Effect of Immunosuppressive Drugs on T and B Lymphocytes in Guinea Pigs

By Alan Winkelstein

Changes in the number of T and B lymphocytes were assessed in guinea pigs following administration of immunosuppressive drugs. T cells were measured by rosetting with papainized rabbit erythrocytes, B cells by complement rosettes. After a single intraperitoneal dose of cyclophosphamide (150 mg/kg), the number of T and B cells in both peripheral blood and lymph node suspensions decreased by 60%-70%. The reduction of both elements persisted through day 5 after drug administration. There was a differential rate of recovery; T cells were restored by day 8; B cells remained subnormal until day 14. Chlorambucil (10 mg/kg) produced less striking changes in the number of both types of lymphocytes; the reductions ranged from 25%-35%. Neither 6-mercaptopurine (100 mg/kg) nor methotrexate (75 mg/kg) altered the number of either cell type in the blood or lymph node samples. Hydrocortisone acetate (400 mg/kg) reduced lymph node T and B cells within 2 days of administration; blood values did not decrease until day 5. Additional studies indicated that five consecutive doses of cyclophosphamide (20 mg/kg/day) and chlorambucil (1 mg/kg/day) caused both T and B lymphopenia; none of the other drugs significantly depleted blood lymphocytes. These results suggest that the initial effects of cytotoxic agents and hydrocortisone are relatively nonselective in terms of changes in functional classes of lymphocytes. The cell-cycle specificity of cytotoxic drugs, rather than a specific toxicity for a particular class of lymphocytes, appears to be the prime determinant in the killing of immunologically competent cells.

INTERACTION of multiple factors contributes to the therapeutic effectiveness of immunosuppressive drugs. This concept is particularly true for cytotoxic agents, drugs whose primary mode of action, in this context, is to kill antigen-responsive cells. In previous studies we have shown that two factors, the proliferative activity of the target cells and the cell-cycle specificity of the drug, are of prime importance in determining the “killing” activities of these agents.1,2

A third criterion for effective immunosuppression is the relative selectivity of an agent for T and B lymphocytes. Any drug with a preferential toxicity for one population would be particularly useful. Of the commonly used immunosuppressive agents, only cyclophosphamide has been extensively evaluated; it has been suggested by some investigators that this agent has a selective activity against B cells.3,7 Others have found that both cell types are simultaneously depressed.8 In order to determine systematically the relative effect on these
lymphocyte populations, we have quantitatively assessed the number of residual T and B cells in guinea pigs receiving cytotoxic therapy. Results indicate that following the administration of one of four agents, cyclophosphamide, chlorambucil, 6-mercaptopurine (6-MP) or methotrexate, the initial changes are primarily determined by the drug's pharmacologic activity and are not an expression of a preferential killing effect on a selected population of lymphocytes.

Parallel studies were performed in order to assess the lymphocytotoxic activities of corticosteroids, noncytotoxic agents widely used as immunosuppressants. In guinea pigs, steroids appeared to cause a modest but nonselective reduction in both types of immunologically competent elements.

MATERIALS AND METHODS

Drug Administration Protocols

Male Hartley-strain guinea pigs, weighing 400-450 g, were used throughout these investigations. Animals received a single intraperitoneal (i.p.) dose of one of the following agents: cyclophosphamide (150 mg/kg), chlorambucil (10 mg/kg), 6-MP (100 mg/kg), methotrexate (75 mg/kg) or hydrocortisone acetate (400 mg/kg). The dose of each agent, except hydrocortisone, was selected because of a similar degree of toxicity. The mortality rate at 14 days was approximately 15%. To solubilize chlorambucil, 2 mg were dissolved in 0.5 ml of 10% NaHCO₃ and subsequently diluted with 4.5 ml distilled water. As described previously, 6-MP was dissolved in 1 N NaOH and the solution was partially neutralized with HCl-glycerine buffer. For each drug treatment group, between 7 and 14 animals were evaluated; in each assay, untreated controls were studied simultaneously. The day of drug administration was designated as day 0.

Groups of animals were sacrificed by ether asphyxia 2 or 5 days after receiving the test agent. In addition, animals receiving cyclophosphamide were evaluated on days 8, 11, and 14. At the time of sacrifice, heparinized cardiac blood was collected for total white cell count, a 200-cell white cell differential, and T- and B-lymphocyte rosette assays. T cells were measured by their ability to rosette with unsensitized, papainized rabbit red cells, and B cells by complement rosettes. For both rosette assays, peripheral blood was initially subjected to Ficoll-Hypaque density gradient centrifugation to separate mononuclear cells. An aliquot of 5 ml whole blood was mixed with 15 ml Hank's balanced salt solution (HBSS) and layered over 10 ml of a Ficoll-Hypaque solution. This preparation was centrifuged for 40 min at 4°C; the mononuclear layer was aspirated and washed three times in HBSS. Cell recovery was 70%-90%. In order to identify monocytes, test cells were incubated with a 0.1% suspension of latex particles (average diameter, 1.1 μ) prior to performing rosette assays.

Lymph nodes were obtained from the cervical and mesenteric areas. All visible nodes were removed, any surrounding connective tissue was carefully dissected from the nodes, and the pooled nodes were weighed to the nearest 0.01 g. Total nodal cellularity was determined by finely mincing all excised nodes, repeatedly aspirating the suspension through a 21-gauge needle, and counting the total number of cells in a hemocytometer. In both lymph node and peripheral blood preparations, viability, as assessed by trypan blue exclusion, was at least 90%.

Additional experiments were performed to evaluate the effects of chronic daily drug administration. In these studies, the following agents were injected i.p. for 5 consecutive days: cyclophosphamide (20 mg/kg/day), chlorambucil (1 mg/kg/day), 6-MP (20 mg/kg/day), methotrexate (12.5 mg/kg/day), or hydrocortisone acetate (50 mg/kg/day). For consistency, the last dose of drug administration was designated as day 0. Groups of animals were killed on days 1, 2, or 5, and both blood and lymph nodes were subjected to the assays listed above. It should be noted that these doses were similar in their acute toxicity: the mortality rate was approximately 15%.

Rosette Assays

The number of T lymphocytes was measured by virtue of their capacity to form rosettes with rabbit red cells, a test analogous to the formation of sheep red cell rosettes with human T lymphocytes. In order to increase the sensitivity of the assay, rabbit red cells were pretreated...
### Table 1. Changes in Blood Lymphocytes After Administration of Immunosuppressive Drugs

<table>
<thead>
<tr>
<th>Drug</th>
<th>T Cells Day 2</th>
<th>B Cells Day 2</th>
<th>T Cells Day 5</th>
<th>B Cells Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>$\times 10^9$/liter</td>
<td>%</td>
<td>$\times 10^9$/liter</td>
</tr>
<tr>
<td>Controls</td>
<td>61.0 ± 2.6</td>
<td>12.7 ± 1.2</td>
<td>58.1 ± 5.3</td>
<td>10.6 ± 2.3</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>50.6 ± 4.5*</td>
<td>7.2 ± 1.8*</td>
<td>0.14 ± 0.03*</td>
<td>0.12 ± 0.03*</td>
</tr>
<tr>
<td>Chlorambucil</td>
<td>59.2 ± 5.4</td>
<td>13.3 ± 3.0</td>
<td>0.34 ± 0.09*</td>
<td>8.3 ± 0.9*</td>
</tr>
<tr>
<td>6-Mercaptopurine</td>
<td>51.6 ± 6.4</td>
<td>13.0 ± 2.9</td>
<td>0.52 ± 0.17</td>
<td>10.8 ± 2.1</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>60.8 ± 5.1</td>
<td>12.0 ± 3.7</td>
<td>0.45 ± 0.16</td>
<td>11.4 ± 2.4</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>57.2 ± 6.0</td>
<td>13.8 ± 1.8</td>
<td>0.54 ± 0.10</td>
<td>8.6 ± 1.5*</td>
</tr>
</tbody>
</table>

Values are means ± SE.

*Significantly different from controls (minimum significance $p < 0.05$).
with papain prior to their incubation with lymphoid cell suspensions. Tests were performed according to the method described by Wilson et al.\textsuperscript{12} EAC rosettes were measured by using human type A red cells coated with a subagglutinating dose of anti-A serum (Spectrum Biologicals, Oxnard, Calif.). Coating was accomplished by incubating cells with the antiserum for 1 hr at 37°C. The red cells were washed three times in saline and reconstituted to a 50% solution in saline. Fresh guinea pig serum (1 ml) was then incubated with 0.1 ml red cell suspension for 40 min at 37°C. The coated cells were washed three times and resuspended to a final concentration of 2.5% in saline. To form EAC rosettes, 1 ml guinea pig lymphocytes (0.75 x 10\(^6\) cells/liter) was incubated with 0.1 ml of the indicator cells for 90 min.

To enumerate both E and EAC rosettes, cells were resuspended with a Pasteur pipette, three drops of toluidine blue were added, and rosettes were counted in a hemocytometer. The criterion for a rosette-forming lymphocyte was the presence of three or more red cells adherent to the cell membrane. In each specimen, 200 lymphocytes were counted; monocytes were excluded from the final count. Neither unsensitized nor antibody-coated human red cells without complement formed rosettes.

**RESULTS**

Despite the variability of drug dosages, the overall toxicities in those groups receiving cytotoxic drugs were similar. The mortality rate with each agent was approximately 15%. Furthermore, normal hematopoiesis was markedly suppressed; blood neutrophils on day 5 were consistently less than 10% of normal. Based on these criteria, it appeared that each treatment group received approximately equivalent doses of each drug, thus allowing for valid comparisons with respect to the agent’s toxicity for lymphocytes. An arbitrary quantity of hydrocortisone was selected; there was no mortality or suppression of hematopoiesis with this agent.

In normal animals, the total number of T and B lymphocytes in the peripheral blood averaged 2.70 ± 0.15 x 10\(^9\)/liter and 0.50 ± 0.05 x 10\(^9\)/liter, respectively. As shown in Table 1, a single, large dose of cyclophosphamide (150 mg/kg) caused a marked reduction in both cell types. Lymphopenia involving T and B cells was apparent on day 2 and the reductions persisted through day 5. At the later time point, T cells were reduced by 69% and B cells by 75%. These values suggested that this alkylating agent had a similar cytotoxic effect on both populations of lymphocytes; the initial manifestations of this agent were nonselective in terms of particular categories of lymphoid cells.

Sequential studies (Fig. 1) indicated that in animals treated with cyclophosph-

![Fig. 1. Sequential changes in B and T cells after cyclophosphamide (150 mg/kg) administered on day 0. As noted, both types were profoundly depleted by this alkylating agent. T cells recovered more rapidly than B cells. Asterisks indicate that the value was significantly less than controls; vertical bars represent 1 SE.](image-url)
### Table 2. Effects of Varying Doses of Cyclophosphamide on Blood T and B Lymphocytes

<table>
<thead>
<tr>
<th>Drug Dose (mg/kg)</th>
<th>Day 2</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T cells × 10⁹/liter</td>
<td>B cells × 10⁹/liter</td>
</tr>
<tr>
<td>0</td>
<td>2.52 ± 0.29</td>
<td>0.43 ± 0.06</td>
</tr>
<tr>
<td>25</td>
<td>1.99 ± 0.30</td>
<td>0.25 ± 0.03*</td>
</tr>
<tr>
<td>50</td>
<td>1.65 ± 0.42*</td>
<td>0.18 ± 0.03*</td>
</tr>
<tr>
<td>100</td>
<td>1.45 ± 0.40*</td>
<td>0.09 ± 0.03*</td>
</tr>
<tr>
<td>150</td>
<td>1.03 ± 0.14*</td>
<td>0.14 ± 0.03*</td>
</tr>
<tr>
<td>300</td>
<td>0.90 ± 0.32*</td>
<td>0.02 ± 0.01*</td>
</tr>
</tbody>
</table>

Values are means ± 5E.

*Significantly different from controls (minimum significance p < 0.05).
phamide, T cells recovered at a more rapid rate than B cells. By day 8, the number of T cells had risen to the normal range; B cells continued to be markedly reduced through day 11. By day 14, the number of bone marrow–derived cells in the peripheral blood had returned to normal. Additional studies, shown in Table 2, indicated that cyclophosphamide's lymphocytotoxic effect was a dose-dependent phenomenon.

Chlorambucil, another alkylating agent, caused a less striking but also nonselective reduction in both lymphoid populations (Table 1). By day 2, both T and B cells were reduced by approximately 30%; these changes persisted through day 5. Thus, chlorambucil had a lymphocytotoxic effect which was qualitatively similar to cyclophosphamide, but the magnitude of the reduction was considerably less pronounced. In contrast to the alkylating agents, neither 6-MP nor methotrexate caused any appreciable changes in blood lymphocytes. At both time points, the number of both T and B cells remained equivalent to controls.

The lymphopenic effect of a large dose of hydrocortisone was also assessed. In peripheral blood samples there was no change in either cell type on day 2. However, both T and B lymphopenia was apparent on day 5; the former was reduced by 36% and the latter by 40%. As the reduction of both elements was of similar magnitude, it appears that the initial effects of steroids are nonselective with respect to different classes of lymphocytes.

In general, the changes in lymph node T and B cellularity paralleled those observed in the peripheral blood (Table 3). The most pronounced effects occurred in animals receiving cyclophosphamide. Two days after administration of this alkylating agent, the number of T cells had decreased by 57%, and B cells by 66%. The reduction in both elements persisted through day 5. At that time, T cells were reduced by 65% and B cells by 74%. Thus, nodal changes closely correlated with those observed in the peripheral blood specimens.

Despite the profound reduction in lymphocytes caused by cyclophosphamide, the percentage of T and B cells in both the node and the blood varied only slightly from control values. The relatively constant ratios between the two types is additional evidence suggesting that the initial cell-depleting effect is comparatively nonselective with respect to different functional classes of lymphocytes.

In accord with the blood findings, T-cell restoration in lymph nodes occurred earlier than that of B lymphocytes. The recovery paralleled blood lymphocytes: T cells were within the normal range by day 8; B cells remained decreased until day 14.

Chlorambucil caused a significant reduction in lymph node B cells on day 2, but these cells were within the normal range by day 5. T cells, although slightly decreased, were within the normal range at both time points. The maximum reduction in B cells was 46%. Neither 6-MP nor methotrexate reduced either cell population in lymph node suspensions, a finding in accord with those seen in the peripheral blood.

With hydrocortisone, changes in the lymph node appeared to precede those in the peripheral blood. Both T and B cells were decreased in the nodal suspensions on day 2. At this time, the peripheral blood did not show alterations
Table 3. Changes in Lymph Node Cellularity After Administration of Immunosuppressive Drugs

<table>
<thead>
<tr>
<th>Drug</th>
<th>Wt. (mg)</th>
<th>T Cells</th>
<th>B Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>0.77 ± 0.07</td>
<td>54.5 ± 2.1</td>
<td>77.1 ± 7.8</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>0.49 ± 0.04</td>
<td>61.5 ± 3.7</td>
<td>33.1 ± 6.5</td>
</tr>
<tr>
<td>Chlorambucil</td>
<td>0.60 ± 0.06*</td>
<td>56.8 ± 2.1</td>
<td>61.2 ± 12.0</td>
</tr>
<tr>
<td>6-Mercaptopurine</td>
<td>0.72 ± 0.02</td>
<td>53.1 ± 6.2</td>
<td>65.8 ± 16.1</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>0.70 ± 0.05</td>
<td>56.8 ± 7.4</td>
<td>68.0 ± 7.3</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>0.55 ± 0.04*</td>
<td>44.3 ± 2.8*</td>
<td>33.2 ± 6.3*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Drug</th>
<th>Wt. (mg)</th>
<th>T Cells</th>
<th>B Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>0.48 ± 0.05*</td>
<td>55.5 ± 4.2</td>
<td>26.7 ± 9.4*</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>0.67 ± 0.06</td>
<td>55.5 ± 4.6</td>
<td>63.7 ± 18.1</td>
</tr>
<tr>
<td>Chlorambucil</td>
<td>0.76 ± 0.04</td>
<td>56.9 ± 6.2</td>
<td>72.7 ± 13.7</td>
</tr>
<tr>
<td>6-Mercaptopurine</td>
<td>0.78 ± 0.07</td>
<td>54.3 ± 2.2</td>
<td>74.0 ± 23.2</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>0.62 ± 0.09</td>
<td>47.8 ± 4.9</td>
<td>46.2 ± 7.2*</td>
</tr>
</tbody>
</table>

Values are means ± SE.
*Significantly different from controls (minimum significance p < 0.05).
Fig. 2. Effects of five daily doses of immunosuppressants on the number of T and B cells in the peripheral blood. Values are expressed as percent of normal; asterisks indicate the mean is significantly different from controls. The top panel refers to determinations made 2 days after the last dose of each drug, the lower, 5 days. Values for lymph nodes paralleled those for the blood and there were no significant differences in numbers of residual cells 1 day after cessation of drug therapy as compared to 2 days. Abbreviations: Cy, cyclophosphamide (20 mg/kg/day); Chl, chlorambucil (1 mg/kg/day); 6MP, 6-mercaptopurine (20 mg/kg/day); MTX, methotrexate (12.5 mg/kg/day); HC, hydrocortisone acetate (50 mg/kg/day).

in either cell type. By day 5, the number of B cells in the nodes had increased to the normal range; T cells were still moderately decreased. As noted previously, both elements were decreased in the blood samples analyzed on day 5.

Additional studies evaluated the effects of daily drug administration. Groups of guinea pigs received a 5-day course of one agent; lymphocyte studies were performed 1, 2, and 5 days after the last dose. Results indicated that the changes in blood lymphocytes were qualitatively similar to those observed after a single large dose. Two days after discontinuation of drug administration cyclophosphamide (20 mg/kg/day) reduced the number of T cells by 61% and B cells by 91%. T cells were normal by day 5; B cells continued to show a moderate depression (38% decrease from controls). Chlorambucil (1 mg/kg/day) caused a less pronounced reduction in both cell populations and the numbers of each were restored by day 5.

Consecutive doses of the two antimetabolites, 6-MP and methotrexate, did not cause significant alterations in either functional type of lymphocyte. Furthermore, hydrocortisone did not cause any decreases in peripheral blood lymphocytes. In fact, the number of both elements 1 and 2 days after the last injection of this steroid was slightly increased. Figure 2 depicts the percentage surviving T and B cells in animals evaluated on day 2 (upper panel) and day 5 (lower panel). Values determined on day 1 were not significantly different from those measured on day 2. Changes in T and B lymph node cellularity in all test groups were similar to those observed in the peripheral blood.

DISCUSSION

Results of these studies indicate that in guinea pigs the initial lymphocytoxic activity of immunosuppressants is nonselective with respect to the cell type. Rather, the major factor influencing changes in both peripheral T and B cells appears to be the cell-cycle specificity of the drug. Antimetabolites, such as 6-MP and methotrexate, do not significantly deplete either population. By
contrast, the alkylating agents cyclophosphamide and chlorambucil, which are toxic to both cycling and intermitotic (G0) cells, cause a generalized but initially nonselective reduction in all lymphocytes. The previously reported selectivity of cyclophosphamide for B cells appears to reflect differences in the recovery rate; T cells are reconstituted at a more rapid rate than are B cells.

It has been shown that methotrexate and 6-MP are maximally effective as immunosuppressants when utilized in the interval immediately following initial antigenic stimulation. This time period is characterized by the rapid proliferation of antigen-sensitive cells. These drugs are comparatively ineffective as immunosuppressants once the reaction enters an established phase, a time when most reactive cells are nonreplicating. As shown in this study, following administration of 6-MP or methotrexate there is no reduction in either population of small lymphocytes. These data are in accord with a lack of toxicities for immunologically competent cells which are in an intermitotic phase of their cycle.

In contrast to the antimetabolites, both chlorambucil and cyclophosphamide cause significant reductions in the number of small intermitotic lymphocytes in both the blood and lymph nodes. Data from the present study indicate that the initial lympholysis does not preferentially affect either T or B cells; this finding suggests that these agents are not selectively toxic to one type of lymphocytes.

Using histologic criteria, Turk et al. reported that cyclophosphamide in guinea pigs appeared to deplete preferentially B-cell areas of lymph nodes. As the dose of drug used (300 mg/kg) and the means of assessing individual cells differed from those in the present study, direct comparisons are not possible. Our study showed that cyclophosphamide caused lympholysis of both cell types at all drug quantities greater than 25 mg/kg; the degree of cellular depletion was a dose-dependent phenomenon. In animals receiving 150 mg/kg, the apparent B-cell selectivity was primarily due to differences in the cell recovery rate. Restoration of B-cells was considerably delayed in comparison to the T-cell system.

These results may explain, in part, the different capacities of cyclophosphamide to suppress cell-mediated and humoral immunity. This agent is highly effective in inhibiting primary humoral responses, whereas cell-mediated reactions may be either suppressed or augmented. The more rapid restitution of T cells would account for its variable effects on cellular reactions. B cells, however, remain subnormal for a longer interval—an observation in accord with the drug's more pronounced inhibition of humoral immunity. The augmented T-cell responses may be due to cyclophosphamide's toxicity for a population of suppressor lymphocytes.

Because of the lack of certain undesirable side effects, chlorambucil has been proposed as a substitute for cyclophosphamide in immunosuppressive protocols. Although chlorambucil causes a modest reduction in lymphocytes, it appears that this agent does not have the same lymphocytotoxic activity as cyclophosphamide. This observation is in agreement with its lesser immunosuppressive activities. The differences in effectiveness cannot be explained by dose differences; in the present study, drug therapy has produced similar rates of mortality, and normal hematopoiesis is suppressed to the same extent with both agents.
The lymphocytotoxic effects of cytotoxic drugs have been further compared with corticosteroids. It has been shown previously that with respect to the sensitivity of lymphocytes to steroids, the guinea pig, like man, is a resistant species. Furthermore, Fauci and Dale have shown that a major effect of corticosteroids in both guinea pigs and man is to alter the distribution of lymphocytes. The present study also indicates that in extremely large quantities hydrocortisone has an additional effect in that it causes a modest, non-selective lympholysis. These data may be relevant to certain clinical situations in that they provide a basis for the use of massive steroid doses in the therapy of certain life-threatening immune reactions. In contrast, smaller quantities, administered daily, do not reduce either lymphocyte population.

These results may have applicability to the selection of a cytotoxic agent and, possibly, its mode of administration in the treatment of ongoing immune responses. The major determinant to changes in total numbers of peripheral lymphocytes is the pharmacologic activity of a cytotoxic drug, particularly its toxicity for intermitotic cells. As such, cyclophosphamide has proved to be a highly effective lymphocytotoxic drug. The initial effects, which are dose dependent, are nonselective for T and B cells. Selectivity appears to result because B-cell recovery is prolonged in comparison to that of T cells. These data suggest that large intermittent doses might achieve a somewhat selective inhibition of humoral reactions, whereas chronic daily administration can impair both immune responses.

ACKNOWLEDGMENT

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REFERENCES

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