mice that survived the injections of formalinized I_b cells were referred to as immune or immunized mice.

V. Quantitation of Adoptive Cellular Immunity

Two weeks after injection with formalinized I_b cells the spleens of 5 or more immunized mice were gently dispersed into a single cell suspension in cold Hanks balanced salt solution (HBSS). Cells were washed three times in cold HBSS (centrifugation at 500 g for 5 min).

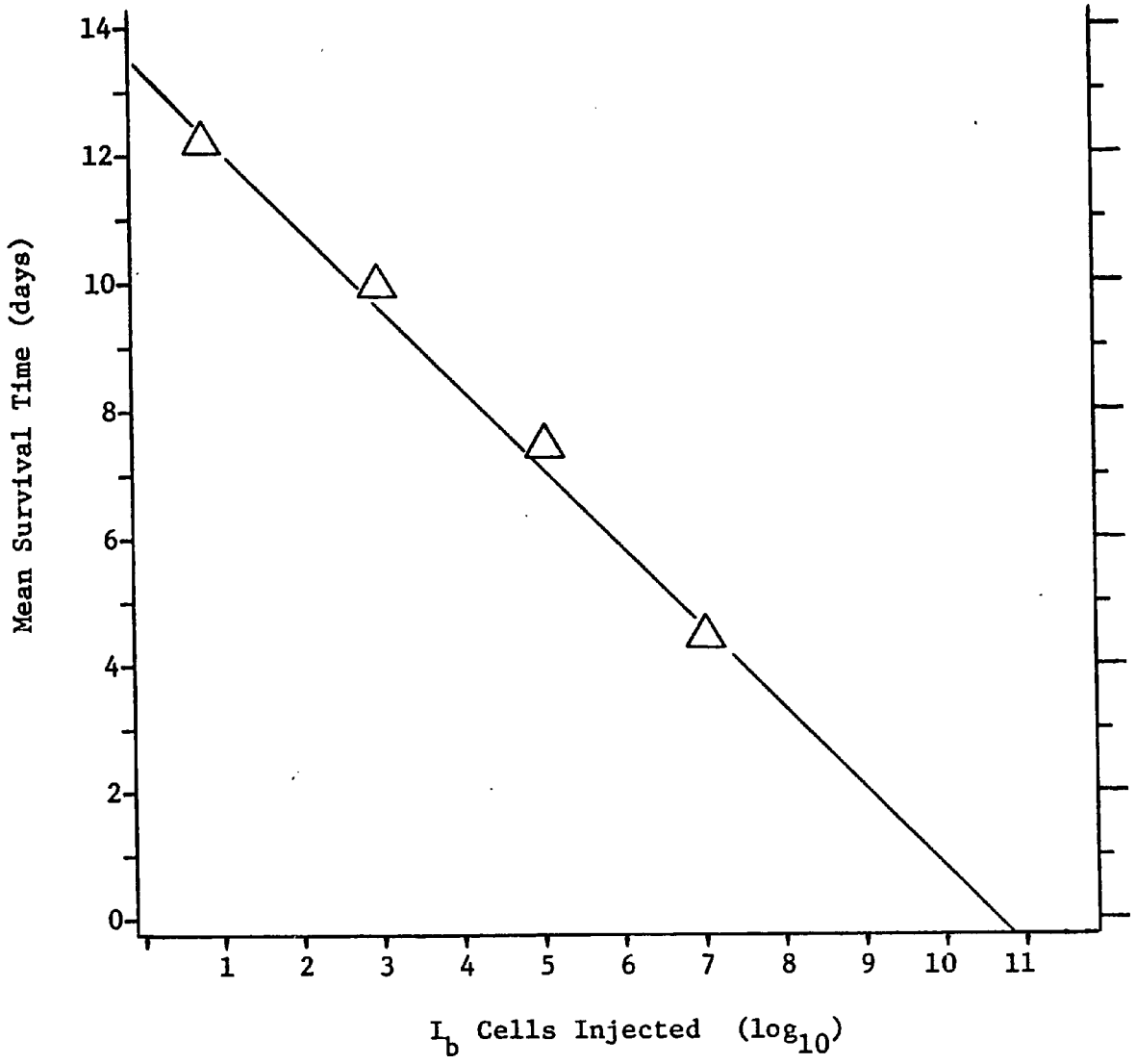


Figure 1. DOSE-RESPONSE OF MICE TO LINE I_b CELLS. Groups of 10 mice each were injected i.p. with graded numbers of viable I_b cells. Mice were observed for deaths at 12-hr intervals.

Viability of cell suspensions (trypan-blue dye exclusion) ranged from 80 to 90 %. Differential counts using Wright's stain showed that 90% of the cells were of lymphoid morphology; 5% had the morphologic properties of macrophages. Viable immune spleen cells were diluted appropriately and injected i.p. (1 ml volume) into normal C58 mice. 24 hr later recipient and control mice received i.p. injections of 10^3 viable I_b cells. Figure 2 gives the results of a representative experiment. The proportion of recipient mice surviving I_b cells increased in proportion to the number of immune spleen cells injected. Mice that received 10^4 immune spleen cells or less were not protected. The estimated median protective dose of immune spleen cells was $10^{6.3}$. The median protective dose ($\log_{10}$) in three replicate experiments was 6.5 ± 0.2 immune spleen cells.

VI. Administration of Immunosuppressive Agents

A. Whole-Body X-Irradiation

X-rays were generated by a Coronado X-Ray Therapy Machine operated at 250 kv accelerating voltage and 15 mA current. Filtration was 0.5 mm Cu and 1.0 mm Al. Dosimetry was performed with Victoreen equipment calibrated by the National Bureau of Standards. Mice restrained in a 1" ceiling cardboard box, were placed on 3" of fresh wood 71 cm from the x-ray source (70 cm distance between source and target center) and given whole-body x-irradiation at 67 r/min. Absorbed dose was 0.95 rads/r in muscle; 1.41 rads/r in bone (interpolated from Table I a 1 of reference 107).

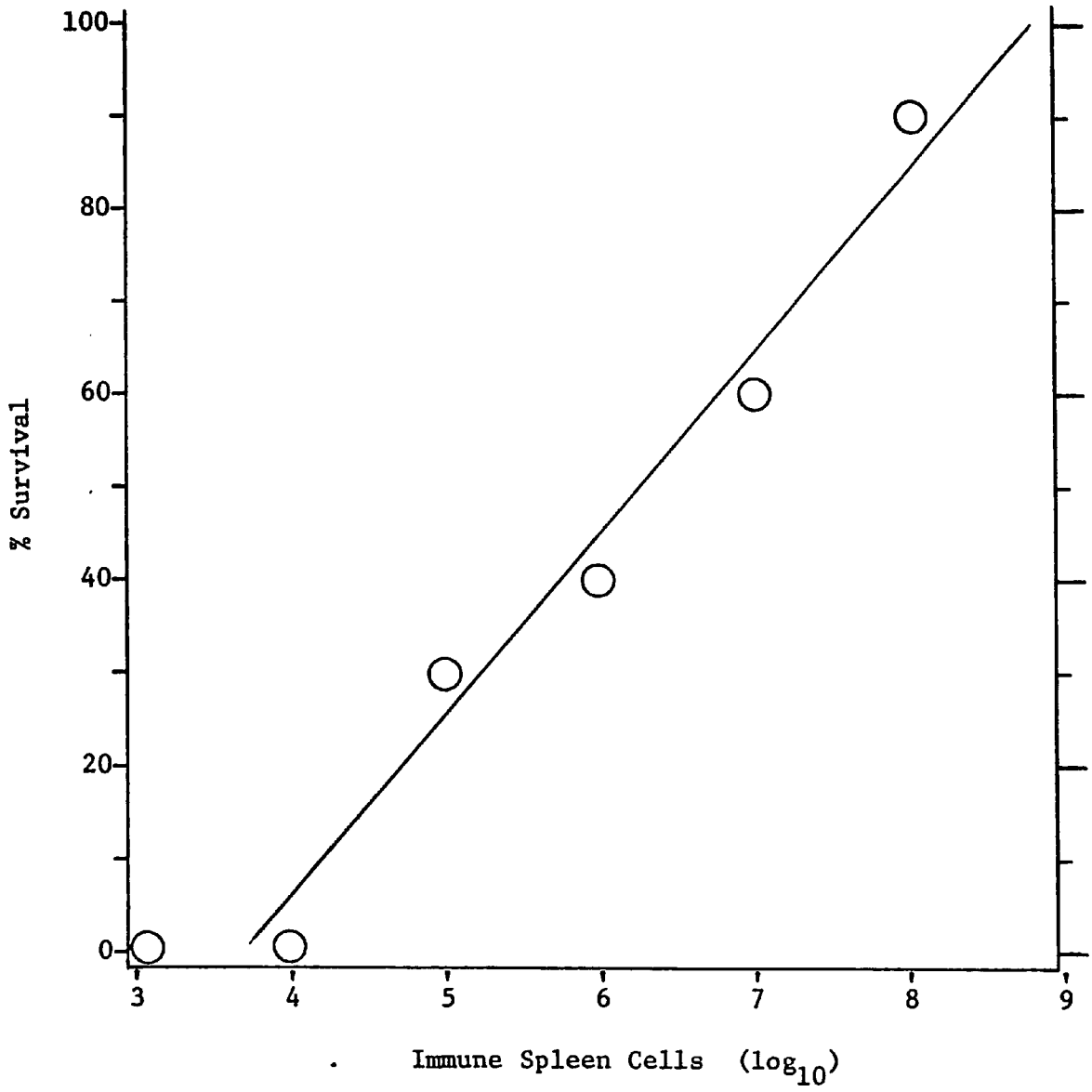


Figure 2. TITRATION OF PROTECTIVE ACTIVITY OF IMMUNE SPLEEN CELLS. Groups of 10 normal mice each were given graded numbers of viable immune spleen cells 24 hr before the injection of 10^3 viable I_b cells. Control mice given I_b cells alone did not survive.

B. Drugs

Cyclophosphamide (CYTOXAN) (Mead Johnson & Co., Evansville, Ind.) was disolved in saline. Methotrexate (Lederle, Pearl River, N.Y.) and prednisolone sodium phosphate (HYDELTRASOL) (Merck, Sharp & Dohme, West Point, Pa.) were diluted in saline. The appropriate concentrations were injected i.p. as a single dose, 0.1 ml/gm body weight.

EXPERIMENTAL RESULTS

I. Maximum Tolerated Dose of Drugs and X-Ray

To quantitatively rank the immunosuppressive effects of drugs and x-ray *in vivo* some equally weighted parameter must be used. As recommended by Skipper and Schmidt (133), the lethal dose for 10% of mice (LD10), read from plotted dosage-mortality data, was employed as the endpoint and considered the maximum tolerated dose. To determine the LD10 for each drug, separate groups of mice received a single i.p. injection of each test dose. Separate groups of mice were likewise exposed to graded doses of x-ray. Figure 3 gives the 30-day mortality data obtained for cyclophosphamide (CY), methotrexate (MTX), prednisolone (PRD), and whole-body x-irradiation (XR). LD10 values were 300 mg CY/kg, 28 mg MTX/kg, 600 mg PRD/kg, and 625 r XR, respectively.

II. Effects of Drugs and X-Ray on First-Set Immune Reaction

A. Suppression of First-Set Immune Reaction

To determine whether each test agent suppressed the first-set immune reaction of C58 mice to I_b cells, mice received approximately an LD10 dose of drug or x-ray either 24 hr before or 24 hr after the injection of 10^7 formalinized I_b cells. The formalinized cell inocula contained approximately 10^3 viable I_b cells. 100% of untreated control mice survived (Table 1) the formalinized I_b cells. Mice treated with CY, PRD, or XR 24 hr before injection of formalinized I_b cells did not survive. However, 4 of 10 MTX-treated mice survived

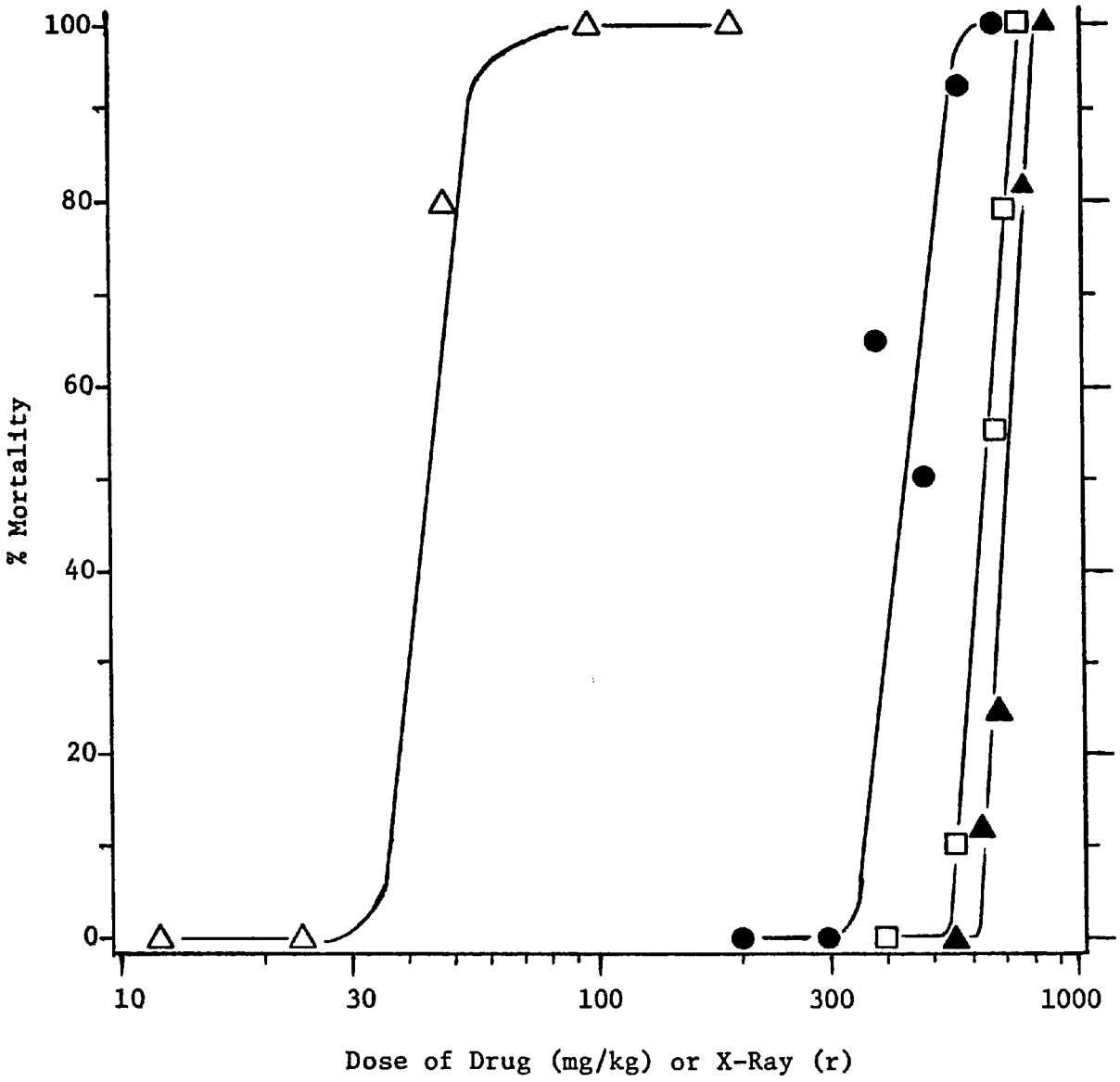


Figure 3. DOSE-RESPONSE OF MICE TO DRUG OR X-RAY. Groups of 10 or more mice were given graded doses of either MTX (△—△), CY (●—●), PRD (□—□), or XR (▲—▲) and observed for 30 days.

TABLE 1

SUPPRESSION OF FIRST-SET IMMUNE REACTION TO FORMALINIZED LINE I_b CELLS

Agent	Dose	Fraction Surviving[a]		
		Agent Before	Agent After	No Agent
X-Ray	625 r	0/11	3/15	39/40
CY	300 mg/kg	0/10	10/10	39/40
PRD	600 mg/kg	0/10	8/10	20/20
MTX	30 mg/kg	4/10	6/10	127/127

[a] Mice received ca an LD10 dose of the indicated agent either 24 hr before or 24 hr after the injection of 10^7 formalinized I_b cells. All deaths observed were due to leukemia.

the formalinized cell preparation. These results suggested that the administration of these agents 24 hr before injection of formalinized I_b cells suppressed the immune response of mice thus permitting the viable cells in the formalinized preparation to cause fatal transplanted leukemia.

When the immunosuppressive agents were given 24 hr after the injection of formalinized I_b cells immunosuppression also occurred but the mortality rate was lower. The complete survival of mice given an LD10 of CY suggested that the drug may have prevented the growth *in vivo* of the viable I_b cells contained in the formalinized cell preparation.

B. Growth Kinetics of Line I_b cells in Normal and Immunosuppressed Mice

To assess the effect of CY on the fate of viable I_b cells in C58 mice, the spleens of treated and untreated mice were assayed for viable I_b cells over a 20-day period following the injection of the formalinized I_b cell preparation. CY (300 mg/kg) was given either 24 hr before or 24 hr after the injection of formalinized preparations. Figure 4 shows that viable I_b cells were detectable in the spleens of normal recipient mice 4 days after the injection of formalinized I_b cells. The number of I_b cells per spleen increased at a rate of 3.32 doublings/day (doubling time = 7.25 hr) up to day 6 and decreased thereafter; none were detectable by day 8. When mice received CY 24 hr before the injection of formalinized cell preparations the number of viable I_b cells per spleen increased at the same rate as in control mice, but the I_b cells continued to proliferate. These results showed

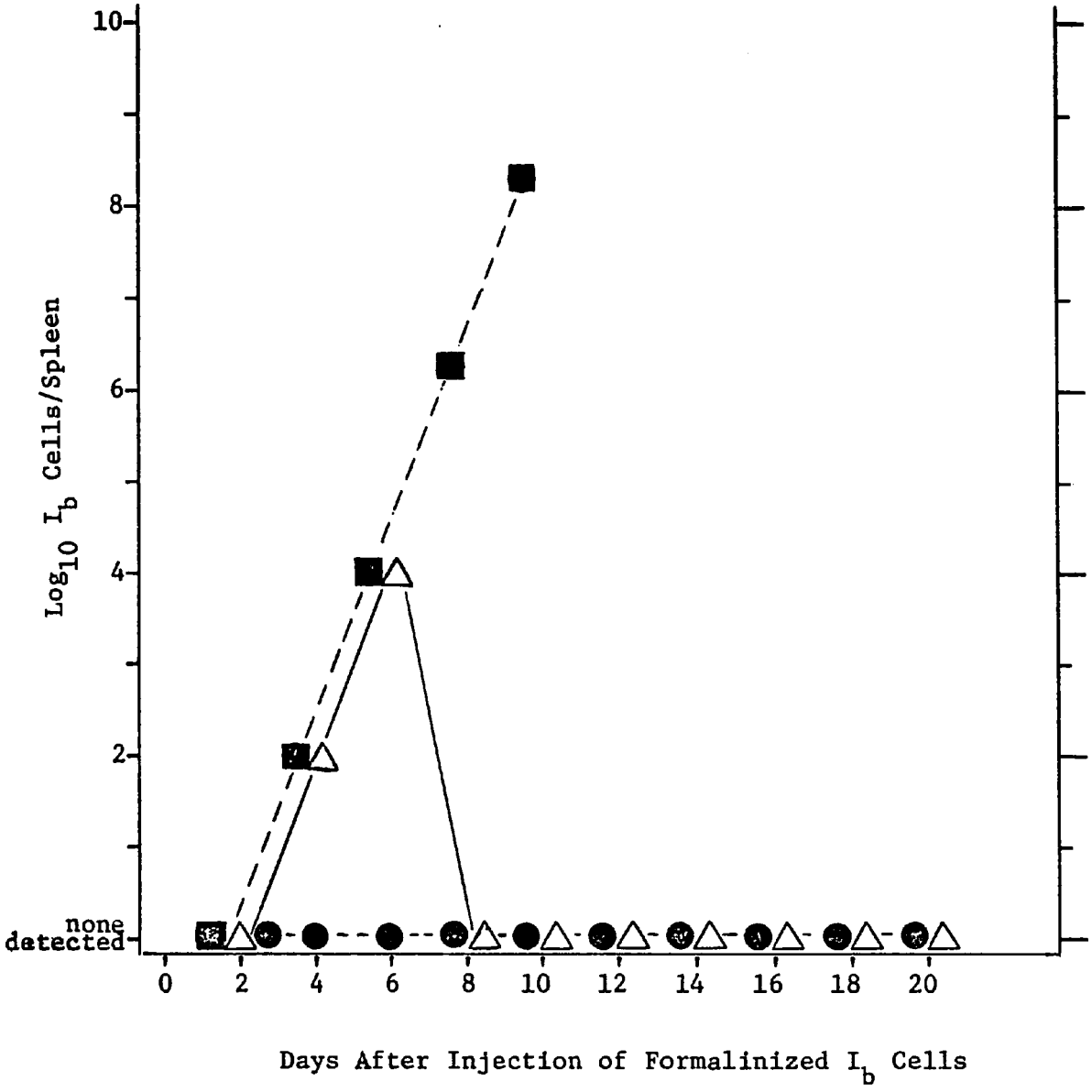


Figure 4. GROWTH OF LINE I_b CELLS IN NORMAL AND CYCLOPHOSPHAMIDE TREATED MICE. Mice received 10^7 formalinized I_b cells alone (△—△) or 300 mg CY/kg 24 hr before (■ --■) or after (●--●) the injection of formalinized cells. Assays for viable I_b cells were done on the spleens of 5 mice from each group.

that CY, given before I_b cells, did not inhibit the growth of I_b cells in vivo. The results also provided evidence that the decline of I_b cells in untreated mice was due to an immunologically mediated rejection and that CY functionally eliminated this immunologic response.

No viable I_b cells were found in the spleens of mice given CY 24 hr after the injection of formalinized I_b cells. Thus the 300 mg/kg dose of CY apparently killed the viable I_b cells in the mice and therefore failed to provide a test for immunosuppression.

C. Dose of Agent and Suppression of First-Set Immune Reaction

To quantitatively determine the relationship between the dose of test agent given and the degree of suppression of the first-set immune reaction, groups of C58 mice received graded doses of drug or x-ray 24 hr before the injection of 10^7 formalinized I_b cells. The median immunosuppressive dose (dose of agent required to suppress 50% of the mice) was determined from the mortality data obtained (Figure 5): 45 mg CY/kg; 300 r XR; 200 mg PRD/kg; 30 mg MTX/kg. The relative immunosuppressive efficacy of the agents can be expressed by the median immunosuppressive dose as fractions of LD10, viz., CY 0.15, XR 0.30, PRD 0.40, and MTX 1.00.

D. Time After Injection of Formalinized Line I_b Cells and Immunosuppression by Cyclophosphamide

An experiment was performed to determine the effect of a single injection of CY (50 mg/kg) on the first-set immune reaction when the drug was given at different time intervals after the injection

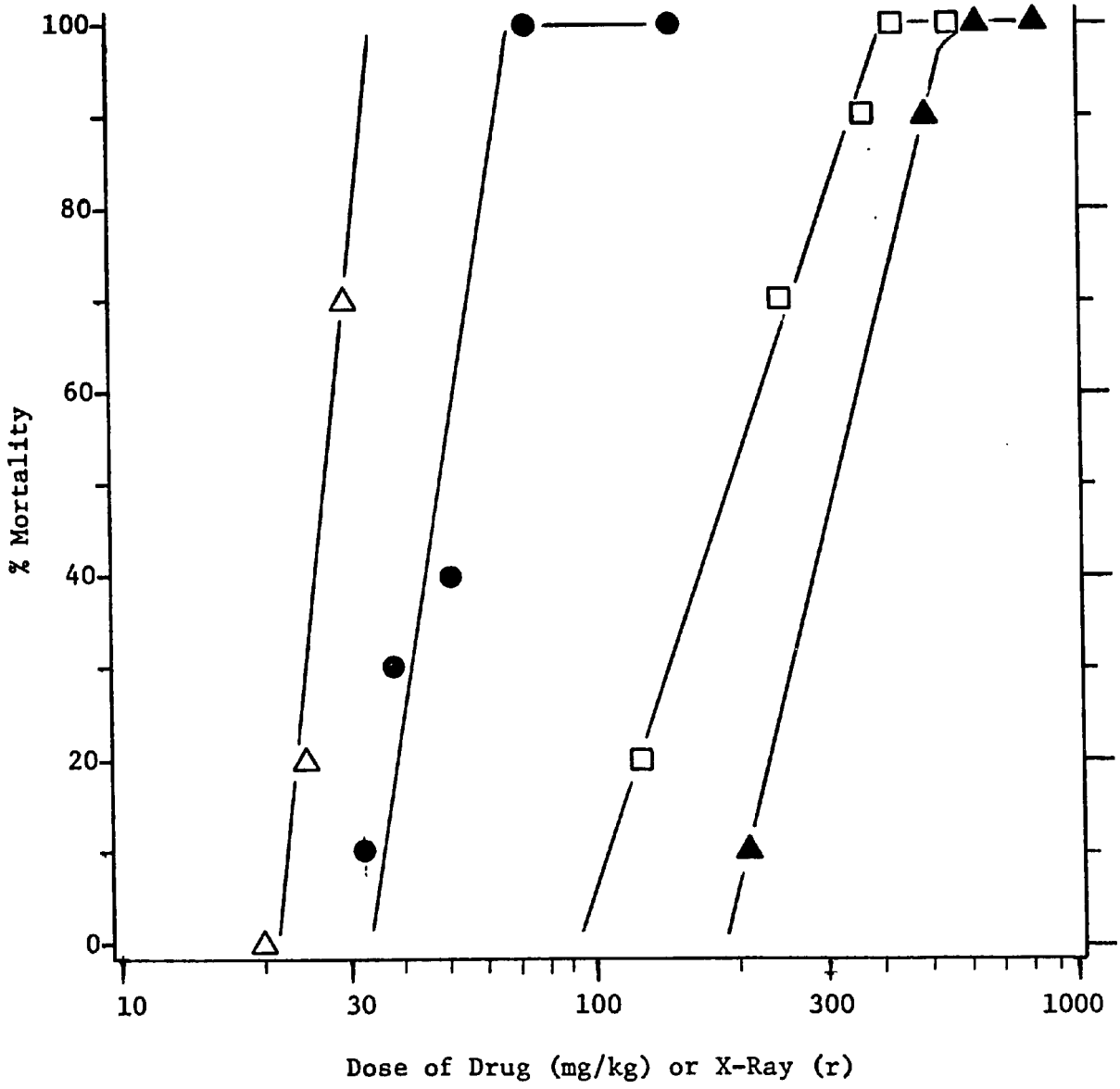


Figure 5. DOSE EFFECTS OF IMMUNOSUPPRESSIVE AGENTS ON THE FIRST-SET IMMUNE REACTION. Groups of 10 mice were given graded doses of either MTX (△—△), CY (●—●), PRD (□—□), or XR (▲—▲) 24 hr before the injection of 10^7 formalinized I_b cells.

of 10^7 formalinized I_b cells. Figure 6 shows that the later CY was given, the less it was immunosuppressive. There were no deaths when CY was given after day 7. These results indicate that the first-set immune reaction to I_b cells became resistant to CY once the immune response was initiated.

E. Recovery of First-Set Immune Reaction Following Immunosuppression

Since CY, PRD, and XR suppressed the first-set immune response of C58 mice to I_b cells, experiments were carried out to define the duration of immunosuppression. Mice were given approximately an LD10 dose of drug or x-ray at varied time intervals before the injection of 10^7 formalinized I_b cells. The fraction of mice that survived the formalinized cell preparation increased with the time allowed for recovery (Figure 7). The median recovery time (days required for recovery of 50% of the mice) was essentially 30 days for XR, 25 days for CY, and 3.5 days for PRD. When a smaller dose of CY (75 mg/kg) was used the median recovery time was decreased to 5 days.

III. Effects of Drugs and X-Ray on Second-Set Immune Reaction

A. Suppression of Second-Set Immune Reaction

To determine whether each test agent suppressed the second-set immune reaction of C58 mice to I_b cells, mice immunized with formalinized cells two weeks previously were given approximately an LD10 dose of drug or x-ray either 24 hr before or 24 hr after the injection of 10^7 viable I_b cells. Table 2 shows that control immune mice uni-

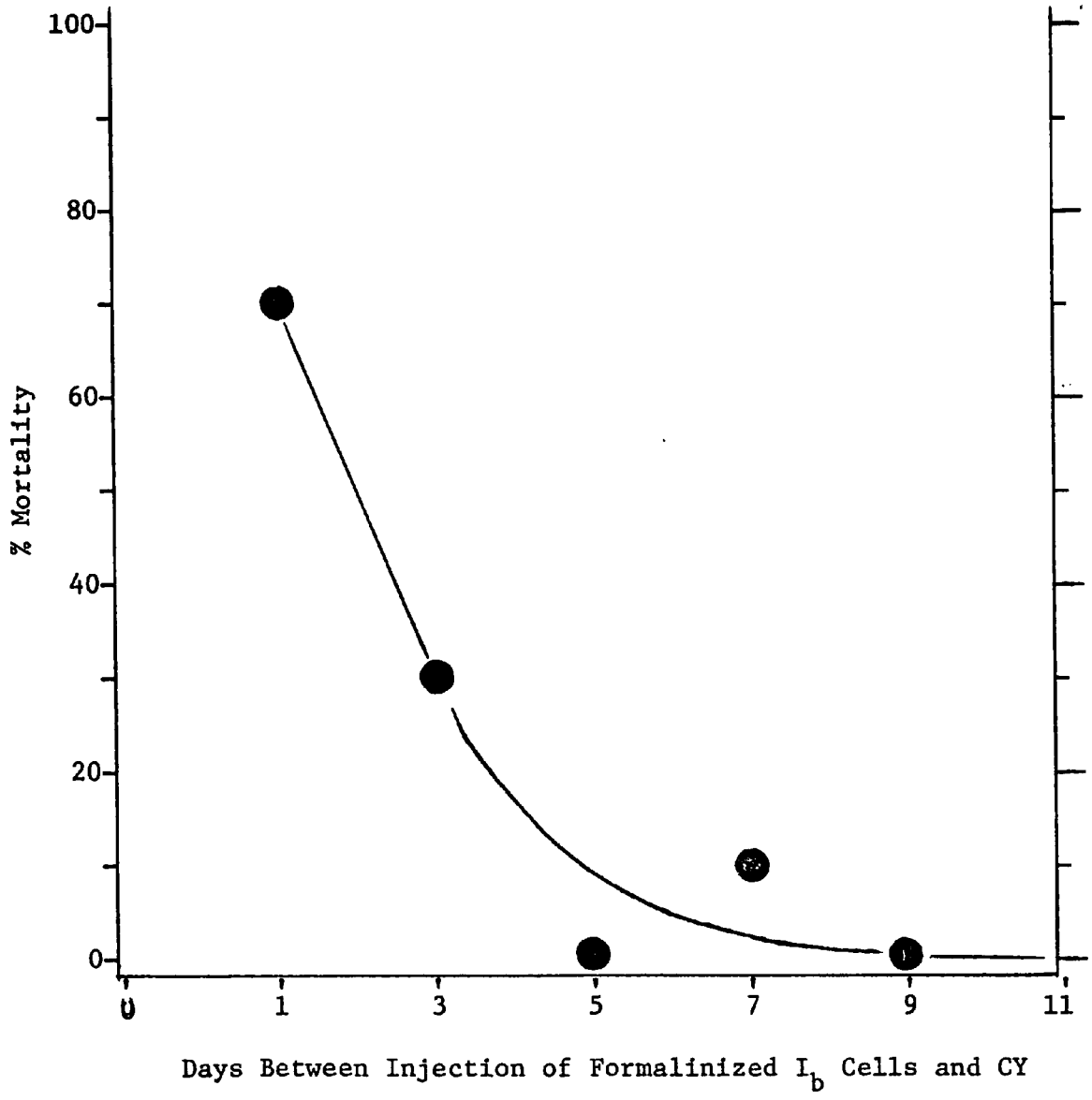


Figure 6. IMMUNOSUPPRESSION BY CYCLOPHOSPHAMIDE WHEN GIVEN AFTER FORMALINIZED LINE I_b cells. Groups of 10 mice each were given 50 mg CY/kg at different time intervals after the injection of 10^7 formalinized I_b cells.

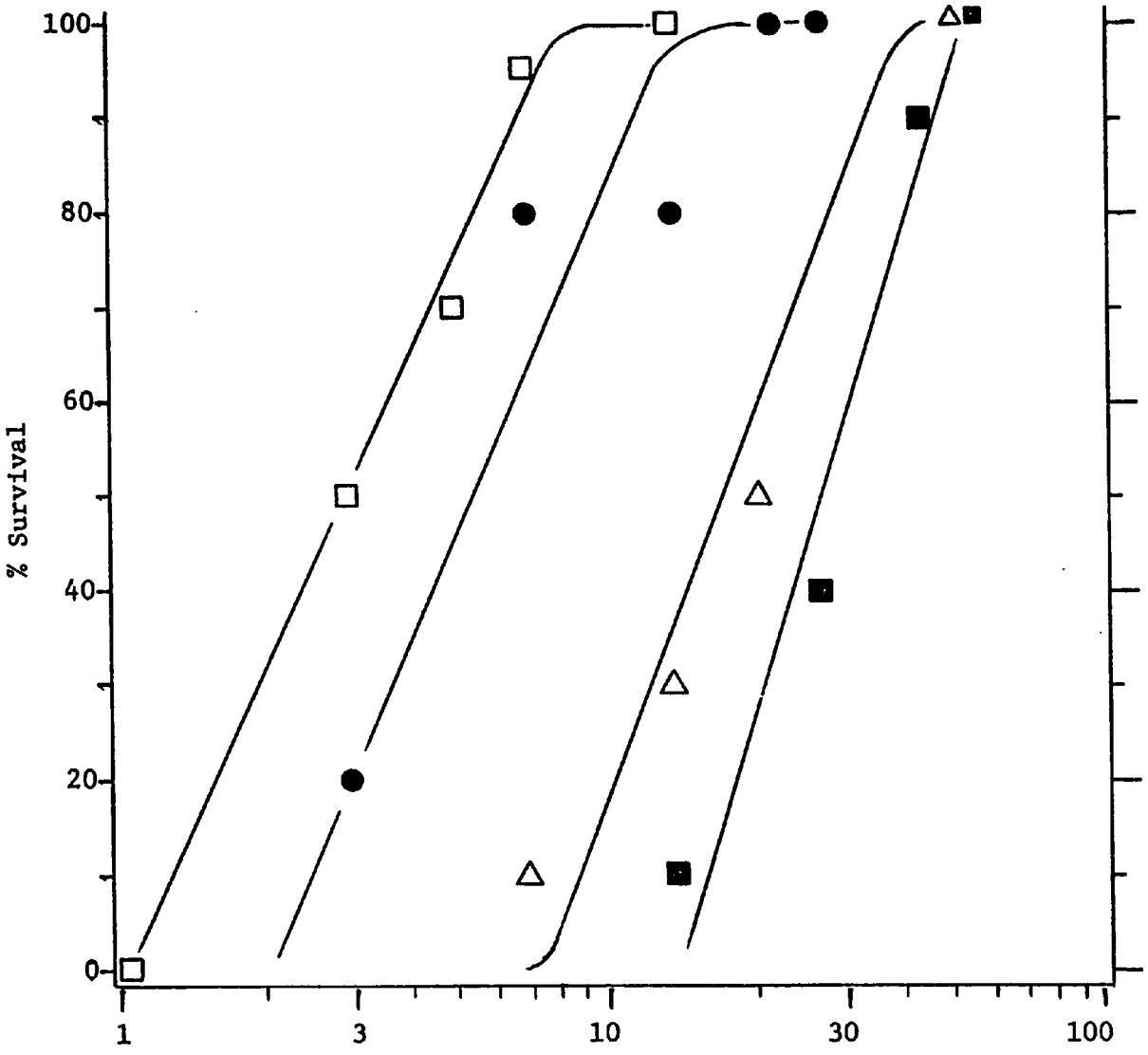


Figure 7. RECOVERY OF FIRST-SET IMMUNE REACTION FOLLOWING TREATMENT WITH IMMUNOSUPPRESSIVE AGENTS. Groups of 10 mice each were given either 600 mg PRD/kg (□—□), 75 mg CY/kg (●—●), 300 mg CY/kg (△—△), or 625 r XR (■—■) at different time intervals before the injection of 10^7 formalinized I_b cells.

TABLE 2

SUPPRESSION OF SECOND-SET IMMUNE REACTION TO VIABLE LINE I_b CELLS

Agent	Dose	Fraction Surviving[a]		
		Agent Before	Agent After	No Agent
X-Ray	625 r	3/11	11/11	9/9
CY	300 mg/kg	2/9	10/10	10/10
PRD	600 mg/kg	1/10	12/12	10/10
MTX	30 mg/kg	10/10	10/10	20/20

[a] Immunized mice received ca an LD10 dose of the indicated agent either 24 hr before or 24 hr after the injection of 10^7 viable I_b cells. All deaths were due to leukemia. Control groups of non-immunized mice succumbed to leukemia.

formly survived the injection of viable I_b cells. Most of the immune mice treated with CY, PRD, or XR 24 hr before they received injections of viable I_b cells did not survive. Thus these agents also suppressed the second-set immune response. MTX was unique in that all the mice given MTX survived challenge with the viable I_b cells. However, when the agents were given 24 hr after injection of I_b cells there were no deaths. The uniform survival of these mice may have been due to the cytotoxicity of the agents for I_b cells and/or resistance of the second-set immune response to suppression after it had been initiated.

The following experiment was performed to determine whether the second-set immune reaction became resistant to XR after mice were injected with viable I_b cells. Two groups of immune mice received injections of 10^7 viable I_b cells. 24 hr later one group received 625r XR. The next day both groups received an additional injection of 10^7 viable I_b cells. If XR were immunosuppressive under these conditions, the XR-treated mice would succumb to the second I_b cell injection. As shown in Table 3 both of these groups survived the double I_b cell injection. However, control immune mice given XR before I_b cell injection did not survive. In the previous experiment (p. 27) it was found that the first-set immune reaction to I_b cells progressively became resistant to immunosuppression once it was initiated. The present results indicate that the second-set immune reaction likewise became resistant to immunosuppression once the immune response was underway.

B. Dose of Agent and Suppression of Second-Set Immune Reaction

To quantitatively determine the relationship between the

TABLE 3

RESISTANCE TO X-RAY INDUCED IMMUNOSUPPRESSION

AFTER INJECTION OF VIABLE LINE I_b CELLS

Group	Treatment of Immune Mice[a]			Fraction[b] Surviving
	Day 0 I_b Cells	Day 1 X-Ray	Day 2 I_b Cells	
1	+	+	+	10/10
2	+	-	+	10/10
3	-	+	+	0/10
4	+	+	-	10/10

[a] Immune mice were given 10^7 viable I_b cells and/or 625 r X-Ray on the days indicated.

[b] All deaths due to leukemia. Control groups of non-immunized mice succumbed to leukemia.

dose of test agent and the degree of suppression of the second-set immune reaction, groups of immunized C58 mice received graded doses of drug or x-ray 24 hr before the injection of 10^7 viable I_b cells. The median immunosuppressive dose of each agent (dose of agent required to suppress 50% of the mice) was determined from the mortality data (Figure 8), viz., 350 r XR, 200 mg CY/kg, 550 mg PRD/kg, and > 30 mg MTX/kg. Similarly, the relative immunosuppressive efficacy of the agents can be expressed by the median immunosuppressive dose as fractions of LD10, viz., XR 0.45, CY 0.70, PRD 0.90, and MTX > 1.00.

C. Titration of Immunity Remaining After Immunosuppression

These experiments were done to quantitate the immunity remaining in immunized mice after they had been treated with either CY, PRD, or XR. Thus, immunized mice received approximately an LD10 dose of agent 24 hr before they received injections of graded numbers of viable I_b cells. Figure 9 shows that the mortality from leukemia decreased with decreasing I_b cell dose. Therefore, after immunosuppression there was residual immunity that could be detected provided that the mice were challenged with appropriate levels of viable I_b cells. The median lethal dose of I_b cells (LD50) in the immunosuppressed immune mice was a quantitative measure of the residual immunity and also a measure of the relative immunosuppressive activity of the agents. The LD50 doses of viable I_b cells estimated from the data were CY 10^3, XR 10^5, PRD $10^{6.5}$, and MTX > 10^7.

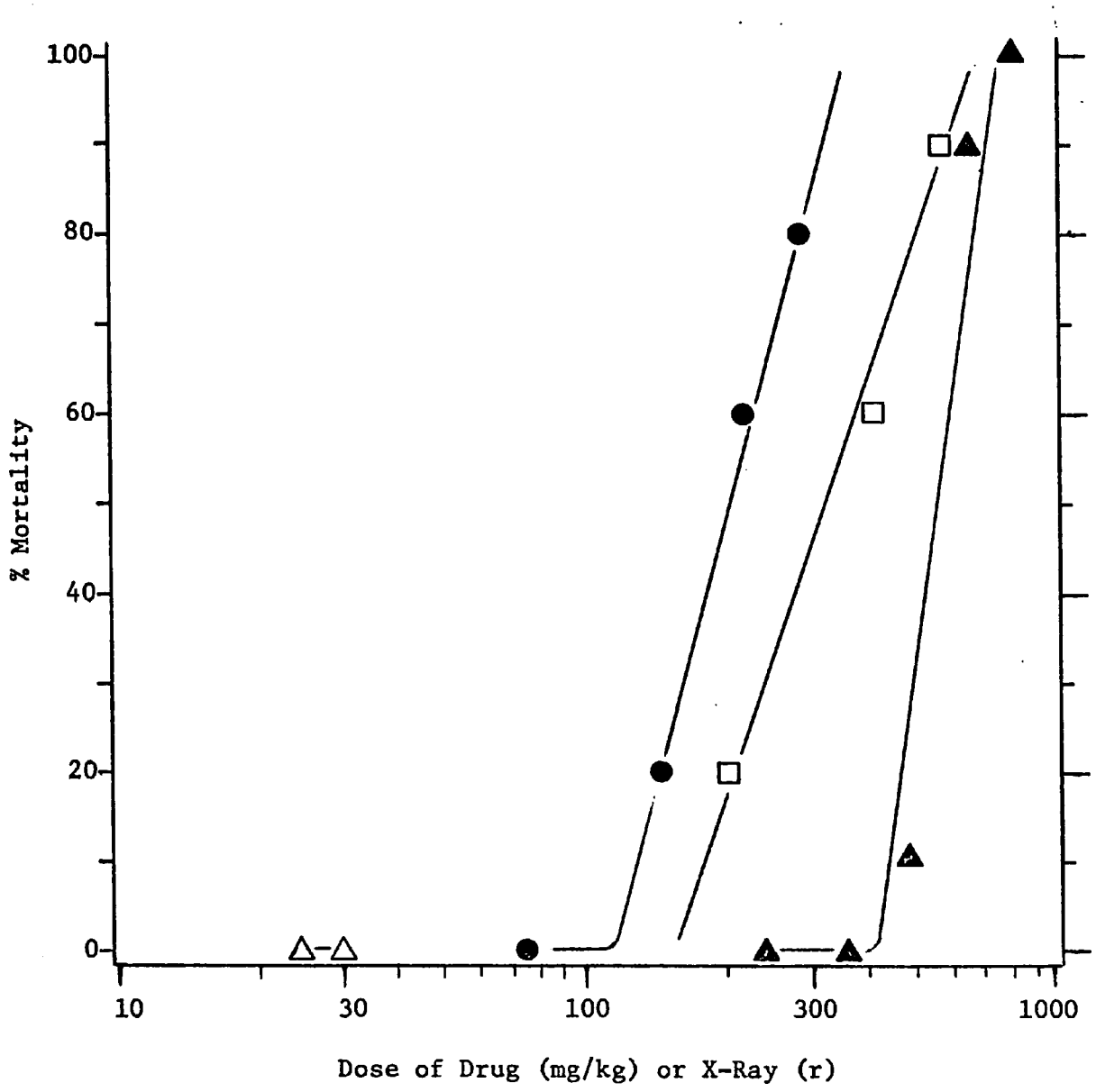


Figure 8. DOSE EFFECTS OF IMMUNOSUPPRESSIVE AGENTS ON THE SECOND-SET IMMUNE REACTION. Groups of 10 mice each were given graded doses of either MTX (△—△), CY (●—●), XR (□—□), or PRD (▲-▲) 24 hr before the injection of 10^7 viable I_b cells.

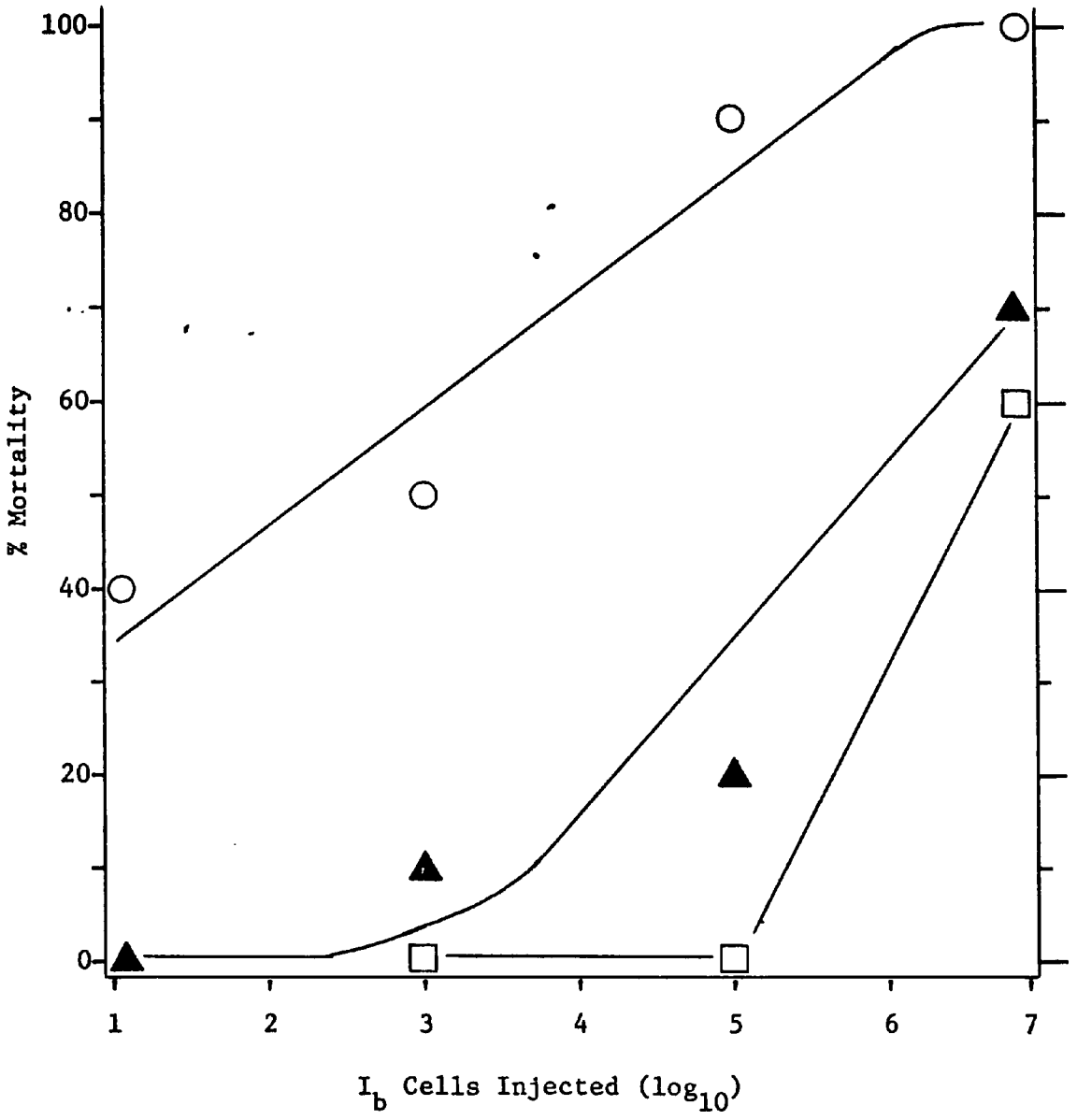


Figure 9. DOSE-RESPONSE OF IMMUNIZED MICE TO LINE I_b CELLS AFTER IMMUNOSUPPRESSION. Groups of 10 immunized mice each were given either 300 mg CY/kg (○—○), 625 r XR (▲—▲), or 600 mg PRD/kg (□—□) 24 hr before the injection of graded numbers of viable I_b cells. Untreated control immune mice survived all challenge doses.

IV. Effects of Drugs and X-Ray on Immunocompetent Cells: Reduction Of Immunocyte Number and Competence

This section presents data on the effects of CY, PRD, and XR on the number of immunocytes per spleen and on the capacity of immunocytes to protect normal mice against I_b cells. The word immunocyte is used in the following context: differential counts showed that 90% of the cells in the spleen were of lymphoid morphology; 5% had the morphologic properties of macrophages. These cells were used for assays of adoptive immunity (see below).

To determine the effects of these immunosuppressive agents on the number of cells per spleen, separate groups of C58 mice, immunized two weeks previously, were given ca an LD10 dose of each agent. One day later their spleen cells were harvested, pooled, and total and viable counts were determined. Table 4 shows that the immunosuppressive agents reduced the number of cells per spleen, viz., XR 75%, CY 74%, and PRD 40%. The reduction in immunocyte number caused by these agents correlated with their relative immunosuppressive effects. Both the total and viable cell number was decreased by XR.

To determine the effects of CY, PRD, and XR on the immunocompetence of immune spleen cells, a comparison was made of the ability of cells from treated and control immune mice to protect recipient mice against I_b cells. Graded numbers of viable spleen cells from immunosuppressed and control immune mice were injected i.p. into groups of normal recipient mice. 24 hr later they were injected with 10^3 viable I_b cells. Figure 10 gives the fraction of recipient mice that survived I_b cells when given the various doses of spleen cells from immunosuppressed and control immune mice. The median protective doses estimated

TABLE 4

REDUCTION OF SPLEEN CELL NUMBER IN DRUG TREATED MICE

Number of Donor Mice	Agent[a]	Dose	Cells/Spleen	% Viability	% Spleen Cell Yield
20	X-Ray	625 r	3.39×10^7	67	25
20	CY	300 mg/kg	3.51×10^7	87	26
10	PRD	600 mg/kg	8.16×10^7	89	60
5	None	-----	13.52×10^7	81	100

[a] Immunized mice were given ca an LD10 dose of the indicated agent 24 hr before spleen cell harvest. Spleens were pooled before making cell counts.

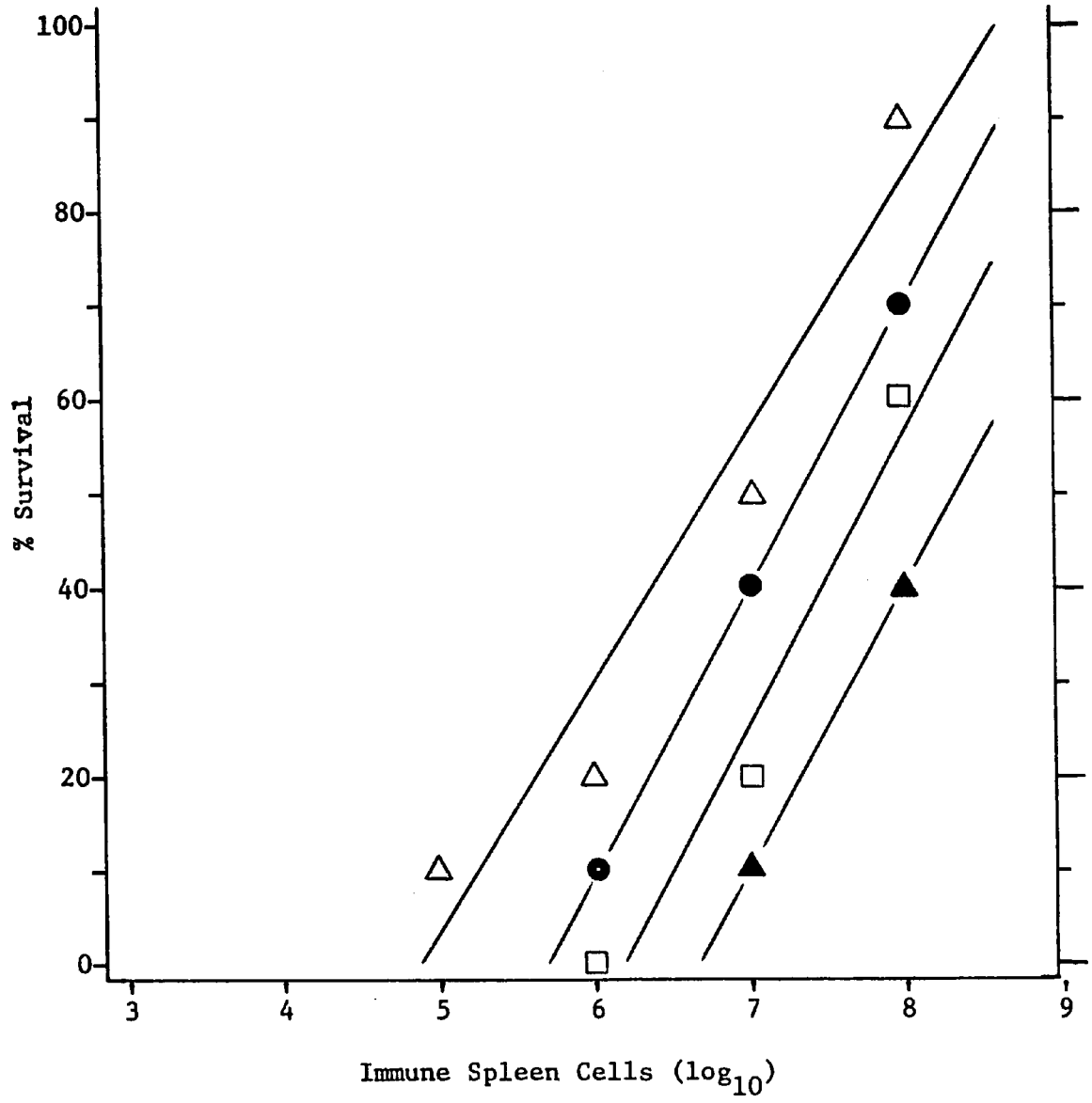


Figure 10. LOSS IN PROTECTIVE CAPACITY OF SPLEEN CELLS FROM DRUG TREATED MICE. Groups of 10 normal mice each were given graded numbers of viable immune spleen cells 24 hr before the injection of 10^3 viable I_b cells. Spleen cell donors were untreated (△—△) or given either 600 mg PRD/kg (●—●), 625 r XR (□—□), or 300 mg CY/kg (▲—▲) 24 hr before spleen cell transfer.

for the cells from the various cell donors were: CY $10^{8.5}$, XR $10^{7.7}$, PRD $10^{7.4}$, and nontreated $10^{6.7}$. Thus the effects of these agents on both immunocyte number and individual cellular immunocompetence correlated with their relative immunosuppressive activity as determined by bioassay in mice.

DISCUSSION

A first-set immune reaction occurs when C58 mice receive an injection of 10^7 formalinized I_b cells containing residual viable I_b cells (76). In the present study, it was determined by bioassay that the viable I_b cells in the formalinized preparation (ca. 10^3) proliferated in the mice until they were immunologically rejected 6 days after injection. Similarly, Lin and Murphy (77) showed that C58 mice inoculated with 10^3 viable I_b cells survived providing they were given additional antigenic stimulation in the form of membrane fractions of I_b cells. Also under these conditions, viable I_b cells proliferated until rejected.

Lin et al. (76) found that C58 mice which had survived an inoculum of formalinized I_b cells withstood a subsequent injection of up to 10^7 viable I_b cells. Lin (75) also showed that when the viable I_b cells were injected into these immunized mice, I_b cells proliferated until they were immunologically rejected. The time when rejection occurred was dependent upon the number of I_b cells injected, i.e., the larger the I_b cell dose, the earlier the immune rejection. These results suggested that a relative antigenic threshold of I_b cells was reached more quickly with larger inocula and thus the second-set immune reaction was triggered sooner. Most likely, a similar immune response is induced in adoptively immunized mice injected with I_b cells.

In essence, the first- and second-set immune reactions have the same outcome, i.e., killing of I_b cells by sensitized immunocytes. Presumably, the rejection of I_b cells procedes in a manner similar to

that proposed by Gowans (47) for the homograft reaction. Thus, target cell-specific antigen interacts with specific receptor-bearing small lymphocytes and causes these lymphocytes to grow and divide, thereby increasing the number of specifically instructed lymphocytes and priming the effector mechanism of rejection. Receptor-bearing lymphocytes then destroy the target cells, possibly with the assistance of macrophages.

In the present study, the first-set immune reaction to I_b cells was indicated by the survival of normal mice injected with 10^7 formalinized I_b cells (containing ca. 10^3 viable I_b cells). The second-set immune reaction was demonstrated by survival of sensitized mice given a subsequent inoculum of 10^7 viable I_b cells.

When a maximum tolerated dose (LD10) of either CY, PRD, or XR was given 24 hr before the I_b cell inoculum, the ability of C58 mice to mount either a first- or a second-set immune response to I_b cells was suppressed. As a result, the mice died of transplanted leukemia. An LD10 dose of MTX detectably suppressed only the first-set immune reaction. As shown by bioassay, the viable I_b cells in the formalinized I_b cell preparation proliferated in the CY-treated mice at the same rate as in the normal mice (3.32 divisions/day) but the immunosuppressed mice were unable to reject the proliferating cells. The observation that CY did not interfere with the growth of I_b cells in vivo when given before the inoculum was somewhat expected since the in vivo half-life of CY (4 hr) is such that it would have been essentially cleared by the time the I_b cells were injected; i.e., there would be only 1.5% of the original dose remaining at the end of 24 hr. The same is true of MTX with its' half-life of 3.6 hr (57).. PRD remains at biologically significantly levels in the blood for at least 24 hr after a single parenteral dose

(18). This is in part due to specific and non-specific binding to plasma proteins (46).

Since CY, PRD, and XR are very potent immunosuppresants when given before antigenic stimulation (84 ,93 ,139), it was expected that they might suppress the immune response of C58 mice to syngeneic I_b cells when given 24 hr before the inoculum. Moreover, Werder et al. (148) were able to reduce the strong first-set allograft reaction against 500 I_b cells in BALB, A, STOLI, and CWF-Swiss strain mice by giving them cortisone (multiple-dose regimen) or XR (400 r). However, they were not able to suppress the second-set allograft reaction to 500 I_b cells with these agents, singly or in combination. In the present study, 60% of the sensitized C58 mice were immunosuppressed by an XR dose of 400 r. The difference in these results suggests that the stronger allograft response may be more resistant to immunosuppression. Possibly there are more immunocyte clones responding in the allogeneic system than in the syngeneic system. As a result, a greater number of reactive immunocytes may remain in the allogeneic system after immunosuppression.

The first-set immune reaction of C58 mice was also suppressed when an LD10 of any of the agents was given 24 hr after the injection of formalinized I_b cells. However, the LD10 of CY killed all of the viable I_b cells in vivo. The effectiveness of CY as an antileukemic agent is well documented. Skipper et al. (130) estimated that a 200 mg/kg dose killed approximately 99.9988% of L1210 leukemic cells in vivo. Still, the immunosuppressive effects of CY could be demonstrated when given after the formalinized I_b cells if a smaller (1/6 LD10) dose was employed. In addition, it was found that the later CY

was given (1 to 7 days) after the injection of formalinized I_b cells, the smaller its immunosuppressive effect. The increase in resistance to immunosuppression is probably due to the expansion in specific immunocyte number and differentiation of the effector cells following antigenic stimulation. For example, antibody precursor cells are inactivated by as little as 25 r, while the antibody-synthesizing and -secreting capacities of the more numerous mature plasma cells cannot be altered by very high doses of ionizing radiation (83 ,114,147). After exposure to 10,000 r of XR, they survive for 4 days or more and have an undiminished rate of antibody synthesis and secretion. Thus, the most difficult phase of the antibody response to suppress is that involving the terminal antibody-synthesizing cells.

The tremendous resilience of the plasma cell emphasizes the stability of the peptide-synthesizing polyribosome system, which includes the messenger RNA, as well as the enzymes required for the synthesis and secretion of the antibody. Perhaps the production of the mediator of cell-mediated immunity (25) is similarly resistant to immunosuppressive agents.

Immunized mice were not detectably immunosuppressed by any of the agents employed when given 24 hr after the injection of viable I_b cells. Thus, the second-set immune reaction also was resistant to immunosuppression once the immune response was underway. Presumably the more rapid development of resistance to immunosuppression in immunized mice following antigenic stimulation reflects the greater numbers of specifically instructed lymphocytes participating in the second-set immune reaction. The explanation that the survival of these mice was due to the toxicity of the agents for I_b cells is not adequate since they

survived re-inoculation with viable I_b cells. However, since the immune response becomes resistant to immunosuppression when it is well underway, chemotherapeutic agents given at this time would probably work in concert with the immune response against the leukemic cells. In fact, Mihich (94) found that more than 60% of DBA/2 mice survived more than 50 days as a result of drug treatment given after the inoculation of syngeneic L1210 leukemic cells. The increased survival of the mice apparently was due to the combined effects of chemotherapy and immunity against the leukemic cells, since the cured mice were immune to L1210 cells. Furthermore, the therapeutic effect was reduced in pre-irradiated hosts. Similar data were obtained in studies with methylcholanthrene-induced sarcomas in syngeneic mice (97, 112). Therefore, it is important to have an idea of how chemotherapeutic agents and immunostimulants interact to be able to capitalize on the therapeutic effects of each and avoid interference.

Because the agents employed in the current studies killed leukemic cells it was not possible to determine the optimum time for immunosuppression in relationship to I_b cell injection. Possibly, the immunosuppressive effects of the agents given before or after the inoculum could be compared if one used killed I_b cells as the primary stimulus and assayed the immunity engendered when the drugs have been cleared. The present experiments also do not reveal whether immunological tolerance was induced or whether the agents produced an adjuvant effect. However, assays for immunocompetence are presented which could be employed for detecting such phenomena.

Several sets of experiments were designed to determine the relative immunosuppressive activity of the chemotherapeutic agents. For

clarity, the experiments were set up so that the drugs were given before the I_b cell inoculum. Thus, the immunosuppressive effects of the agents were quantified in terms of the median immunosuppressive dose, the median recovery time from an LD10 dose, the median lethal dose of I_b cells, and the median protective dose of immune spleen cells.

The median immunosuppressive dose (fraction of LD10 required to immunosuppress 50% of the mice) revealed the relative immunosuppressive activity of the drugs. The same relative order of effectiveness was obtained in the first- and second-set immune reactions, viz., CY > PRD > MTX. But the median immunosuppressive doses of the drugs were much larger for the second-set immune response. The increased resistance of immunized mice to immunosuppression could be explained in terms of an increased number of reactive immunocytes in the sensitized animals. For example, in antibody systems it has been shown that there can be a 10 to 100-fold increase in the number of specific precursor cells in the spleen upon immunization with an antigen (3 , 82 ,106 ,124). Presumably, a similar increase in cell number occurs in cell-mediated immunity. As a consequence, a larger number of immunocytes would remain in sensitized mice after immunosuppression.

It is also possible that the immunocytes in immunized and normal mice may have different sensitivities to the immunosuppressive agents. However, sensitized immunocytes, capable of mediating allograft rejection, do not appear quantitatively different from nonsensitized immunocytes (47). Furthermore, in antibody systems the precursor cells from sensitized mice are as radiosensitive as those from nonsensitized mice (82), although the immune response of sensitized animals is more resistant than that of nonsensitized animals (64 ,80 ,82 ,106). In the

present study, an assay for cell-mediated immunocompetence was available for lymphoid cells from immunized mice, but not for lymphoid cells from nonimmunized animals. Therefore, the sensitivity of these two cell classes to the various agents was not compared.

The median recovery time for immunocompetence was determined for the first-set immune reaction of C58 mice given an LD10 dose of either CY, PRD, or XR. Mice treated with XR or CY had a much longer median recovery time than those given PRD. This probably reflects the more severe effect of XR (26, 93, 100, 101) and alkylating agents,such as CY (56, 78, 128), on the hematolymphopoietic tissue. Thus it took longer to regenerate the immunocytes reactive to I_b cells when XR or CY was given. The duration of XR-induced immunosuppression was the longest, probably because it has the more lasting effect on lymphoid tissue (93). On the other hand, adrenocortical steroids are known to have relatively little toxicity for bone marrow cells (56). Therefore, the immunocytes took less time for regeneration. It also was noted that the median recovery time after a 1/6 LD10 dose of CY was much less than that for the LD10 dose. Furthermore, repopulation of the lymphoid organs takes longer with higher doses of immunosuppressants (93). These results indicate that the immunological recovery time was dependent on the number of mature immunocytes and stem cells destroyed.

The median lethal dose of I_b cells was determined for immunized C58 mice given an LD10 dose of either CY, PRD, or XR. This LD50 reflects the residual immunity in the immunosuppressed mice and it thus served as an indicator of the relative immunosuppressive activity of the agents. The relative effectiveness of the agents was CY > XR > PRD, respectively. CY also decreases antibody production to a greater extent

than equitoxic doses of XR (118). A similar assay for immunosuppression could probably be developed for the first-set immune reaction to I_b cells. Graded doses of viable I_b cells could be given to mice receiving additional antigenic stimulation (in the form of killed I_b cells or membrane fractions of I_b cells) and the immunocompetence of the mice measured in terms of the LD50 of viable I_b cells. Thus, the residual immunocompetence of immunosuppressed mice could be titrated.

CY, PRD, and XR decreased the number of cells in the spleen of immunized C58 mice. This indicates that the immunosuppression was at least partly due to the reduction in immunocyte number. Also, greater numbers of spleen cells from immunosuppressed donor mice were required to protect 50% of recipient mice against a challenge of 10^3 I_b cells. Therefore, the immunosuppressive effects of the agents were also partly due to the impairment of immunocyte functions. Such damaged cells may die, remain inactivated, or repair the damage and regain their functional capacity. It was also observed that the reduction in spleen cell number and competence correlated well with the relative immunosuppressive activity of the agents. Thus, this experimental approach might be utilized to analyze the effects of chemotherapeutic agents on subpopulations of immunocytes, e.g., thymus-derived lymphocytes and bone marrow-derived lymphocytes. Information concerning the effects of chemotherapeutic agents and immunostimulants on subpopulations of immunocytes is important for designing chemo-immunotherapeutic approaches to the treatment of leukemia. For example, application of the appropriate adjuvants may restore the immunocompetence of patients after they have received chemotherapy.

The immunosuppressive agents used in these studies are toxic

for lymphoid cells but are relatively nontoxic for macrophages (169,162). Some agents have a differential effect on subpopulations of lymphoid cells. For example, a given dose of cortical steroid kills a greater proportion of immature thymocytes (166) and bone marrow-derived lymphocytes (158) than mature thymus-derived (helper) lymphocytes. After activation of mature B and T cells with antigen, their functions become resistant to immunosuppressive agents. The resistance of antibody production by plasma cells to drugs and XR was described above. The resistance of "helper activity" of primed mature T cells to XR has been clearly established (167,165). Similarly, there have been several demonstrations of functionsl radioresistance of T cells participating in cell-mediated immune reactions, e.g., in vitro killing of target cells (168) and in vivo delayed hypersensitivity (161). Also, activated T cells responsible for GVH reactivity are resistant to cortisone (159).

Since cell-mediated immune reactions are predominantly a function of T cells (160), one might expect that suppression of cellular immunity to I_b leukemia reflects killing of immature T cells and quiescent mature T cells; the immature cells being the most sensitive. However, other cell-mediated reactions such as skin graft rejection (163), delayed hypersensitivity (170), and GVH (164) require bone marrow-derived cells in addition to T cells. Therefore, the immunosuppressive effects are likely to be due to killing of both B and T cells. Possibly the effect on the B cells contributes most to the immunosuppression, since the agents employed appear to have a greater effect on these cells (160,137).

The in vivo methods presented in this study for quantifying immunity to I_b leukemia can be used in conjunction with in vitro assays to determine which in vitro phenomena correlate best with the in vivo

situation. Such information would be invaluable to the physician desiring to monitor the immunological status of leukemic patients receiving chemotherapy (157). These *in vivo* methods also can be used to determine the best combinations of chemotherapy and immunotherapy. As was shown in these studies, the relative time of administration of chemotherapeutic agents and immunostimulants is a very important consideration in developing combination therapy, i.e., chemotherapeutic (immunosuppressive) agents should be administered at the resistant phase of the immune response so that they do not interfere with immunotherapy. By using this approach, it is even possible to get the immune response and chemotherapy to work in concert.

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