Differential Effects of Immunosuppressants on Lymphocyte Function

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ABSTRACT In vitro and in vivo parameters of T lymphocyte function were evaluated in guinea pigs following treatment with the "cycle-active" drugs, 6-mercaptopurine (6MP) and methotrexate, and the "noncycle-active" alkylating agent, cyclophosphamide. Commencing at the time of sensitization to tuberculin protein, animals were treated with an 8 day course of one of the cytotoxic drugs. This regimen either reduced or abolished the cutaneous response to PPD. The two cycle-active drugs inhibited the in vitro lymphoproliferative response to PPD and suppressed the elaboration of migration inhibition factor (MIF) by lymph node cells. However, these agents did not reduce blood lymphocytes, deplete the cellularity of the thymic dependent areas of peripheral tissues, or alter the in vitro response of lymph node cells to the nonspecific mitogen PHA. In contrast, treatment with cyclophosphamide was associated with a reduction in peripheral blood and tissue lymphocytes and impaired responses to PHA by residual lymph node cells. In vitro proliferative responses to PPD were inhibited but the capacity of lymph node cells to elaborate MIF was not suppressed. In addition to their effects on antigen-reactive lymphocytes, all three drugs significantly reduced the number of macrophages in induced peritoneal exudates. With respect to immunosuppressive activities, results of these investigations suggest that the noncycle-active agents affect both intermitotic and dividing T lymphocytes without impairing certain intermitotic functions of residual cells. The cycle-active drugs have a more restricted toxicity limited to those T lymphocytes which have been stimulated to undergo active DNA synthesis by antigenic challenge.

INTRODUCTION

The effective use of immunosuppressive agents should involve considerations of their pharmacologic activities in relation to the generative cycle characteristics of the immune effector cells. These drugs were originally introduced as anti-neoplastic agents; accordingly, those factors which pertain to the inhibition of tumor cell replication may apply to immunologically competent lymphocytes. Based on their cycle specificities, cytotoxins are subdivided into two classes: those primarily active against cells in the S (DNA synthetic) or M (mitotic) phase of their cycle (cycle-active) and those which are toxic for both proliferating and nonproliferating (G_0) cells (noncycle-active) (1, 2). Of the most commonly used immunosuppressive agents, 6-mercaptopurine (6MP)[1] and methotrexate are classified as cycle-active drugs while the alkylating agent cyclophosphamide is categorized as a noncycle-active agent.

In the present investigations, the cell specificities of these three immunosuppressants were related to a defined immunological response, the development of a delayed hypersensitivity reaction to tuberculin protein in guinea pigs. This cell-mediated immune response depends on the functional activity of a specific subpopulation of lymphocytes, the thymic dependent (T) cells. Previous studies have established the kinetic characteristics of this population of lymphoid cells; they are long-lived, slowly-replicating elements which recirculate between the blood and the thymic dependent areas of peripheral lymphatic tissues. Following appropriate immunologic stimulation, antigen-committed T cells transform into actively replicating elements (3). Concomitantly, these responsive lymphocytes elaborate a spectrum of soluble mediators which are believed to expand the number and types of cells responding to the antigenic challenge (4).

In addition to defined in vivo characteristics, the activities of sensitized T lymphocytes can be monitored by

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[1] *Abbreviations used in this paper:* [^{3}H]Tdr, tritiated thymidine; MIF, migration inhibition factor; 6MP, 6-mercaptopurine; PHA, phytohemagglutinin; T cells, thymic dependent cells.

TABLE I

Effects of Immunosuppressants on Cutaneous Response to PPD

	No. of animals initially sensitized	No. surviving treatment	Diameter of induration (mm)		
			<5	6–15	>15
Controls	45	43	0	0	43
6-Mercaptopurine					
12 mg/kg per day	6	6	0	1	5
16 mg/kg per day	6	5	1	1	3
20 mg/kg per day	25	21	2	18	0
24 mg/kg per day	6	2	1	1	0
Methotrexate					
12.5 mg/kg per day	32	30	28	2	0
12.5 mg/kg per every other day	5	5	2	2	1
Cyclophosphamide					
20 mg/kg per day	60	55	53	2	0
10 mg/kg per day	6	6	1	5	0

several in vitro assays. Current concepts suggest that the cellular responses in culture may simulate those of committed T lymphocytes following antigenic challenge in the intact animal (5). In the present studies, both in vitro assays and in vivo correlates were related to the immunosuppressive activity of the three aforementioned drugs, 6MP, methotrexate, and cyclophosphamide. Results indicate that the kinetic activites of lymphoid cells and the cycle specificities of immunosuppressants represent important considerations in developing programs of effective immunosuppressive therapy.

METHODS

Male, Hartley-strain guinea pigs were sensitized to tuberculin protein by the injection of 1 ml complete Freund's adjuvant containing H-37Ra mycobacteria. Starting on the day of immunization, animals received either cyclophosphamide (20 mg/kg per day), methotrexate (12.5 mg/kg per day) or 6MP (20 mg/kg per day). The latter was prepared by dissolving the powdered drug in 1.0 N NaOH followed by partial neutralization with a HCl-glycerine buffer. Details of this procedure have been reported previously (6). Drug therapy was continued for 8 days following sensitization.

On day 7, all animals were skin tested with second strength, purified protein derivative (PPD). The cutaneous responses were recorded on the basis of the size of the induration 24 h after challenge; a reaction of 5 mm or less in diameter was considered negative. In all experiments, sensitized controls were simultaneously evaluated. These animals received either sterile saline or buffer used to prepare the 6MP solution. No significant differences were noted in any tests between the groups receiving saline and those treated with buffer, and, as such, data from all controls were pooled. In additional studies, other doses of each agent were tested for their activity in suppressing delayed hypersensitivity responses in vivo. These are listed on Table I. The dose of each agent used in in vitro studies was selected to provide maximum inhibition of the skin tests without causing excessive mortality.

Following interpretation of the cutaneous reactions to PPD, animals were killed by ether asphyxia. Cardiac blood was obtained for a total leukocyte count and a 200 cell differential; these values were used to determine the number of circulating lymphocytes. In representative animals, the spleen and a sample of lymph nodes were removed, fixed in 10% formalin, stained with hematoxylin and eosin, and examined histologically.

To evaluate cell-mediated immunity by in vitro assays, cervical and mesenteric lymph nodes were aseptically removed and minced to make a single cell suspension. These cells were then used in three measures of T cell function, the proliferative responses to the nonspecific mitogen, phytohemagglutinin (PHA), and to the specific antigen used to initially sensitize the test animals, PPD, and their ability to elaborate migration inhibition factor (MIF). In each experiment, groups of 9–27 animals were evaluated.

The response to PHA and PPD was measured by culturing 10×10^6 lymph node cells in 5 ml media RPMI 1640 containing 20% fetal calf serum and supplemented with glutamine (300 μg/ml), bicarbonate (250 μg/ml), penicillin (100 U/ml), and streptomycin (100 μg/ml). For each determination, at least three replicate cultures were incubated with an optimal concentration of PHA (Burroughs Wellcome & Co., Tuckahoe, N. Y.); two or three unstimulated cultures were established as controls.

To determine the response to this nonspecific mitogen, cultures were incubated for 2 days in a 5% CO_2 atmosphere at 37°C. 2 μCi of tritiated thymidine ([^{3}H]Tdr) (New England Nuclear Corp., Boston, Mass.; spec act 6.7 Ci/mM) was added 4 h prior to the termination of the incubation period. Cultures were harvested by washing the cell buttons twice in phosphate buffered saline (pH 7.4) followed by three washes with cold 5% trichloroacetic acid. The residual insoluble material was fixed in methanol, dissolved in Soluene (Packard Instrument Co.), and added to Liquifluor-toluene scintillation fluid (Liquifluor, New England Nuclear Corp.). Incorporated radioactivity was determined by counting each sample for 5 min in a Packard Tricarb Liquid Scintillation Counter (Packard Instrument Co., Downers Grove, Ill.). Results of each culture were corrected for background and expressed as counts per minute per million cells (cpm/10^6 cells). Isotope uptake for replicate cultures were averaged and the degree of stimulation determined by subtracting the mean uptake in unstimulated replicates from the average value in those containing the mitogen. In general, the uptake in replicate cultures did not vary by more than 20% from the mean.

Proliferative responses to PPD were measured using a similar culture system. In these determinations, an optimal concentration of tuberculin protein (4 μg PPD/ml) was employed, and the incubation period was extended to 5 days. Results of antigen stimulated cultures were expressed as a stimulation ratio which was determined by dividing the average isotope uptake in stimulated cultures by that determined in unstimulated replicates. No PPD positive control showed less than a twofold increase in [^{3}H]Tdr incorporation in the stimulated cultures.

Inhibition of macrophage migration was determined by techniques previously described (7). Exudate cells were obtained from unsensitized control animals by the intraperitoneal injection of 30 ml light mineral oil 4 days prior to sacrifice. Cells were harvested by washing the peritoneal cavities with 120 ml Hanks' balanced salt solution contain-

ing heparin (1 ml of a 1% solution). The exudate cells were centrifuged, washed several times in HBSS to remove traces of oil, enumerated, and resuspended in minimal essential media with 15% guinea pig serum. Lymph node cells from test animals were added in a fixed ratio to the unsensitized peritoneal exudate cells. As in previous studies (7), a ratio of 1 lymph node cell to 40 peritoneal exudate cells proved optimal; lesser concentrations of lymph node cells did not produce significant inhibition and a greater concentration yielded only a slight decrease in migration. Capillary tubes were filled with this mixture of lymph node cells and peritoneal exudate cells; these tubes were centrifuged, cut at the cell-supernate interphase, and the cellular portions attached to the cover glass of a Sykes-Moore culture chamber. Each capillary tube contained 10 $\times 10^6$ peritoneal exudate cells and 2.5×10^5 lymph node cells. Individual chambers were filled with MEM containing 15% guinea pig serum, penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin (0.25 μg/ml), glutamine (300 μg/ml), bicarbonate (250 μg/ml), and PPD (10 μg/ml). The chambers were incubated at 37°C and the migration measured at 24 h by planimetry. For each test animal, at least four replicate capillary tubes were evaluated; the area of migration usually varied by less than 10% from the mean. Inhibition was determined by the following formula: % migration = (area of migration in tubes containing lymph node cells from test animals)/(area of migration in tubes containing lymph node cells from unsensitized controls) $\times$ 100. Lymph node cells from all PPD responsive control animals reduced migration at least 20% when compared with similar cultures containing an equal number of lymphocytes from unsensitized animals.

The effects of drug therapy on inflammatory cells were evaluated by assessing the total number of cells in induced peritoneal exudates four days after the intraperitoneal injection of 30 ml light mineral oil. Recoverable exudate cells were enumerated and a 300 cell differential performed on Wright-Giemsa stained smears. The course of drug therapy was identical to that used in the immunosuppressive studies; each animal received an 8 day course of a drug terminating 1 day prior to sacrifice. In order to avoid nonspecific exudation of cells by concomitantly injecting the immunosuppressive agent into the peritoneal cavity, all drugs were administered by the subcutaneous route.

RESULTS

As an initial evaluation of the immunosuppressive activity of each cytotoxic agent, animals were tested for cutaneous responses to PPD. All controls showed strongly positive tuberculin reaction 1 wk after immunization; the induration of individual tests measured from 15 to 35 mm in diameter (Table I). A course of either cyclophosphamide or methotrexate, initiated at the time of immunization, effectively inhibited cutaneous responses. Only 2 of the 55 cyclophosphamide-treated recipients showed induration greater than 5 mm in diameter; most showed no response. Similarly 28 of the 30 animals receiving methotrexate were rendered anergic; the other two showed minimally positive tests (7 and 10 mm, respectively). Treatment with 6MP reduced but did not abolish skin reactivity. In all but one animal, the responses were less than 12 mm in diameter.

TABLE II

Effects of Immunosuppressants on Lymphocytes

	No. of animals	Lymphocytes	Cellularity of thymic dependent areas*
		cells/mm³	
Controls	15	3,600±490‡	Normal
6-Mercaptopurine (20 mg/kg per day)	16	2,931±387	Normal
Methotrexate (12.5 mg/kg per day)	16	2,830±290	Normal
Cyclophosphamide (20 mg/kg per day)	16	1,200±130§	Decreased

* Based on histologic criteria.
‡ Mean±SE.
§ Significantly less than controls ($P < 0.001$).

With each agent, other dose schedules were evaluated. Treatment with both cyclophosphamide (10 mg/kg per day) and methotrexate (12.5 mg/kg per every other day) resulted in a reduction in the size of the cutaneous response compared with controls but did not completely inhibit reactivity. 6MP was tested in four dose schedules (12, 16, 20, and 24 mg/kg per day). The lower two doses did not result in significant impairment in responses; the higher dose was lethal to a majority of animals.

To assess the effects of therapy on lymphocytes, peripheral blood cells were enumerated and the cellularity of thymic dependent areas of peripheral lymphatic tissues estimated by histologic criteria (8). Neither 6MP nor methotrexate caused significant lymphopenia (Table II). Further, the thymic dependent areas of lymph nodes and spleens were of normal cellularity. As an additional measure of recirculating elements, the number of lymphocytes migrating into induced peritoneal exudates was not reduced by either of the cycle-active drugs (Table III). In contrast, cyclophosphamide therapy was associated with a significant lymphopenia ($P < 0.001$), reduction

TABLE III

Effects of Cytotoxic Drugs on Peritoneal Exudate Cells

	No. of animals	Total no. of exudate cells $\times 10^{-6}$	No. of monocytes and macrophages $\times 10^{-6}$	No. of lymphocytes $\times 10^{-6}$
Controls	16	98.8±16.1*	84.9±5.6	13.1±1.8
6-Mercaptopurine	9	45.0±11.3‡	22.9±5.8§	15.1±3.7
Methotrexate	17	56.6±8.4‡	34.0±6.4§	18.8±5.0
Cyclophosphamide	17	7.2±1.0‡	5.4±0.8§	0.9±0.2§

* Mean±SE.
‡ Significantly different from controls ($P < 0.01$).
§ Significantly different from controls ($P < 0.001$).

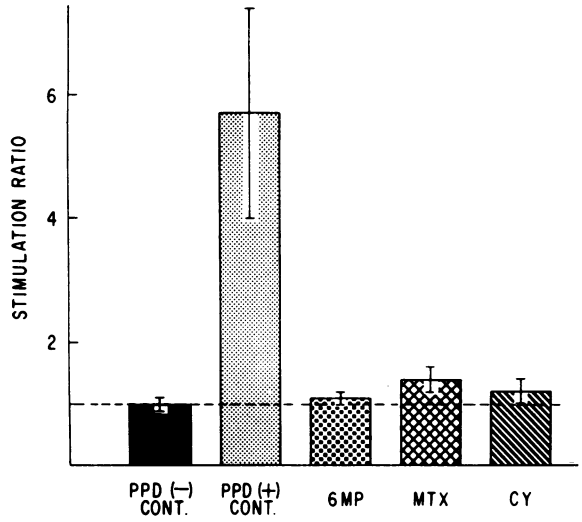

FIGURE 1 The lymphoproliferative responses to PPD in animals receiving the three cytotoxic drugs. Results, expressed as a stimulation ratio, indicate that a course of therapy with each of the agents initiated at the time of sensitization to tuberculin protein virtually abolishes antigen-induced proliferation. Each point represents the mean of 9–16 animals ±1 standard error. MTX, methotrexate; CY, cyclophosphamide.

of the cellularity of the thymic dependent areas of peripheral lymphatic tissues, and a marked reduction in the number of lymphocytes found in peritoneal exudates.

Although this study was not primarily directed at assessing alterations in the number of B cells in peripheral tissues, it is noteworthy that histological evaluation of these tissues following cyclophosphamide indicated a pronounced reduction in the cellularity of the thymic independent areas. Treatment with either 6MP or methotrexate reduced the cellularities of germinal centers but did not significantly deplete either subcapsular lymphocytes or the number of lymphoid cells in the medullary cords.

To further assess the effects of each treatment program, peritoneal exudate macrophages were quantitated following a standard i.p. challenge of mineral oil. Results (Table III) indicate that therapy with cyclophosphamide had a profound effect on the total number of macrophages localized to these induced exudates. 6MP and methotrexate also significantly reduced the number of these phagocytic cells but not to the same extent as observed with cyclophosphamide.

Lymph node cells from drug-treated animals were evaluated in three assays indicative of the status of cell-mediated immunity; the proliferative responses to PPD and PHA and the ability of lymph node cells to elaborate MIF. A clear distinction was observed in PHA responses between animals treated with cyclophosphamide and those receiving either of the cycle-active agents (Table IV). As measured by the incorporation of [^{3}H]Tdr, the lymphoproliferative response was not impaired by treatment with either 6MP or methotrexate. Compared with sensitized controls, the average isotope uptake was 78% in the former and 90% in the latter; these values are not statistically different from controls. By contrast, cyclophosphamide markedly impaired the response to PHA; the average was reduced to 16% ($P < 0.001$).

All three agents reduced the proliferative response to PPD, the specific antigen used to sensitize these animals. Results, expressed as a stimulation ratio ([^{3}H]Tdr incorporation in cultures stimulated with PPD divided by that observed in unstimulated replicates), indicate that cells from sensitized controls showed an average of 5.7±1.7. In contrast, treatment with each of the cytotoxic agents during the induction phase of this immune response virtually abolished in vitro antigen induced proliferative responses. The stimulation ratios in cultures obtained from these drug-treated animals were not significantly greater than those observed in nonimmunized controls (Fig. 1).

Lymph node cells from each of the drug-treated animals were evaluated for their ability to elaborate MIF. In this assay system, lymphocytes from test animals were mixed with peritoneal exudate cells from unsensitized donors in a fixed ratio. As in previous studies (7), using lymph node cells from sensitized controls, a ratio of 1:40 consistently inhibited migration. The average area of migration was 55±11% compared with that observed in capillary tubes containing lymph node cells from unsensitized controls. Lymphocytes from animals treated with either 6MP or methotrexate failed to significantly inhibit migration. The average value was 84±13% for the 6MP-treated donors and 98±13% for the methotrexate animals (Fig. 2). In contrast, a similar

TABLE IV
Effects of Cytoxins on Lymphoproliferative Responses to PHA

	No. of animals	PHA response (net [^{3}H]Tdr incorporation cpm/10^6 cells)*
Controls	11	1,542±285
6-Mercaptopurine	16	1,197±281
Methotrexate	16	1,391±303
Cyclophosphamide	16	243±55‡

* Response expressed as [^{3}H]Tdr incorporation in PHA stimulated cultures less that in unstimulated replicates ±1 SE.
‡ Significantly different from controls ($P < 0.001$).

number of lymph node cells from cyclophosphamide-treated animals proved highly effective in this test; migration was reduced to 45±7%—a value equivalent to that observed with lymph node cells from sensitized controls. Lymph node cells from unsensitized animals treated with a similar course of cyclophosphamide did not inhibit migration, thereby indicating that drug therapy itself did stimulate these cells to elaborate MIF.

DISCUSSION

Owing to differences in pharmacologic activities, immunosuppressive agents have variable effects on both lymphocyte kinetics (10, 11) and immune responses (12–16). In these reports, the comparative effects of drugs were defined on selective parameters of lymphocyte function. They did not compare in vitro with in vivo measures of cellular immunity, nor did they correlate results with the cell-cycle specificities of the agents. The present investigations detail and contrast the effects of three potent cytotoxic agents on both the number and functional activities of T lymphocytes in a developing cell-mediated immune response. These observations indicate that the cycle specificities (1, 2, 9) of these agents are important determinants of their immunosuppressive activities; differential effects are observed on T lymphocytes, including those specifically sensitized by antigenic challenge.

As shown in this study, 6MP and methotrexate, two cycle-active agents which are primarily effective against cells in DNA synthesis (9), have a limited toxicity. They do not significantly reduce the total number of T lymphocytes or inhibit the lymphoproliferative responses to PHA, a measure of T cell function (17). As judged by the lack of reactivity in assays which evaluate the lymphocytic response to PPD, these agents appear to be toxic to those cells which have been stimulated to proliferate following initial sensitization.

By contrast, cyclophosphamide, a noncycle active drug effective against both dividing and intermitotic cells, exhibits a more generalized influence on T lymphocytes. This alkylating agent not only interferes with those elements initially stimulated to respond to tuberculin protein but also affects a proportion of intermitotic cells present in both the blood and peripheral tissues. Administration of this agent is associated with a partial depletion of T cells, and residual lymphocytes show impaired proliferative responses to PHA. These results are in accord with previous studies from this laboratory (17, 18). In addition to its effects on T cells, cyclophosphamide markedly reduced the cellularity of the thymic independent areas. Other studies suggest that this alkylating agent is more toxic for the B cell compartment than T lymphocytes (20–22).

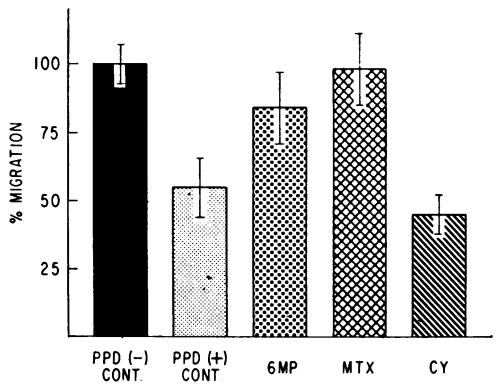

FIGURE 2 The activity of lymph node cells in assays for migration inhibition factor. In each test, a fixed number of lymph node cells from the test animals were added to peritoneal exudate cells from nonsensitized donors. Lymphocytes from sensitized controls inhibited migration to 55% compared with that observed using a comparable number of lymph node cells from unsensitized controls. No significant inhibition of migration was noted with cells from either 6MP or methotrexate-treated animals; in contrast, lymph node cells from animals receiving cyclophosphamide were highly active in this assay. Results are expressed as percent migration in comparison to PPD negative controls and each point represents the mean ±SE of 10–27 animals.

As assessed by the cutaneous response to PPD, all three drugs inhibit expressions of delayed hypersensitivity reactions. However, in this model, 6MP was less potent than the other two agents. Antimetabolite therapy decreased but did not abolish cutaneous reactivity; even at doses lethal to a majority of animals, a positive reaction was elicited in one survivor. In contrast, most animals treated with either cyclophosphamide (20 mg/kg per day) or methotrexate (12.5 mg/kg per day) failed to show a response. Nonreactivity was dose dependent; lesser quantities of either of the latter two drugs reduced the size of skin tests but did not abolish them. On the basis of these results, the dose of each agent employed for in vitro studies was selected to provide maximum impairment of cutaneous responses without causing excessive mortality.

The administration of each immunosuppressant impaired the in vitro proliferative response to PPD. In concert with this finding, treatment with the two cycle-active agents was associated with a failure to elicit a positive MIF response. The lack of activity in both assays suggests that cycle-active drugs specifically eliminate a proportion of those lymphocytes initially stimulated to proliferate by immunization. Inhibition of in vitro proliferative responses to PPD by 6MP is probably of short duration; Zweiman and Phillips showed that lymphocyte reactivity to this antigen was equivalent to controls one week after cessation of therapy (23).

In contrast, cyclophosphamide inhibited lymphoproliferative responses to PPD but did not impair the ability of these cells to elaborate MIF. These data suggest that antigen sensitive cells capable of mediator production are present in the tissues despite drug therapy. However, these cells are restricted in their in vitro replicate capacity. A similar dissociation between cell proliferation and other measures of T cell function has been reported in other assays (24–27). Using specific inhibitors of cell division, Bloom, Gaffney, and Jimenez concluded that the elaboration of MIF is a function of intermitotic cells (24). As the primary pharmacologic activity of cyclophosphamide is directed at the integrity of DNA templates (9), the dichotomy of in vitro results observed in this study may result from injuries to some T lymphocytes which do not effect either the viability or function of cells in the intermitotic phase but prove lethal when they are in cycle.

Previous investigations support conclusions regarding the importance of cell-cycle specificities of cytotoxic agents in determining both their effects on lymphocytes and their ability to suppress immune responses. In a study comparing the lymphotoxic and mitostatic effects of different immunosuppressive agents, Petrov, Manyko, Khaitov, and Seslavina (10) observed that cyclophosphamide was highly effective against lymphocytes. In contrast, the toxicity of 6MP was limited to replicating cells; this agent did not impair the function of immunologically competent elements. Lemmel, Hurd, and Ziff (11) showed that cyclophosphamide but not 6MP depleted long-lived lymphocytes. The effect of this antimetabolite was primarily limited to actively replicating cells; as such, there was a marked decrease in short-lived blood neutrophils.

In another study, Santos and Owens (13) compared the immunosuppressive effects of 6MP, methotrexate, and cyclophosphamide. All three drugs inhibited production of sheep cell agglutinins in the rat when therapy was initiated shortly after antigen challenge. The effective immunosuppressive interval coincided with the phase of proliferative expansion of cells destined to develop into antibody producing elements (28). However, only cyclophosphamide impaired this response when administered prior to immunization. This latter finding suggests that this agent is effective against potentially responsive lymphocytes present in the tissues prior to stimulation; these elements are presumably in an intermitotic phase of their cell cycle (6).

In considering the impaired cutaneous reactivity in animals treated with these cytotoxic agents, it is known that only a few sensitized lymphocytes are required to initiate a cell-mediated immune response (29, 30). Most of the cells present at the local site are macrophages which appear to function as nonspecific effectors of this reaction (31). These cells arise from rapidly replicating progenitors in the marrow (32) and have been shown to be susceptible to cytotoxic agents (33, 34). In the present investigations, treatment with each of these agents resulted in a significant decrease in the number of peritoneal exudate macrophages. Similarly, a reduced number of these cells may be available for participation in cell-mediated reactions; this deficit may be a major cause for decreased PPD reactivity. Of note, only cyclophosphamide reduced the total number of lymphocytes in these exudates, further evidence indicating a differential effect of cycle and noncycle-active drugs on these cells.

Although results of this study cannot be directly applied to the clinical use of immunosuppressants, they suggest that the cycle specificities of these agents may be important in determining optimal therapeutic programs. Cycle-active drugs appear to be maximally active in eliminating proliferating lymphoid cells. In contrast, noncycle-active agents, because of their effect on intermitotic cells, may have immunosuppressive effects on phases of the immune response other than those involving the replication of immunologically active lymphocytes.

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REFERENCES

1. Bruce, W. R., B. E. Meeker, and F. A. Valeriote. 1966. Comparison of the sensitivity of normal hematopoietic and transplanted lymphoma colony-forming cells to chemotherapeutic agents administered in vivo. *J. Natl. Cancer Inst.* **37**: 233.
2. Bergsagel, D. E. 1971. An assessment of massive-dose chemotherapy of malignant disease. *Can. Med. Ass. J.* **104**: 31.
3. Craddock, C. G., R. Longmire, and R. McMillan. 1971. Lymphocytes and the immune responses. *N. Engl. J. Med.* **285**: 324.
4. David, J. R. 1971. Mediators produced by sensitized lymphocytes. *Fed. Proc.* **30**: 1730.
5. In vitro methods in cell-mediated immunity. 1971. B. R. Bloom and P. R. Glade, editors. Academic Press, Inc., New York.
6. Winkelstein, A., C. G. Craddock, and J. S. Lawrence. 1971. Cell replication in the primary hemolysin response: the effect of six mercaptopurine. *J. Reticuloendothel. Soc.* **9**: 307.
7. Winkelstein, A. 1972. Cellular immunity in vitro: elaboration of MIF by thymic and marrow lymphoid cells. *Proc. Soc. Exp. Biol. Med.* **140**: 93.

8. Parrott, D. M. V., M. A. DeSousa, and J. East. 1966. Thymus-dependent areas in the lymphoid organs of neonatally thymectomized mice. *J. Exp. Med.* **123**: 191.
9. Berenbaum, M. C. 1967. Immunosuppressive agents and allogeneic transplantation. *J. Clin. Pathol.* **20**: 471.
10. Petrov, R. V., V. M. Manyko, R. M. Khaitov, and L. S. Seslavina. 1971. An experimental system for the simultaneous estimation of mitostatic and lymphotoxic effects of immunosuppressants and cytostatics *J. Exp. Med.* **133**: 640.
11. Lemmel, E., E. R. Hurd, and M. Ziff. 1971. Differential effects of 6-mercaptopurine and cyclophosphamide on autoimmune phenomena in NZB mice. *Clin. Exp. Immunol.* **8**: 355.
12. Maguire, Jr., H. C., and H. I. Maibach. 1961. Effect of cyclophosphamide, 6-mercaptopurine, actinomycin D and vincaleukoblastine on the acquisition of delayed hypersensitivity (DNCB contact dermatitis) in the guinea pig. *J. Invest. Dermatol.* **37**: 427.
13. Santos, G. W., and A. H. Owens, Jr. 1964. A comparison of the effects of selective cytotoxic agents on the primary agglutinin response in rats injected with sheep erythrocytes. *Bull. Johns Hopkins Hosp.* **114**: 384.
14. Owens, Jr., A. H., and G. W. Santos. 1971. The effect of cytotoxic drugs on graft-versus-host disease in mice. *Transplantation.* **11**: 378.
15. Maibach, H. I., and H. C. Maguire, Jr. 1966. Studies on the inhibition of antibody formation in the guinea pig with cyclophosphamide, azathiopurine, 5-fluorouracil and urethane. *Int. Arch. Allergy.* **29**: 209.
16. Berenbaum, M. C. 1962. The effect of cytotoxic agents on the production of antibody to T.A.B. vaccine in the mouse. *Biochem. Pharmacol.* **11**: 29.
17. Oppenheim, J. J. 1968. Relationship of in vitro lymphocyte transformation to delayed hypersensitivity in guinea pigs and man. *Fed. Proc.* **27**: 21.
18. Winkelstein, A. 1973. Mechanisms of immunosuppression: effects of cyclophosphamide on cellular immunity. *Blood.* **41**: 273.
19. Winkelstein, A., J. M. Mikulla, H. R. Nankin, B. H. Pollock, and B. L. Stolzer. 1972. Mechanisms of immunosuppression: effects of cyclophosphamide on lymphocytes. *J. Lab. Clin. Med.* **80**: 506.
20. Lerman, S. P., and W. P. Weidanz. 1970. The effect of cyclophosphamide on the ontogeny of the humoral immune response in chickens. *J. Immunol.* **105**: 614.
21. Turk, J. L., and L. W. Poulter. 1972. Selective depletion of lymphoid tissue by cyclophosphamide. *Clin. Exp. Immunol.* **10**: 285.
22. Stockman, G. D., L. R. Heim, M. A. South, and J. J. Trentin. 1973. Differential effects of cyclophosphamide on the B and T cell compartments of adult mice. *J. Immunol.* **110**: 277.
23. Zweiman, B., and S. M. Phillips. 1970. In vitro lymphocyte reactivity during depression of tuberculin hypersensitivity by 6-mercaptopurine. *Science (Wash. D. C.).* **169**: 284.
24. Bloom, B. R., J. Gaffney, and L. Jimenez. 1972. Dissociation of MIF production and cell proliferation. *J. Immunol.* **109**: 1395.
25. Rocklin, R. E. 1973. Production of migration inhibitory factor by non-dividing lymphocytes. *In* Proceedings of the Seventh Leucocyte Culture Conference. F. Daguillard, editor. Academic Press, Inc., New York. 381.
26. Moller, G., and E. Moller. 1965. Plaque-formation by non-immune and X-irradiation lymphoid cells on monolayers of mouse embryo cells. *Nature (Lond.).* **208**: 260.
27. Wallen, W. C., J. H. Dean, and D. O. Lucas. 1973. Interferon and the cellular immune response: separation of interferon-producing cells from DNA-synthetic cells. *Cellular Immunol.* **6**: 110.
28. Craddock, C. G., A. Winkelstein, Y. Matsuyuki, and J. S. Lawrence. 1967. The immune response to foreign red blood cells and the participation of short-lived lymphocytes. *J. Exp. Med.* **125**: 1149.
29. Lubaroff, D. M., and B. H. Waksman. 1967. Delayed hypersensitivity: bone marrow as the source of cells in delayed skin reactions. *Science.* **157**: 322.
30. McCluskey, R. T., B. Benacerraf, and J. W. McCluskey. 1963. Studies on the specificity of the cellular infiltrate in delayed hypersensitivity reactions. *J. Immunol.* **90**: 466.
31. Mackaness, G. B. 1970. The monocyte in cellular immunity. *Semin. Hematol.* **7**: 172.
32. Van Furth, R. 1970. Origin and kinetics of monocytes and macrophages. *Semin. Hematol.* **7**: 125.
33. Page, A. R. 1964. Inhibition of the lymphocyte response to inflammation with antimetabolites. *Am. J. Pathol.* **45**: 1029.
34. Arinoviche, R., and G. Lowei. 1970. Comparison of the effects of two cytotoxic drugs and of antilymphocytic serum. *Ann. Rheum. Dis.* **29**: 32.