Effects of Cytotoxic Immunosuppressants on Tuberculin-Sensitive Lymphocytes in Guinea Pigs

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ABSTRACT The immunosuppressive activities of two phase-specific drugs, 6-mercaptopurine (6-MP) and methotrexate, and a cycle-specific agent, cyclophosphamide, were evaluated on the lymphocytic component of established tuberculin hypersensitivity in guinea pigs. In these animals, purified protein derivative (PPD)-sensitive lymphocytes are in an intermitotic phase of their proliferative cycle. Neither phase-specific drug significantly altered either the number or functional activities of these lymphocytes. By two in vitro criteria, PPD-induced lymphoproliferation and elaboration of migration inhibition factor (MIF), the responses of lymph node cells were equivalent to sensitized controls. In addition, these agents did not deplete pools of T lymphocytes, impair responses to phytohemagglutinin (PHA), nor inhibit cutaneous reactivity if employed before sensitization.

In contrast, cyclophosphamide showed broader immunosuppressive effects including significant toxicities for intermitotic lymphocytes. This drug depleted pools of T cells and markedly impaired the in vitro proliferative responses of residual lymphocytes. The latter occurred with both PHA and PPD. Suppression of PHA reactivity was a dose-dependent phenomenon but was evident even with small quantities of this alkylating agent. The suppression of antigen-induced responses was independent of the proliferative status of target lymphocytes in vivo, and, after a single large dose, it persisted for more than 3 wk. In total, these results indicate that the effective use of cytotoxic drugs as immunosuppressants must include consideration of both the cycle specificities of the agent and the proliferative activities of the target lymphoid population.

INTRODUCTION

Despite the increasing use of cytotoxic drugs in clinical situations designed to suppress manifestations of autoimmune processes, conditions required for maximum effectiveness have not been fully delineated. In animal studies, several cytotoxic compounds have been shown to have potent immunosuppressive activities (reviewed in reference 1). However, in these studies, the period of drug administration often coincided with the induction phase of the immune response, a period characterized by the rapid proliferation of antigen-responsive lymphocytes. Thus, these situations cannot be considered as analogous to the autoimmune disorders seen clinically; the latter are mediated by clones of intermitotic (G_0) lymphocytes and plasma cells (2). These nonproliferating cells would be expected to differ in the susceptibility to cytotoxins. To more precisely define these differences, the present studies assessed the effects of three commonly used cytotoxins, 6-mercaptopurine (6-MP),[1] methotrexate, and cyclophosphamide, on the number and functional activity of tuberculin-sensitive intermitotic lymphocytes in guinea pigs.

As judged by their activities against both bone marrow stem cells and neoplastic cells, cytotoxic agents can be subdivided into three groups (3-5): (*a*) nonspecific agents which are equally effective against dividing and intermitotic cells, (*b*) cycle-specific agents which are toxic to cells in all phases of their cycle but show a preferential effect on actively dividing elements, and (*c*) phase-specific drugs, the toxicity of which are restricted to a discrete period during cell division. Cyclophosphamide is considered a cycle-specific agents; 6-MP and methotrexate are phase-specific. The latter two are primarily active against cells in the DNA synthetic (S) phase of their cycle. Although it has not been proven, these same principles may apply to immune lymphocyte populations.

The results of this study indicate that the capacity of a particular drug to deplete a population of antigen-

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[1] *Abbreviations used in this paper:* MIF, migration inhibition factor; 6-MP, mercaptopurine; PHA, phytohemmagglutinin; PPD, purified protein derivative; [^{3}H]Tdr, tritiated thymidine.

sensitive lymphocytes is primarily determined by two factors: the cycle specificity of the agent, and the proliferative activity of the target lymphocytes. As previously shown, all three drugs exert a lymphocytotoxic effect if the period of drug administration coincides with the induction phase of a delayed hypersensitivity response (6). However, in the established phase, only cyclophosphamide has significant toxicities for antigen-sensitive cells. The two phase-specific agents did not significantly alter either the number or activity of these lymphocytes.

METHODS

Male, Hartley-strain guinea pigs were sensitized to H37Ra mycobacteria in Freund's adjuvant (day 0). Animals were inoculated with a total of 1.0 ml, divided between foot pad and intradermal sites. Except as noted below, tuberculin skin tests, using 0.1 ml second strength purified protein derivative (PPD) injected into the skin of the flank, were performed on day 14. Skin tests were interpreted the following day. Responses were recorded by measuring the sum of the perpendicular diameters of induration. A reaction was considered negative if the value was less than 10 mm. For tabulation of results, responses in all animals with negative skin tests were arbitrarily considered as 10 mm.

Schedules of drug administration. After initial testing with PPD, animals were divided into four treatment groups: (Fig. 1) group I served as controls, group II received 6-MP, 20 mg/kg per day. As previously reported (4), this drug was solubilized in 1.0 N NaOH and neutralized with HCl-glycerine buffer (6). Group III was treated with methotrexate, 12.5 mg/kg per day, and group IV, cyclophosphamide, 20 mg/kg per day. All drugs were administered daily by the intraperitoneal route, from day +15 through +22. Second strength PPD skin tests were applied on day 22 and interpreted 24 h later. The same criteria as listed above were used to score cutaneous reactivity.

Immediately after interpretation of tuberculin skin tests, animals were killed by ether asphyxia. As such, there was a 1-day interval between the last dose of the immunosuppressant and sacrifice. Cardiac blood was obtained for leukocyte counts and differentials. Lymph nodes from the cervical, axillary, mesenteric, and periaortic areas were aseptically removed and used in two in vitro assays of cellular immunity. These were the lymphoproliferative responses to PPD and the activities of lymph node cells in assays for migration inhibition factor (MIF). Suspensions of these lymph node cells were also evaluated for their response to the nonspecific lymphocyte mitogen phytohemagglutinin (PHA). In representative animals, lymph nodes and spleen sections were evaluated histologically for the cellularity of T-cell areas. These were processed using routine fixation and sectioning techniques and staining with hematoxylin and eosin.

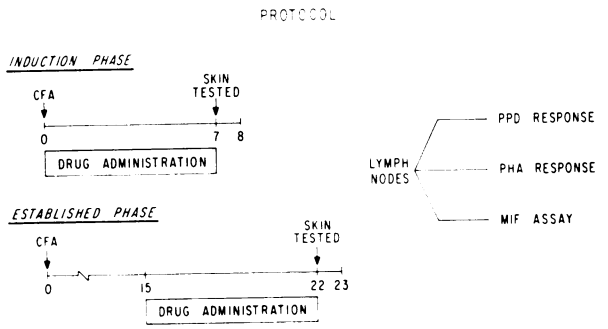

FIGURE 1 Diagrammatic representation of protocols used to evaluate the effects of daily administration of drugs on the induction or established phases of immune responses to tuberculin protein. CFA, complete Freund's adjuvant. Note: The last injection of each drug was administered 24 h before sacrifice. In these experiments, the following daily doses were employed: 6-MP, 20 mg/kg; methotrexate, 12.5 mg/kg; cyclophosphamide, 20 mg/kg.

In other experiments, similar courses of therapy were administered to animals starting 7 days before immunization (day −7 to 0). The last injection was given at the time of sensitization with Freund's adjuvant. Skin tests were performed on day +7. Other animals were treated with courses of each agent, starting either at the time of sensitization (day 0) or on day +3. Skin tests were applied on day +7 in the former and day +10 in the latter.

The effects of a single large dose of cyclophosphamide, (150 mg/kg), administered at the time of initial sensitization with complete Freund's adjuvant, were also measured. Groups of sensitized controls and drug-treated animals were killed on days 5, 8, 11, 14, 21, and 28. PPD-induced proliferative responses were measured at all time intervals; activities in MIF assays were assessed through the day 14 point. In each group, skin tests with second strength PPD were applied 1 day before sacrifice. As a corollary study, the sequential responses to PHA were measured in animals treated with a single injection of cyclophosphamide (150 mg/kg). Groups of guinea pigs were killed on days 1, 2, 3, 5, and 8; at each time interval, lymph node cells were tested for their response to this nonspecific mitogen.

The effects of varying doses of cyclophosphamide on the PHA reactivity of lymph node cells were evaluated in additional experiments. Groups of guinea pigs were treated with a 7-day course of this alkylating agent. The following daily dose schedules were employed: 1, 5, 10, 15, and 20 mg/kg. Lymph node suspensions were prepared 24 h after the last injection of cyclophosphamide.

In vitro assays of proliferative responses. Technical aspects of the in vitro lymphoproliferative response (6) have been previously reported in detail; these are summarized below: Lymph nodes were minced to make a single cell suspension. 10×10^6 cells were cultured in 5 ml media RPMI 1640 containing 20% fetal calf serum and supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), glutamine (300 μg/ml), and bicarbonate (250 μg/ml). For each animal, three cultures were incubated with 25 μg PPD; two to three cultures were established as unstimulated controls. These antigen-stimulated cultures were incubated for 5 days at 37°C in a 5% CO_2 atmosphere. Dose-response studies in sensitized controls indicated that this quantity of PPD provided optimal stimulation. Proliferative responses were assessed by the addition of tritiated thymidine ([^{3}H]Tdr), New England Nuclear (Boston, Mass.) sp act 6.7 Ci/M, 2.0 μg/culture 4 h before harvest. Incorporated isotope was determined by precipitating DNA with cold 5% TCA, dissolving the insoluble residue in Soluene and adding it to 10 ml of a liquid scintillation cocktail (Liquifluor-tolene). Isotope measurements were performed in a Packard Tri-Carb liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.); all results were corrected for background. Values for repli-

cate cultures were averaged and their response determined by calculating a stimulation ratio (counts per minute in PPD-stimulated cultures per counts per minute in unstimulated cultures).

PHA cultures and appropriate controls were incubated for 2 days and harvested in a similar fashion. In most experiments, the optimal concentration of PHA (0.1 ml of a standard solution) was employed. Additional studies in both control and drug-treated animal were undertaken to assess responses at suboptimal concentrations of this mitogen. The drug protocols consisted of seven daily injections, using the following dose schedule: 6-MP, 20 mg/kg, methotrexate, 12.5 mg/kg, and cyclophosphamide, 20 mg/kg. Animals were killed 24 h after the last injection. The PHA response of lymph node cell cultures was tested using 10-fold serial dilutions of the standard solution.

MIF assays. Assays for MIF were performed using peritoneal exudate cells from unsensitized guinea pig as the indicator system (6). These were obtained by the intraperitoneal injection of 30 ml light mineral oil 3 days before sacrifice. The exudate cells were thoroughly washed with Hank's balanced salt solution containing 1% heparin. Residual red cells were lysed by incubating the cell suspension in NH_4Cl-Tris buffer for 15 min. After additional washings with Hank's balanced salt solution, exudate cells were enumerated. An aliquot of lymph node cells from test animals were added to the exudate cells (7.5×10^5 lymph node cells mixed with 10×10^6 peritoneal exudate cells). In each assay, similar suspensions were prepared using lymph node cells from nonsensitized animals. The latter served as negative controls.

The mixture of lymph node cells and peritoneal exudate cells was added to microhematocrit capillary tubes; these were fixed at one end with a mixture of Vaseline and petroleum jelly, centrifuged, and cut at the cell-supernate interphase. Cellular portions were attached to cover slips of Sykes-Moore chambers with stopcock grease; the chambers were filled with minimal essential medium supplemented with heat-inactivated guinea pig serum, glutamine, bicarbonate, and antibiotics. The final concentration of PPD was 20 μg/ml. Migration was measured by planimetry after 24 h of incubation at 37°C. Values were expressed as the percent migration compared to control chambers containing lymph node cells from unsensitized animals.

For each MIF assay, lymph node suspensions from individual animals were evaluated separately. A single experiment included two to three animals treated with each drug and a similar number of both tuberculin-sensitized and unsensitized controls. The percent migration was calculated by comparing the average area in each test group with that measured in chambers containing unsensitized cells. Thus, the results of each experiment are based on the mean of two to three animals. Variability in the areas of migration between individual animals in each treatment group generally did not exceed 15%. The effect of each cytotoxin was determined by averaging values from four to six separate experiments.

Results of all experiments were evaluated by comparing the mean value for each drug protocol with those determined in comparable controls. Statistical significance was determined by the Mann-Whitney U test (7).

RESULTS

In vivo responses to PPD. In initial studies, the effects of timing of drug administration were examined on delayed hypersensitivity responses to tuberculin protein (Table I). The day of sensitization with complete Freund's adjuvant was designated as day 0; the periods of drug administration were: day −7 to 0; day 0 to +7; day +3 to +10, and day +15 to +22. Skin tests, using 0.1 ml second strength PPD, were applied on the last day of drug administration in all groups except for those animals treated before sensitization (day −7 to 0). In the latter group, skin tests were performed on day +7.

TABLE I

*Effects of Immunosuppressive Therapy on Cutaneous Responses to PPD**

	Treatment interval			
	Day −7 to 0	Day 0 to +7	Day +3 to +10	Day +15 to +22
6-MP, *20 mg/kg*	34±4 (4)	19±2§ (8)	24±3 (5)	34±2‡ (16)
Methotrexate, *12.5 mg/kg*	27±8 (6)	11±1§ (20)	17±4‡ (7)	25±3§ (16)
Cyclophosphamide, *20 mg/kg*	17±5‡ (7)	11±1§ (30)	17±3§ (7)	25±6§ (18)
Controls	31±3 (5)	29±2 (40)	32±3 (5)	42±3 (15)

* Second strength PPD; tests were interpreted on the day +8 in the groups treated on day −7 to 0 and day 0 to +7, and 1 day after termination of drug therapy in the other two groups. Values are the means ±1 SE. The numbers in parentheses indicate the number of animals in each group. Reaction sizes were measured as the sum of the perpendicular diameters.
‡ Significantly different from controls ($P < 0.025$).
§ Significantly different from controls ($P < 0.01$).

Animals treated before sensitization (day −7 to 0) with either 6-MP or methotrexate, the two phase-specific agents, showed no suppression of in vivo tuberculin responses. Compared to sensitized controls, the average reaction size with each of these drugs was 110 and 87%, respectively. Maximal inhibition with both 6-MP and methotrexate was observed when the agents were administered between day 0 and +7. 6-MP caused a 34% reduction in diameters while methotrexate reduced skin test size by 62%. If drug treatment was initiated on day +3, methotrexate was effective in suppressing the in vivo response (47% reduction); 6-MP did not cause significant inhibition. In animals treated between day +15 and +22, methotrexate impaired skin test reactivity by 40%; 6-MP caused only a 17% decrease in test diameters. It should be noted that before initiating drug therapy in the latter group, all animals had positive tuberculin reactions; the mean diameter was 32±4 mm.

In those guinea pigs treated with cyclophosphamide, a cycle-specific agent, significant inhibition of cutaneous reactivity to PPD was noted at all four time intervals.

Treatment before sensitization reduced reaction sizes by an average of 55%. Maximal inhibition occurred in those guinea pigs treated during the induction phase of this cell-mediated immune response (day 0 to + 7); the mean reaction size during this interval was reduced by 62%. In animals in which tuberculin hypersensitivity was already present (days + 15 to + 22) cyclophosphamide therapy caused a 40% decrease in the diameters of skin tests.

Effects on lymphoproliferative responses. A primary emphasis of these investigations concerns the effects of these cytotoxic drugs on the in vitro proliferative responses of lymphocytes obtained from guinea pigs with established tuberculin hypersensitivity. Using the dose schedule described above, animals received a 7-day course of one of the three agents between days + 15 and + 22. Lymph node suspensions, obtained 24 h after the last injection, were tested for responses to both PPD and PHA. To contrast the effects of timing of immunosuppressive therapy, these results were compared to previously reported data (6) which described the effects of treatment during the induction phase (day 0 to + 7) of this hypersensitivity reaction. The results of treatment during the induction phase are depicted in the left panel Fig. 2; those from animals with established responses in the right panel.

In animals with established tuberculin hypersensitivity, only cyclophosphamide inhibited the in vitro proliferative response to PPD. Stimulation ratios in the group receiving this cycle-specific agent averaged 1.2±0.2; the mean value in sensitized, untreated controls was 3.0±0.3 ($P < 0.001$). The reduced reactivity reflects a true impairment in antigen-induced proliferation, as the [^{3}H]-Tdr incorporation in unstimulated cultures was similar in both cyclophosphamide and control groups.

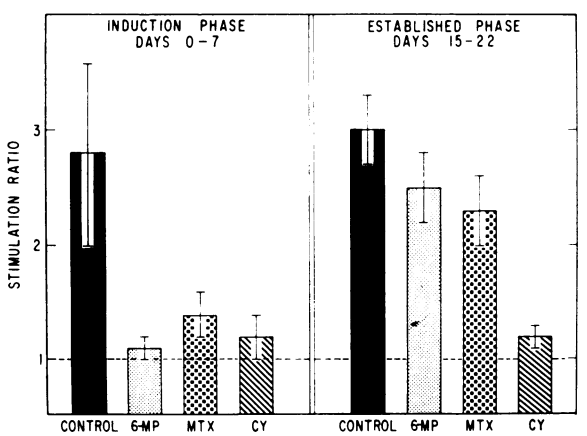

FIGURE 2 In vitro proliferative responses to PPD. Results are expressed as a stimulation ratio ±SE; the dotted line indicates a ratio of one. The panel in the left depicts responses in animals treated during the induction period (days 0 to + 7); that on the right represents measurements when treatment courses were employed in guinea pigs with established tuberculin responses (days +15–+22).

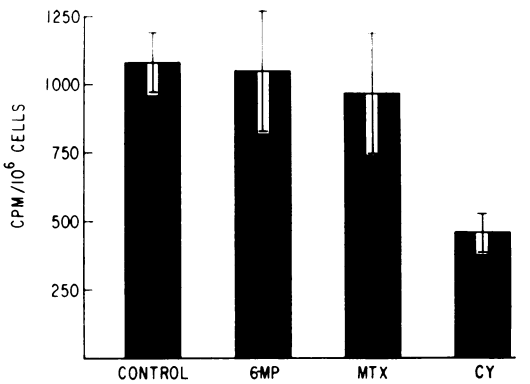

FIGURE 3 Lymphoproliferative responses to PHA using animals treated with the indicated cytotoxic agent. The value for lymph node cells from cyclophosphamide-treated animals is significantly less than those from controls ($P < 0.01$). In contrast, neither 6-MP nor methotrexate suppressed responsiveness to this nonspecific mitogen.

Stimulation ratios in animals treated with cyclophosphamide during the course of an established response were similar to those observed when this drug was administered during the induction phase (day 0 to + 7). In this latter group, the average ratio was 1.2±0.2. These results suggest that, in the doses employed in these assays, cyclophosphamide interferes with the in vitro proliferative response of T lymphocytes. Further, this effect may occur at any time after sensitization with Freund's adjuvant; it is not dependent on the target population of sensitized lymphocytes being in an active proliferative phase.

In contrast to the inhibitory activity of cyclophosphamide on antigen-induced lymphoproliferation, neither 6-MP nor methotrexate significantly suppressed in vitro lymphocyte reactivity in animals with established tuberculin responses. Stimulation ratios in lymph node cultures from guinea pigs treated between days + 15 and + 22 were: 6-MP, 2.5±0.3; methotrexate, 2.3±0.3. The normal responses in these groups can be contrasted with those determined when the treatment period coincided with the induction phase. During this initial period, both phase-specific agents markedly suppressed in vitro PPD reactivity.

The lymphoproliferative response of the nonspecific mitogen, PHA, paralleled those observed with PPD in animals with established responses to tuberculin protein (Fig. 3). In PHA-stimulated cultures, the mean isotope incorporation was significantly reduced by cyclophosphamide. After a 7-day course of this drug (20 mg/kg per day), isotope uptake was only 58% of controls. In contrast, neither 6-MP nor methotrexate impaired this

response. Activity using lymph node cells from the 6-MP-treated animals was 97% and those receiving methotrexate, 90%.

MIF assays. Lymph node cells from animals with established tuberculin hypersensitivity were evaluated for their reactivity in MIF assays. Using peritoneal exudate cells from unsensitized donors as the indicator system, addition of a constant number of lymph node cells from any of the treatment groups resulted in an inhibition of migration which was similar to that calculated in sensitized controls. The average areas of migration were: PPD-positive controls 36±6%; 6-MP-treated animals, 57±13%; methotrexate, 50±11%; and cyclophosphamide, 48±7% (Fig. 3). For comparison, the data of MIF assays performed in animals with developing tuberculin responses is also presented in Fig. 4. During this induction period, cell suspensions from 6-MP and methotrexate groups failed to inhibit migration, whereas those from the cyclophosphamide group showed activity which was similar to PPD-positive controls.

Effect on lymphocyte number. Differential effects of these drugs were also observed on quantitative estimates of the number of blood lymphocytes (Fig. 5) and the histology of thymic-dependent areas of peripheral lymphoid tissues. All measurements were made 24 h after the last dose of each agent. The number of blood lymphocytes was significantly reduced in the cyclophosphamide-treated group (67%); by contrast, lymphopenia was not observed in animals treated with either of the phase-specific agents. Further, by morphologic evaluation, the cellularity of thymic-dependent areas of lymph nodes and spleens from animals receiving cyclophosphamide was moderately decreased. No comparable reduction was observed in animals receiving either 6-MP or

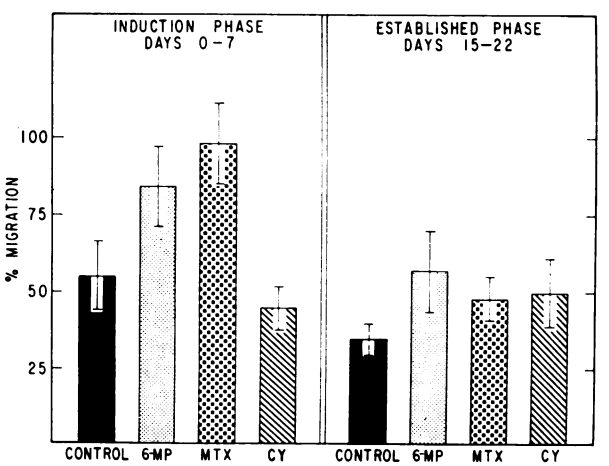

FIGURE 4 MIF assays in guinea pigs treated with the indicated cytotoxic agent. Treatment periods are indicated above. Values represent means±SE in four to six experiments; each experiment consisted of two to three animals.

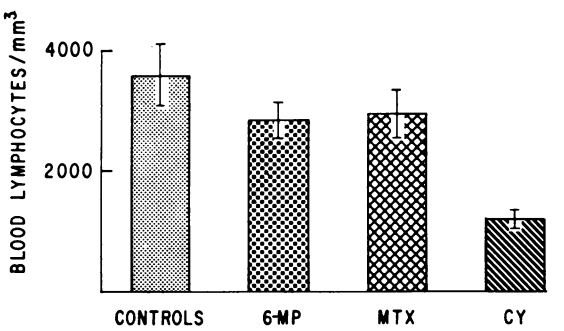

FIGURE 5 Blood lymphocyte counts. Only cyclophosphamide significantly reduced the number of these cells ($P < 0.001$).

methotrexate. In all treatment groups, both the number of germinal centers and the size of individual centers were markedly reduced.

Sequential effects of cyclophosphamide. To further delineate the toxicities of cyclophosphamide, sequential studies were performed in guinea pigs after a single large dose of this agent. The drug (150 mg/kg) was administered at the time of sensitization with Freund's adjuvant (Table II). Compared to controls, cutaneous responses to PPD were delayed in onset, and the intensity of reactions was reduced through day 14. Sensitized controls showed strongly positive tests on day 8; in contrast, half of the treated animals failed to show measurable reactions (> 10 mm) at this point. In the remainder, only minimally positive tests (15 mm) were elicited. By day 21, there was no significant difference in reaction size.

Activity in MIF assays generally paralleled skin test responses. By day 8, lymph node suspensions from controls were highly reactive. Cells from treated animals did not significantly inhibit migration until day 11. Responses in the two groups were equivalent by day 14. Thus, by both criteria, skin tests reactivity and responses in MIF assays, a single dose of cyclophosphamide caused only a transient delay in responses to tuberculin protein.

A more prolonged inhibition of PPD-induced lymphoproliferative responses was observed in cyclophosphamide-treated animals. Significant reactivity was observed in lymph node cultures from controls on day 8; maximal activity occurred on day 21. By contrast, there was no demonstrable response in PPD-stimulated cultures from drug-treated animals through the day 21 point. Mean stimulation ratios were consistently below 1.5; this value was the minimum observed in all positive controls. These results indicate that impairment of in vitro lymphoproliferative responses to a specific antigen persists for longer periods than the other measures of T-cell function.

In a parallel study, PHA responses were sequentially measured in animals treated with cyclophospha-

TABLE II
Serial Responses to PPD

Days after cyclophosphamide	Skin tests (MM)		MIF assays (% migration)		Proliferative responses (stimulation ratios)	
	Controls	Cyclo. dose	Controls	Cyclo. dose	Controls	Cyclo. dose
5	<10 (5)	<10 (5)	103±9% (3)	103±17 (3)	0.8±0.1 (7)	0.8±0.1 (5)
8	31±2 (8)	13±2‡ (12)	53±3 (4)	91±3‡ (4)	2.8±0.2 (12)	1.2±0.2‡ (5)
11	39±4 (5)	19±5* (8)	46±7 (5)	68±6* (5)	2.2±0.3 (6)	1.2±0.2‡ (5)
14	46±4 (7)	29±3‡ (13)	40±3 (6)	54±6 (5)	2.6±0.3 (7)	1.1±0.2‡ (8)
21	51±3 (7)	41±5 (9)	ND	ND	4.8±0.8 (10)	1.4±0.4‡ (10)
28	60 (4)	60 (4)	ND	ND	3.8±0.7 (4)	2.6±1.1 (6)

Numbers in parentheses refer to number of animals used in each assay except for MIF assays where it represents the number of experiments. Values are means ±1 SE. In recording skin tests, no variance term is recorded when all measurements for a single group were identical. ND = not done.
* Significantly different from PPD-positive controls ($P < 0.05$).
‡ Significantly different from PPD-positive controls ($P < 0.01$).

mide. As measured by [^{3}H]Tdr incorporation, reactivity was markedly suppressed 1 day after drug administration (26% of control values). Maximum inhibition occurred on day 2 (15%). By day 5, responses were equal to untreated controls.

Dose-response effects of cyclophosphamide. Additional studies were undertaken to determine the relationship between the daily dose of cyclophosphamide and suppression of PHA reactivity. Results, summarized in Table III, indicate that a modest degree of suppression was observed with as little as 1 mg/kg per day. More significant inhibition was noted in animals treated with the higher daily doses. The mean blood lymphocyte counts in the group receiving 1 mg/kg per day were not significantly different from controls; in all other groups. lymphopenia was a consistent finding. Thus, it appears that reduced responses to PHA reactivity can occur even in animals with normal blood lymphocyte counts. Preliminary studies indicate that, at all doses tested, PPD reactivity in animals with established tuberculin reactions was also impaired.

TABLE III
Effects of Varying Doses of Cyclophosphamide on PHA Responses

Daily dose of cyclophosphamide*	No. of animals	Blood lymphocytes	PHA response (stimulation ratios)
mg		*mm*^{3}	
0	11	3,490±420	35.3±8.7
1	9	3,050±630	12.6±4.6‡
5	10	1,850±280§	7.8±2.1§
10	7	1,900±250§	8.7±2.8§
15	6	ND	8.0±2.4§
20	6	950±130§	4.7±1.3§

* The indicated dose of cyclophosphamide was administered for 7 consecutive days. Animals were sacrificed 24 h after last dose.
‡ Significantly different from controls ($P < 0.025$).
§ Significantly different from controls ($P < 0.01$).
ND Not done.

Effects of variable concentrations of PHA. Lymph node cell reactivity to suboptimal concentrations of PHA was measured in both drug-treated and control animals. Data, summarized in Table IV, indicate that the response of lymphocytes obtained from guinea pigs treated with either phase-specific agent did not significantly differ from normal. In contrast, at all concentrations of PHA capable of stimulating normal lymphocytes, cells from cyclophosphamide-treated animals were suppressed in the reactivity.

DISCUSSION

Results of these investigations define two factors of importance in determining the toxicities of different cytotoxic immunosuppressants for antigen-sensitive lymphocytes. These are: (*a*) the cycle specificity of the drug

and (*b*) the proliferative status of the target lymphocytes. The differences in the activity of these drugs are summarized in Table V. In this study, those agents classified as phase-specific proved to have only a limited toxicity for PPD-sensitive lymphoid cells; their inhibitory activity coincided with the proliferative expansion of the stimulated cells. During established phases of this delayed hypersensitivity response, these agents did not significantly alter either the number or functional activity of responsive lymphoid elements. In contrast, cyclophosphamide, a cycle-specific compound, was toxic for both intermitotic and cycling lymphocytes. As such, it showed immunosuppressive activities which extend throughout all phases of this response.

In guinea pigs with established tuberculin hypersensitivity, most antigen-sensitive lymphocytes are in an intermitotic (G_0) phase of their proliferative cycle (2). Data from the present study suggest that neither 6-MP nor methotrexate, two phase-specific drugs (3-5), inhibited the lymphocytic component of this immune response. By two in vitro criteria, PPD-induced lymphoproliferation and the release of MIF, the activity of lymph node cells from treated animals was equivalent to those from sensitized controls. The resistance of intermitotic lymphoid cells to these agents was further supported by preservation of the cellularity of thymic-dependent areas of peripheral tissues (7) and normal lymphocytic responses to PHA, a nonspecific mitogen capable of activating T cells (8).

The lack of toxicity of phase-specific drugs for intermitotic PPD response of lymphocytes can be contrasted to their activity during the induction period (day 0 to +7). This interval is characterized by the rapid proliferative expansion of antigen-sensitive cells. Administration of either 6-MP or methotrexate during this phase effectively limited the development of responsive cells; this is illustrated by the impaired responses in both in vitro assays for PPD-sensitive lymphocytes.

In accord with other studies, neither 6-MP nor methotrexate were immunosuppressive when employed before initial antigen challenge (9-11). Again, this can be attributed to the proliferative characteristics of the target lymphocytes; potentially responsive cells are in an intermitotic interval before initial antigen challenge (2). In total, these results indicate that the immunosuppressive activities of phase-specific drugs are intimately linked to the proliferative status of the target lymphocytes. They are maximally effective only if active lymphopoiesis is occurring during the period of drug administration.

Cyclophosphamide shows toxicities for lymphocytes which significantly differ from those observed with phase-specific agents. These include a reduction of the number of small lymphocytes and an impairment in the in vitro proliferative responses of residual cells. The decrease in small lymphocytes affected both the thymic-dependent and independent areas. In accord with other studies, this agent caused a more pronounced cellular depletion in the areas populated by B lymphocytes (12-14).

In addition, residual lymphocytes with cyclophosphamide-treated animals showed induced in vitro proliferative responses with two stimuli, PHA and PPD. Inhibition of PHA response was dose dependent; in animals treated with 1 mg/kg per day, reactivity was mini-

TABLE V

Differential Effects of Cytotoxic Agents on Responses to PPD

	Phase-specific (6-MP, methotrexate)	Cycle-specific (cyclophosphamide)
Effective immunosuppressive interval (skin test reactivity)		
Before sensitization	N	D
Induction phase	D	D
Established phase	D	D
Blood lymphocytes	N	D
Tissue small lymphocytes		
Thymic dependent	N	D
Thymic independent	D	D
PHA responses	N	D
PPD responses		
Induction phase	D	D
Established phase	N	D
MIF response*		
Induction phase	D	N
Established phase	N	N

N, normal (equivalent to PPD-sensitized controls); D, decreased response.

* Measure on a constant number of lymph node cells.

TABLE IV

Dose-Response Relationships for PHA

	Dilution of PHA			
	Undiluted*	1:10	1:100	1:1,000
Controls	27.6±3.7*	25.1±4.5	1.4±0.1	1.3±0.1
6-MP,§ *20 mg/kg/day*	26.7±3.7	25.8±6.7	1.7±0.2	1.1±0.2
Methotrexate, *12.5 mg/kg/day*	26.0±4.3	21.6±11.1	1.4±0.1	1.1±0.1
Cyclophosphamide, *20 mg/kg/day*	6.1±1.4	7.6±2.1	1.5±0.4	1.0±0.1

* Standard reconstituted solution of PHA.

‡ Stimulation ratio ±1 SE.

§ Each animal received a 7-day course of drug.

mally inhibited. Tests using various concentrations of PHA indicated that residual lymphocytes did not display an altered sensitivity to this mitogen; rather, the impaired reactivity probably reflects intrinsic cellular defects which limit in vitro replicate activity. Of note, the lymphocytic responses to PPD were markedly suppressed in animals treated during the induction or the established phase of tuberculin hypersensitivity. This suggests that this agent has antimitotic activities which can affect both cycling and intermitotic cells.

Cyclophosphamide's antiproliferative effects not only affects cells in all phases of their generation cycle, but temporally persists beyond the time associated with the presence of cytotoxic activity in the circulation. Although the active components of cyclophosphamide are not known, most of the drug and its major metabolic products are rapidly cleared from the circulation and can be recovered in the urine within 24 h after administration (15). However, the suppression of PHA responses was not reversed until the 5th day after a single large dose. A similar suppression of splenic lymphopoiesis also has been observed in vivo. In a murine model, splenic DNA synthesis was markedly inhibited for 5 days after cyclophosphamide administration (16).

A more prolonged depression of in vitro antigen-induced replication was observed in guinea pigs treated at the time of initial sensitization. The response to PPD was inhibited for over 3 wk. Recovery of this parameter of lymphocyte function was appreciably delayed in comparison to responses in MIF assays or restoration of skin test reactivity. It has been postulated that cyclophosphamide causes latent damage to DNA; this is lethal to the cell if it enters mitosis before completion of a repairative process. The latter is comparatively slow (17). In this regard, therapy with cyclophosphamide leads to an increased incidence of chromosome aberrations in lymphocytes (18) including those associated with reproductive nonviability (19). This prolonged inhibition of proliferative responses may be an important factor in the sustained immunosuppressive activity of cyclophospamide (9, 16).

Lymph node cells from guinea pigs treated with cyclophosphamide produced positive MIF responses. In this assay, a constant number of lymph node cells is added to indicator macrophages. As such, the test depends on the number of MIF-synthesizing cells in this lymphoid aliquot rather than the total pool of antigen-sensitive cells remaining after drug administration. In evaluating the effects of this cycle-specific agent, it appears that the reduction in PPD-reactive cells is partially counter balanced by the decrease in lymphoid mass. This serves to maintain a sufficient quantity of reactive cells in the aliquot to effect a positive response. By contrast, during the induction phase, phase-specific drugs are selectively toxic to actively replicating precursors of tuberculin-sensitive cells. They do not significantly reduce lymph node cellularity. Therefore, the test lymphoid suspension will contain decreased numbers of MIF-synthesizing cells. Because this assay does not solely evaluate changes in body pools of PPD-reactive cells, these results alone cannot be used to compare the immunosuppressive potencies of different classes of cytotoxins.

It is noteworthy that PPD-sensitive cells surviving administration of cyclophosphamide can elaborate this lymphokine but are markedly suppressed in their capacity to proliferate. The reasons for these discordant values are not completely known. However, it has been shown that the elaboration of MIF proceeds independent of cell division (20). Thus, the synthesis of this soluble mediator may not be impaired by a drug which acts primarily on the structural integrity of DNA (17).

Findings regarding the activities of cyclophosphamide in guinea pigs with established tuberculin hypersensitivity differ in certain respects for those recently reported by Barlow et al. (21). These investigators found that this agent impaired MIF responses in a dose-dependent fashion. They also did not observe a pronounced suppression of PHA responses. In concert with the present study, antigen-induced proliferative responses were markedly inhibited. Although the reasons for these apparent discrepancies are not clear, they may reflect differences in experimental design and methodology.

Results in the present study extend previous observations regarding the immunosuppressive potency of cyclophosphamide. This drug effectively inhibits both cell-mediated (10, 22, 23) and humoral antibody responses (9, 11, 24). It acts both before and after initial antigenic challenge (9-11) and is capable of suppressing secondary (anamnestic) responses (24). In several studies, it appears to be a more potent imunosuppressant than phase-specific drugs such as 6-MP (25-27) and has a high therapeutic: toxic ratio (1). In other reports, cyclophosphamide has been shown to be more effective in depleting pools of small lymphocytes than phase-specific drugs (25).

Cyclophosphamide has been reported to be maximally effective as an immunosuppressant when administered in the period immediately after primary antigenic challenge (9-11). This probably relates to its greater toxicities for actively cycling cells (28). This agent has a more profound effect on humoral antibody responses; this may be due to its greater effect on B lymphocytes (29, 30). However, its toxicities are not limited to this lymphocyte population. Cyclophosphamide has significant toxicities for T cells. As shown in the present study, it inhibits the lymphocytic component of a delayed hypersensitivity reaction. It also suppresses the activity of T-helper cells (31) and T-suppressor cells (32).

In considering results of this study, it should be recognized that cutaneous responses to PPD may not accurately reflect the toxicities of these drugs for antigen-sensitive lymphocytes. Only a few sensitized cells are required to initiate a delayed hypersensitivity response (33, 34). The expression of the reaction requires cells of the monocyte-macrophage series (35). These arise from rapidly replicating progenitors in the marrow (36), and treatment with these antimitotic agents limits their production (6). Thus, decreased sizes of skin tests may represent a nonspecific anti-inflammatory activity due to suppression of macrophage production. The in vitro measures appear to be a truer indicator of the lymphocytic component of this response. Although macrophages may be required to initiate in vitro responses (37), differential cell counts indicate that impaired proliferative reactivity is not due to a qualitative deficit in these phagocytes. In vivo, these drugs may have an additional effect, that of limiting the function of macrophages in the initial processing of the antigen.

The results of this study appear to have clinical significance. They suggest that a cycle-specific drug will be more effective in suppressing established immune responses than phase-specific agents. In this regard, autoimmune disorders are considered to be manifestations of established reactions and thus will be mediated by intermitotic cells. It appears that these reactions are relatively resistant to short courses of phase-specific agents but can be partially suppressed by cycle-specific compounds.

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