Inhibition of Immune Responses by Glutamine Antagonism: Effect of Azotomycin on Lymphocyte Blastogenesis

Evan M. Hersh and Barry W. Brown

SUMMARY

The effects of the glutamine antagonist azotomycin on the immune response in vitro was studied by measuring the effect of the drug on the blastogenic responses of human lymphocytes to mitogens. Doses of 1.0 µg/ml or greater completely inhibited responses to phytohemagglutinin, streptolysin O, and allogenic leukocytes. Both thymidine-³H incorporation and morphological manifestations of blastogenesis were inhibited. Inhibition could be achieved without cytotoxicity, as demonstrated by stable viable cell counts and restoration of responses by washing the drug from the cultures. Specificity of action was proved by complete reversal of inhibition by L-glutamine. Addition of a wide range of L-asparaginase doses markedly increased the inhibition produced by azotomycin. Certain dosage combinations were synergistic. This study suggests that, depending upon pharmacological factors, azotomycin, either alone or in combination with L-asparaginase, may produce potent immunosuppression in vivo.

INTRODUCTION

The blastogenic and mitotic responses of human peripheral blood lymphocytes in vitro are currently interpreted as manifestations of the individual’s immunological competence and commitment (23). Recent studies of the effects of drugs added directly to lymphocyte cultures have suggested that inhibition of blastogenesis may predict for inhibition of the primary immune response in vivo (20—22). In a continuing search for immunosuppressive agents with minimal myelosuppressive activity, we studied the immunosuppressive effects of the antibiotic azotomycin. This antitumor agent is a specific glutamine antagonist (17) which is not markedly myelosuppressive at maximally tolerable doses (2). It was found to inhibit lymphocyte blastogenesis reversibly without marked lymphocyte cytotoxicity in vitro and to exhibit synergism with L-asparaginase. These results suggested that azotomycin administration, either alone or in combination with other agents, might be a potent immunosuppressive regimen in vivo.

MATERIALS AND METHODS

All lymphocyte cultures were done with the peripheral blood leukocytes of healthy 20- to 50-year-old volunteers. Venous blood was defibrinated with glass beads, and the red cells were sedimented with 0.1 volume of 4% dextran (M.W. 250,000). The white cell count of the leukocyte-rich dextran was determined, and the cell concentration was adjusted to 10⁶ lymphocytes/ml with autologous serum. Lymphocyte cultures were set up containing 10⁶ lymphocytes, 1 ml serum, and 2 ml Spinner modified minimal essential medium (Hyland Laboratories, Los Angeles, Calif.). This was supplemented with penicillin and streptomycin and 0.2 mM L-glutamine. For the mixed leukocyte cultures, 1.0 X 10⁶ lymphocytes from each of the 2 individuals of the mix were set up under the above culture conditions. A typical set of cultures included an unstimulated control culture, a PHA²-stimulated culture, a culture stimulated with SLO, and a mixed leukocyte culture. PHA and SLO were supplied by Difco Laboratories, Detroit, Mich.

 Cultures were incubated at 37° in 5% CO₂ in air. The PHA cultures were harvested at 3 or 5 days, SLO cultures were harvested at 5 days, and mixed cultures were harvested at 7 days. Cultures were incubated with 2 µCi tritiated thymidine (Schwarz BioResearch, Orangeburg, N. Y.) during the last 3 hr of the culture period. The cultures were then harvested, the cells washed with 0.9% NaCl solution, and the incorporated, acid-insoluble radioactivity was collected by trichloroacetic acid precipitation, dissolved in Hyamine (Packard Instruments, Downers Grove, Ill.), diluted in phosphor-toluene solution, and counted in a liquid scintillation counter (Packard). Blastogenesis was measured as cpm/10⁶ cultured lymphocytes in the stimulated cultures minus cpm/10⁶ cultured lymphocytes in the unstimulated control cultures.

Azotomycin (manufactured by Charles Pfizer and Co., Maywood, N. J.) was supplied in lyophilized form as the monosodium salt. It was diluted in sterile, preservative-free water before use. It was used either fresh or frozen and freshly thawed. The diluted material was never frozen and thawed more than once. L-Glutamine (Hyland) was diluted from a stock supplied as a 200 mM solution. It was stored frozen, 

¹The abbreviations used are: PHA, phytohemagglutinin; SLO, streptolysin O.

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thawed immediately before use, and never frozen and thawed more than once. L-Asparaginase (Merck, Sharp and Dohme Research Laboratories, West Point, Pa.) was supplied lyophilized and was diluted with sterile, preservative-free water before use. It was stored frozen and thawed before use. It was previously found that repeated freezing and thawing did not impair the potency of L-asparaginase (22). All reagents were added to the cultures at the beginning of the culture period unless otherwise noted.

In experiments in which one of the above reagents was removed by washing, the following procedure was used. Cultures were centrifuged at 1000 rpm (230 x g) for 10 min, the supernatant was discarded, and the cells were washed twice with 5 ml Hanks' solution (Hyland) containing 5% fetal bovine serum (Hyland). At the end of this procedure, the cells were resuspended in 3 ml Spinner modified minimal essential medium containing 33% human serum (usually but not always autochthonous). Mitogens were either omitted or readded at this point, and cultures were incubated for the remaining portion of the 3-, 5-, or 7-day culture period.

For in vitro lymphocyte survival studies, cultures were set up in the usual manner and incubated at 37° without mitogenic stimulation; total viable cell counts were done at 3, 5, 7, and 10 days. Cell viability was determined by trypan blue dye exclusion.

For assessment of the significant degrees of chemical immunosuppression in vitro, the natural variability of the number of counts in replicated samples and hence the smallest difference which would be unlikely to arise from chance alone was estimated. Data from 41 preliminary experiments, each of which contained 3 to 4 sets of 4 to 10 replicate cultures, were used for this purpose. These experiments covered all commonly encountered conditions including unstimulated cultures and PHA-, SLO-, and WBC-stimulated cultures.

Statistical examination of the data (27) showed that the variability in counts was greater for those experiments in which the mean count was higher. The proportional variation was roughly constant, however, so log values were taken. With this transformation, the variance in counts in most experiments was less than 0.1. This value was taken as a conservative estimate of the variance of the log counts in replicate samples.

If we had 2 samples, then the variance of the difference of the log counts would be 0.2, and the square root of this (the standard error of the difference) is 0.447. The difference should exceed 1.65 times its standard error only 1 time in 20 by chance alone. We thus take 1.65 X 0.477 as the minimal significant log differences. This corresponds in raw counts to a ratio of the values of about 2. Hence the criterion of minimal significant response or inhibition is that the number of experimental counts must be twice or one-half the number of control counts.

RESULTS

The dose-response curves for the effects of azotomycin on the in vitro blastogenic response to PHA is illustrated in Chart 1 and is compared to the effect of the drug on the responses to SLO and allogeneic leukocytes in Chart 2. Blastogenic responses to PHA and SLO usually were significantly inhibited by 0.01 μg/ml azotomycin, but mixed cultures were inhibited only at doses of 0.1 μg/ml and greater. A dose of 1.0 μg/ml inhibited the PHA, SLO, and mixed leukocyte responses over 97%. A dose of 2.5 μg/ml or greater completely inhibited the blastogenic response to each of these mitogens. It can be seen that the mixed leukocyte response was consistently slightly less inhibited than the responses to PHA and SLO.

This inhibition of thymidine incorporation actually reflected an inhibition of blastogenesis. In 2 experiments (Table 1), cultures for thymidine incorporation and slide preparations were set up and harvested simultaneously. A parallel inhibition of morphological and biochemical blastogenesis was noted. In contrast, there was somewhat less inhibition of uridine-3H incorporation (Table 2). This is compatible with the known major inhibitory effects of azotomycin on DNA synthesis.

Onset of azotomycin action was determined by the simultaneous addition of azotomycin and 2 μCi thymidine-3H to 72-hr PHA-stimulated cultures (Table 3). Inhibition of thymidine-3H incorporation was not noted until after 2 hr of incubation with the drug.

This inhibition of blastogenesis was achieved in part without significant cytotoxicity. Experiments were set up to study the in vitro survival of lymphocytes exposed to different doses of the drug. The results are illustrated in Chart 3, which represents a typical experiment. At 5 days, survival was reduced moderately only by the 5-μg/ml dose of drug. At 9
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days, survival was moderately reduced by 0.1 or 1.0 µg/ml, doses that produced vigorous inhibition of blastogenesis. Doses of 5.0 µg/ml produced marked cytotoxicity by 9 days, and only 17% lymphocytes survived at this time.

The relative lack of in vitro cytotoxicity was confirmed by the fact that the drug could be washed from the cultures after at least 24 hr of exposure with subsequent blastogenesis remaining relatively normal (Table 4). Thus cells washed free of drug can still respond to mitogen. In these experiments, mitogen and drug were added simultaneously and both washed from the cultures after various times after which mitogen was readded. At 48 hr, cytotoxicity (manifested by inhibition of

<table>
<thead>
<tr>
<th>Time of azotomycin and thymidine-3H administration</th>
<th>0</th>
<th>0.1</th>
<th>1.0</th>
<th>5.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 min</td>
<td>1.5</td>
<td>1.6</td>
<td>1.6</td>
<td>1.9</td>
</tr>
<tr>
<td>5 min</td>
<td>5.6</td>
<td>4.8</td>
<td>4.3</td>
<td>4.6</td>
</tr>
<tr>
<td>10 min</td>
<td>10.7</td>
<td>9.0</td>
<td>7.0</td>
<td>8.9</td>
</tr>
<tr>
<td>30 min</td>
<td>32.5</td>
<td>28.0</td>
<td>27.0</td>
<td>26.8</td>
</tr>
<tr>
<td>60 min</td>
<td>55.0</td>
<td>54.7</td>
<td>44.9</td>
<td>44.5</td>
</tr>
<tr>
<td>120 min</td>
<td>60.9</td>
<td>37.4</td>
<td>28.4</td>
<td>24.5</td>
</tr>
<tr>
<td>240 min</td>
<td>65.4</td>
<td>51.9</td>
<td>32.2</td>
<td>32.6</td>
</tr>
<tr>
<td>480 min</td>
<td>91.3</td>
<td>57.4</td>
<td>42.2</td>
<td>30.2</td>
</tr>
<tr>
<td>24 hr</td>
<td>172.3</td>
<td>59.6</td>
<td>31.0</td>
<td>23.1</td>
</tr>
</tbody>
</table>

Table 3

Onset of azotomycin action after addition to PHA cultures

Table 1

Relationship between inhibition of blastogenesis measured by morphological and biochemical parameters

<table>
<thead>
<tr>
<th>Azotomycin dose (µg/ml)</th>
<th>% blasts</th>
<th>cpm&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PHA response</th>
<th>% blasts</th>
<th>cpm&lt;sup&gt;a&lt;/sup&gt;</th>
<th>SLO response</th>
<th>% blasts</th>
<th>% blasts</th>
<th>SLO response</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>82</td>
<td>44.1</td>
<td></td>
<td>19</td>
<td>81.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>35</td>
<td>11.5</td>
<td></td>
<td>26</td>
<td>50.7</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>19</td>
<td>3.8</td>
<td></td>
<td>10</td>
<td>1.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>6</td>
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<td>0.00</td>
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<td></td>
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<tr>
<td>20.0</td>
<td>2</td>
<td>0.06</td>
<td></td>
<td>0</td>
<td>0.00</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> cpm/10<sup>6</sup> lymphocytes of thymidine-3H incorporation x 10<sup>3</sup>.

Table 2

Effect of azotomycin on lymphocyte DNA and RNA synthesis responses to PHA stimulation

<table>
<thead>
<tr>
<th>Duration of culture (hr)</th>
<th>Thymidine-3H</th>
<th>Uridine-3H</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0</td>
<td>0.1</td>
</tr>
<tr>
<td>24</td>
<td>0.08</td>
<td>0.09</td>
</tr>
<tr>
<td>48</td>
<td>25.7</td>
<td>45.3</td>
</tr>
<tr>
<td>72</td>
<td>85.6</td>
<td>66.1</td>
</tr>
<tr>
<td>96</td>
<td>73.0</td>
<td>39.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Significant inhibition of isotope incorporation.
In vitro cytotoxicity of azotomycin on lymphocytes after following time of exposure to drug and mitogen

<table>
<thead>
<tr>
<th>Drug dose (µg/ml culture)</th>
<th>Continuous exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 hr</td>
</tr>
<tr>
<td>PHA</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>53,900</td>
</tr>
<tr>
<td>0.1</td>
<td>51,900</td>
</tr>
<tr>
<td>1.0</td>
<td>49,200</td>
</tr>
<tr>
<td>5.0</td>
<td>45,200</td>
</tr>
<tr>
<td>SLO</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>32,900</td>
</tr>
<tr>
<td>0.1</td>
<td>33,600</td>
</tr>
<tr>
<td>1.0</td>
<td>32,700</td>
</tr>
<tr>
<td>5.0</td>
<td>33,200</td>
</tr>
<tr>
<td>Allogeneic WBC</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>3,200</td>
</tr>
<tr>
<td>0.1</td>
<td>6,800</td>
</tr>
<tr>
<td>1.0</td>
<td>3,300</td>
</tr>
<tr>
<td>5.0</td>
<td>7,500</td>
</tr>
</tbody>
</table>

* Mitogen and drug removed, mitogen readded after this time.

Cytotoxic effects of azotomycin: effect of no readdition of PHA after drug removal

<table>
<thead>
<tr>
<th>Dose of azotomycin (µg/ml)</th>
<th>1 hr</th>
<th>4 hr</th>
<th>24 hr</th>
<th>48 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>0.0</td>
<td>66.3</td>
<td>74.0</td>
<td>59.6</td>
<td>107.9</td>
</tr>
<tr>
<td>0.1</td>
<td>76.4</td>
<td>89.8</td>
<td>46.5</td>
<td>138.1</td>
</tr>
<tr>
<td>1.0</td>
<td>92.8</td>
<td>102.2</td>
<td>29.8</td>
<td>83.6</td>
</tr>
<tr>
<td>5.0</td>
<td>78.7</td>
<td>87.6</td>
<td>11.5</td>
<td>77.7</td>
</tr>
</tbody>
</table>

* A, PHA not restored after washing; B, PHA restored after washing.
restored by 120 mM L-glutamine. If the glutamine was added after the addition of the azotomycin rather than simultaneously with the drug, it was significantly less effective in the restoration of blastogenesis. Table 6 shows a series of azotomycin-treated PHA cultures to which 20 mM L-glutamine was added at 0 to 72 hr and which were harvested at 120 hr. When L-glutamine was added after 24 hr, it failed to reverse the effects of 1 µg/ml or more of azotomycin.

In contrast to reversal of the azotomycin effect by L-glutamine, L-asparagine in concentrations up to 10 µM had no effect on the inhibitory effects of even low doses of azotomycin.

Since L-glutamine is a precursor involved in the synthesis of L-asparagine (8), the effects of simultaneous exposure of the cultures to azotomycin and L-asparaginase was explored. The results of a typical experiment are shown in Chart 5. For each dose of azotomycin, the simultaneous addition of L-asparaginase produced further suppression of the blastogenic response. This was true at doses of L-asparaginase, or doses of azotomycin which did not themselves inhibit blastogenesis in vitro. Also, there were dose combinations in which true synergism of the suppressive effects of these drugs were observed. Thus, Table 7 shows the particular dosage combinations from 4 experiments, in which the product of the percentage of reduction in blastogenesis induced by each drug alone yielded a predicted value for blastogenesis inhibited by the combination, which was significantly greater than the actual blastogenesis observed in cultures treated by the combination. This was interpreted as evidence of synergism.

**DISCUSSION**

The in vitro lymphocyte culture system has proved to be a useful method for the identification of a given individual's immunological commitment (3, 6, 10) and for the evaluation of states of immunological deficiency. Progress has been made in defining the defect in primary immunological deficiency states (11, 15, 18) and immunological deficiency associated with cancer (13, 24, 25). In addition, immunological deficiency induced by chemotherapy can be evaluated with this system (14, 21, 30), and it can be used to evaluate chemotherapeutic agents by their direct addition to cultures of normal leukocytes (5, 22, 28).

The current study represents use of this lymphocyte culture system to explore the potential immunosuppressive effects of the glutamine antagonist azotomycin. It was observed that doses as low as 1.0 µg/ml added at the beginning of the culture period completely inhibited all blastogenic responses. Of great
Immunosuppression by Azotomycin

Table 7

Synergism between azotomycin and L-asparaginase

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Azotomycin dose (μg/ml)</th>
<th>Asparaginase dose (i.u./ml)</th>
<th>cpm/10⁶ lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No drug</td>
<td>Azotomycin</td>
<td>Asparaginase</td>
</tr>
<tr>
<td>1</td>
<td>0.1</td>
<td>0.01</td>
<td>74,000</td>
</tr>
<tr>
<td>2</td>
<td>0.1</td>
<td>0.01</td>
<td>56,000</td>
</tr>
<tr>
<td>3</td>
<td>0.1</td>
<td>0.01</td>
<td>24,000</td>
</tr>
<tr>
<td>4</td>
<td>1.0</td>
<td>0.001</td>
<td>22,500</td>
</tr>
<tr>
<td>5</td>
<td>1.0</td>
<td>0.001</td>
<td>22,500</td>
</tr>
</tbody>
</table>

interest was that, at least at this dose, azotomycin showed little direct cytotoxicity to resting lymphocytes maintained in culture for up to 10 days. That the specific mechanism of action of the drug was as a glutamine antagonist was proved by the fact that the lymphocyte function was restored to normal by the simultaneous addition of L-glutamine.

The mechanism of action of the glutamine antagonists is complex as would be expected from the multiple sites of participation of glutamine in biosynthetic pathways and the varying content of glutamine synthetase of various tissues (8, 9, 16, 19). The mechanisms have been worked out mainly with the drugs azaserine and 5-diazo-4-oxo-L-norleucine (7). The major mechanism is the inhibition of purine biosynthesis via irreversible binding to the SH group of formylglycinamide ribotide amidotransferase, preventing conversions of formylglycinamidine ribotide to formylglycinamide ribotide (1). The supply of adenine is thus markedly reduced, decreasing the availability of DPN and TPN. Thus, glutamine antagonists are cell cycle active and would be expected to inhibit mainly dividing cells. In addition, these diazo compounds may have alkylating agent properties. Thus, there is some evidence that azaserine binds to DNA and interferes with template function (26). In the current study, as well as in a number of other systems, the addition of glutamine partially or completely prevented the effects of the glutamine antagonist. The late addition of the L-glutamine was much less effective, and this was to be expected since the binding of the antagonist to the enzyme is irreversible.

The inhibition of blastogenesis by azotomycin was found to be enhanced synergistically by the simultaneous addition of L-asparaginase. This observation is compatible with the fact that glutamine antagonists and L-asparaginase are synergistic in a number of animal tumor systems (29). The mechanism of this synergism probably relates to the fact that L-asparagine is synthesized in vitro from aspartic acid and L-glutamine via the action of asparagine synthetase (8). The simultaneous presence of both azotomycin and asparaginase decreases not only the product of the enzyme reaction but also the substrate.

The results described here suggest that azotomycin alone or the drug in combination with L-asparaginase may be potent immunosuppressants in vivo. Indeed, recent studies indicate that it is immunosuppressive in the mouse, and 1 to 5 mg/kg/day reduce the antibody titer to sheep RBC 80%, when measured 6 days after immunization (E.M. Hersh, unpublished observations). Lymphoid tissue is relatively deficient in glutamine synthetase (9) and more susceptible than other normal tissues to the inhibition of purine biosynthesis by L-glutamine antagonists (19). This probably explains why azotomycin has its major effects in experimental tumor systems or lymphoid cancers (4). Lymphocyte blastogenesis is inhibited completely by low doses of either L-asparaginase or azotomycin in vitro, and the combination is synergistic. The dose-limiting toxicity is not myelosuppressive for either drug but relates rather to gastrointestinal (2) and central nervous system effects (11). Therefore, immunosuppressive therapy with these agents may not be associated with the increased susceptibility to infection characterized by immunosuppressive doses of thiopurines, alkylating agents, and folic acid analogs (12). This should make azotomycin alone or in combination with L-asparaginase a potent immunosuppressive combination in man.

ACKNOWLEDGMENTS

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