The Effect of Autologous Serum on Lymphocyte Response to Human Leukemia Cells

By J. Hugh Bryan, Gloria E. Johnson, and Brigid G. Leventhal

The normal lymphocytes of 11 of 18 patients with acute leukemia in remission showed a positive blastogenic response in vitro to autologous leukemia cells. The effect of autologous serum on this response was studied to determine if there were factors which might abrogate or "block" this response. Serum obtained at diagnosis (i.e., during a tumor-bearing period) was compared with remission serum with the expectation of demonstrating a "blocking" effect of the former and no effect or perhaps even facilitation with the latter. However, this was not the case, and no consistent differences in blastogenesis in the presence of the two sera were seen. In addition, during the period of observation, there was little correlation between the presence or absence of in vitro response to autologous tumor and prognosis.

The past few years have seen the rapid accumulation of evidence for the existence of tumor-associated antigens in human malignancies, including acute leukemia. It appears that both humoral and cell-mediated immune responses to these antigens may be important in the host's natural defense against neoplasia.

Lymphocytes obtained from patients with acute leukemia in remission can be stimulated to undergo blastogenesis in the presence of autologous blast cells. In many patients vigorous positive stimulation correlates with good prognosis.

The pioneering work of the Hellstroms and their co-workers has offered one possible explanation for the failure of cellular immune reactions to prevent the progression of clinical malignancies. They have demonstrated factors in the serum of patients bearing solid tumors that will prevent target-cell destruction...
These “blocking” factors may be either antibodies or possibly complexes of soluble tumor antigen and antitumor antibody. However, “blocking” factors generally disappear as patients become tumor free, and there is further evidence that serum from tumor free patients can “unblock” the “blocking” effect of sera from patients bearing tumors of the respective types.

Recently, Gutterman et al. described positive blastogenic responses to autologous blasts in 19 of 24 patients with acute myelocytic leukemia (AML) and five of ten patients with acute lymphocytic leukemia (ALL) who were tested between courses of initial induction therapy. In eight of the 19 AML patients and in one of five with ALL, the positive blastogenic response was completely or partially abrogated when lymphocytes were cultured in autologous rather than allogeneic serum. In addition, there was an almost exact correlation between the inhibition of blastogenesis by autologous serum and the presence of IgG bound to the surface of the patient’s own blasts. Positive blastogenesis, its inhibition by autologous serum, and the presence of bound IgG on the cell surface were indicative of a good prognosis.

In the present study, the lymphocytes of five patients with AML and 13 patients with ALL in remission were tested for an in vitro response to autologous blasts in the presence of the patient’s own serum from the time of initial diagnosis and in the presence of remission serum obtained on the day the experiment was performed. A majority of patients showed positive blastogenesis in response to their own tumor cells; significant abrogation of this response by autologous serum was rarely seen.

MATERIALS AND METHODS

Patients

Of the 18 patients, 13 had ALL and five had AML. There were ten females and eight males with a median age of 13 yr (range 3½–58½ yr). All 18 of the patients were studied during their first remission, a median of 10 mo (range, 1 day–24 mo) from achieving remission status. Remission is defined morphologically as <5% blasts in a normocellular marrow aspirate. Twelve of the 13 patients with ALL had been induced into remission with prednisolone, vincristine, methotrexate, and 6-mercaptopurine (POMP). The remaining patient with ALL failed to achieve remission with the POMP regimen initially but responded to L-asparaginase therapy. Of the five patients with AML, two achieved remission with POMP, two with cytosine arabinoside and 6-thioguanine, and one with Methyl-CCNU [1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosurea]. In all patients, drugs were discontinued at least 72 hr prior to study.

Storage of Malignant Cells

Leukemia blast cells, from the peripheral blood, were collected by leukophoresis at the time of admission to the NIH before any chemotherapy was given and stored in liquid nitrogen as previously described.

Mixed Leukocyte Cultures (MLC)

Whole blood was collected in heparin (Upjohn), 5–10 U/ml. Lymphocytes were separated from the peripheral blood by the Ficoll-Hypaque gradient separation method as described by Stjernsward et al., and the yield generally was 90%–95% pure lymphocytes. Cultures were set up in triplicate in 12 × 75-mm glass tubes with 2 × 10⁶ responding lymphocytes in 1 cc of Eagle’s medium (Flow Labs, Rockville, Md.), supplemented with 10% pooled normal human serum (Flow Labs, Rockville, Md.). Stimulator lymphocytes or blast cells were added at a concentra-
tion of $2 \times 10^5$ in 0.1 ml after inactivation by 5000 R irradiation from a $^{137}$Cs source (Gamma-M, Kewaunee Scientific Engineering, Adrian, Mich.). One-tenth milliliter of serum to be investigated was added to the reaction mixture. "Original" serum was collected at initial diagnosis prior to therapy and stored at $-20^\circ$ C. "Remission" serum was obtained on the day of study. "Control" serum was from a normal male who had never been transfused. Any possible complement in the serum was inactivated by heating to $56^\circ$ C for 20 min prior to use. Cultures were harvested as previously described.16 The degree of stimulation was estimated by the quantity of $^3$H-thymidine incorporated after six or seven days of incubation. Counts per minute were corrected for counting efficiency and quenching, and are expressed as dpm (disintegrations per minute). The results are expressed as the ratio of the mean dpm in cultures where patient normal lymphocytes (A) are stimulated by x-irradiated blast cells (B) over the mean dpm in control cultures where the normal lymphocytes are added to normal lymphocytes which have been x-irradiated (A). The serum in question was added to both experimental and control cultures for each comparison, i.e.,

$$\text{Stimulation ratio} = \frac{\text{mean dpm } A + B}{\text{mean dpm } A + A + \text{ serum No. 1}}$$

Any ratio of two or greater in our laboratory represents statistically significant stimulation. A technically satisfactory MLC is defined as one in which the frozen blast cells stimulated either the patient or an unrelated donor and in which the patient was capable of responding to at least one set of cells from an unrelated donor.

Statistical Analysis

The differences in reactions in the different sera were compared to one another in the following manner:

$$\Delta 1 = \text{mean log dpm } A + B + \text{ serum No. 1} \text{ minus mean log dpm } A + A + \text{ serum No. 1}.$$  
$$\Delta 2 = \text{mean log dpm } A + B + \text{ serum No. 2} \text{ minus mean log dpm } A + A + \text{ serum No. 2}.$$  

$\Delta 1$ is then compared to $\Delta 2$ by an f test of the interaction terms. This test includes an analysis of variance of the logarithms. Values which are statistically different using an f test will also be different if a t test is applied to the same set of numbers. The logarithms were taken in order to stabilize the variance and because the absence of significant interaction on a log scale corresponds to a constant ratio of the mean counts. This statistical test is equivalent to that suggested by Hersh and Brown17 except that the variance is estimated from the current experiment without use of past data.

RESULTS

Eleven of the 18 patients (61%), nine of 13 with ALL, and two of five with AML, demonstrated positive blastogenesis against their own leukemic cells; one patient (No. 15) was studied twice and reacted on both occasions (Table 1). There was no evidence of an inhibitory effect of "original" serum obtained when the patients were first diagnosed and had florid disease (Table 1) except in patient No. 15. In fact, seven of the 11 patients who reacted to autologous blasts had response ratios that were higher in "original" than in "remission" serum (Table 1), although only one patient reacted significantly better in "original" than in "remission" serum (No. 4). Patient No. 15 (DC4) was the only one whose lymphocytes underwent significantly better blastogenesis in "remission" serum as compared with "original" serum (Table 1). When this study was repeated (DCb), the response in the new sample of remission serum was not so vigorous; indeed, all of his responses had fallen to the previous "blocked" level. He relapsed 1 mo after study b.

Blastogenic responses in "control" serum, were examined to rule out the
Table 1. Effect of Autologous Sera on Blastogenesis

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Disease</th>
<th>Original Serum</th>
<th>Remission Serum</th>
<th>Greatest Blastogenesis</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 JD</td>
<td>AML</td>
<td>0.58</td>
<td>0.96</td>
<td>0.28</td>
<td></td>
</tr>
<tr>
<td>2 MM</td>
<td>AML</td>
<td>1.09</td>
<td>1.63</td>
<td>1.50</td>
<td></td>
</tr>
<tr>
<td>3 AF</td>
<td>AML</td>
<td>0.74</td>
<td>0.79</td>
<td>1.10</td>
<td></td>
</tr>
<tr>
<td>4 DW</td>
<td>AML</td>
<td>5.68</td>
<td>3.16</td>
<td>1.42</td>
<td>Original</td>
</tr>
<tr>
<td>5 JM</td>
<td>AML</td>
<td>5.58</td>
<td>2.01</td>
<td>6.24</td>
<td>Remission</td>
</tr>
<tr>
<td>6 RE</td>
<td>ALL</td>
<td>0.87</td>
<td>0.54</td>
<td>0.74</td>
<td></td>
</tr>
<tr>
<td>7 RP</td>
<td>ALL</td>
<td>0.45</td>
<td>0.78</td>
<td>1.17</td>
<td></td>
</tr>
<tr>
<td>8 DL</td>
<td>ALL</td>
<td>0.36</td>
<td>0.84</td>
<td>1.96</td>
<td></td>
</tr>
<tr>
<td>9 JC</td>
<td>ALL</td>
<td>0.41</td>
<td>0.76</td>
<td>1.15</td>
<td></td>
</tr>
<tr>
<td>10 KS</td>
<td>ALL</td>
<td>2.44</td>
<td>2.32</td>
<td>4.37</td>
<td>Remission</td>
</tr>
<tr>
<td>11 PD</td>
<td>ALL</td>
<td>2.97</td>
<td>2.36</td>
<td>0.89</td>
<td>Original</td>
</tr>
<tr>
<td>12 JO</td>
<td>ALL</td>
<td>1.50</td>
<td>4.36</td>
<td>3.10</td>
<td>Original</td>
</tr>
<tr>
<td>13 AG</td>
<td>ALL</td>
<td>6.26</td>
<td>2.30</td>
<td>6.93</td>
<td>Remission</td>
</tr>
<tr>
<td>14 DG</td>
<td>ALL</td>
<td>0.27</td>
<td>2.70</td>
<td>1.17</td>
<td>Original</td>
</tr>
<tr>
<td>15 DC_A</td>
<td>ALL</td>
<td>13.30</td>
<td>2.42</td>
<td>62.80</td>
<td>Remission</td>
</tr>
<tr>
<td>DC_B</td>
<td>ALL</td>
<td>1.42</td>
<td>4.51</td>
<td>2.26</td>
<td>Original</td>
</tr>
<tr>
<td>16 MG</td>
<td>ALL</td>
<td>0.15</td>
<td>17.43</td>
<td>6.82</td>
<td>Original</td>
</tr>
<tr>
<td>17 DH</td>
<td>ALL</td>
<td>1.21</td>
<td>11.05</td>
<td>12.86</td>
<td>Remission</td>
</tr>
<tr>
<td>18 BK</td>
<td>ALL</td>
<td>2.01</td>
<td>5.77</td>
<td>4.09</td>
<td>Original</td>
</tr>
</tbody>
</table>

The possibility that both "original" and "remission" sera contained "blocking" factors and were therefore inhibitory to the recognition of tumor-associated antigens on blast cells by the patient's own lymphocytes (Table 1). In no instance did a patient react to autologous blasts in the presence of allogeneic "control" serum who failed to react in the presence of autologous serum.

In this group of patients, the presence or absence of a positive in vitro response to autologous blasts seemed to have little prognostic value. All patients were studied in their first remission, although in three patients there had been initial unsuccessful chemotherapy before the remission was achieved with the current therapy. The interval in months between achieving remission and date of study was compared for the two groups, those who responded to their own blasts and those who did not (Table 2). For the 11 reactors the median interval is 8 mo (range, 1 day–15½ mo), and for the seven nonreactors the median interval is 14 mo (range, 2–24 mo); the difference is not significant (p = 0.1). When responders are compared to nonresponders in terms of duration of remission and survival, there is also no significant difference. Only five of the study patients have relapsed, and four have subsequently expired.

Table 2. Relationship of Blastogenic Response to Prognosis

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Disease</th>
<th>Blastogenic Response</th>
<th>Date of Remission to Date of Study Months</th>
<th>Remission Duration Median (range)</th>
<th>Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–3</td>
<td>AML</td>
<td>–</td>
<td>10 (2.14)</td>
<td>14 (3–23+)</td>
<td>21+(11,25+)</td>
</tr>
<tr>
<td>4–5</td>
<td>AML</td>
<td>+</td>
<td>4,12</td>
<td>4,22+</td>
<td>16,22+</td>
</tr>
<tr>
<td>6–9</td>
<td>ALL</td>
<td>–</td>
<td>12.5 (9–24)</td>
<td>21+(15–34+)</td>
<td>22+(16–35+)</td>
</tr>
</tbody>
</table>
The reactors and nonreactors were studied at various times in relation to previous therapy. When the time off therapy was compared in reactors and nonreactors, it was the same. Nonreactors had been off therapy for a median of 20 days (range, 6 days–1 yr). Reactors had been off therapy for a median of 21 days (range, 3–52 days).

**DISCUSSION**

The Hellstroms and their colleagues have demonstrated tumor-specific factors in the serum of patients bearing solid tumors that “block” cell-mediated immune responses of sensitized killer lymphocytes to tumor cells. These “blocking” or “enhancing” factors disappear as the patient becomes tumor free and reappear at the time of relapse. Since “blocking” seems to be a property of serum obtained during a disease-bearing interval, we had hoped to demonstrate the phenomenon by comparing the effect of serum obtained after the patient was in complete remission from his disease.

In a report by Gutterman et al., leukemia patients were studied prior to entering remission, and autologous sera were found to inhibit blastogenesis in MLC in eight of 19 patients with AML and one of five patients with ALL. Gutterman hypothesizes that “blocking” may occur when serum factors (?) soluble tumor antigen) react with immunoglobulins coating malignant cells. He feels that malignant cells from patients with ALL do not produce surface immunoglobulin.

The results of the current study confirm previous reports that the normal lymphocytes of patients with acute leukemia will frequently undergo reactive blastogenesis in the presence of autologous blast cells. However, “blocking” of this response by serum obtained at diagnosis was demonstrable in only one patient, No. 15, with ALL who reacted only at the “blocked” level when re-tested 2 mo later. In addition, in one patient with AML (No. 4), the response was better in relapse than remission serum. The explanation for this result is not clear. Other differences between the effect on blastogenesis of the “original” versus “remission” sera appeared to occur in a random fashion and were not statistically significant.

The present method for performing the MLC, i.e., the addition of 0.1 ml of serum under study to our usual culture system, was chosen because Leventhal et al. had found this a workable experimental design for demonstration of “blocking” activity in maternal plasma which could abrogate the blastogenic response of maternal lymphocytes to the mitomycin C-treated lymphocytes of her child. These maternal factors were hypothesized to be antibodies directed against HL-A loci on the stimulating cells. Tumor-specific antigens are “weak” antigens when compared to HL-A determinants and probably do not elicit as vigorous production of antibody. It may be that with a different culture system a “blocking” effect could be demonstrated.

During the reported period of observation we were unable to correlate a positive blastogenic response with a favorable prognosis as has been done by other investigators. Several possible explanations for this observation have occurred to us. In the first place, the original study by Gutterman et al. was performed during induction therapy. It is quite likely that patients who have
good in vitro responses during induction therapy to autologous blasts, as well as recall antigens, are going to have a better prognosis than those who are more profoundly immunosuppressed and therefore more susceptible to potentially fatal complications such as infection. Indeed, such a correlation between general cellular reactivity and prognosis has been reported before.\textsuperscript{19,20} Since our patients were all studied after they had already achieved remission, this therapeutic advantage of intact cellular immune reactivity during remission induction would no longer be an operative factor, and all of these patients would fit into a relatively good prognostic group.

Second, while the difference in this series is not significant, patients demonstrating positive blastogenesis to their leukemia blasts were studied earlier in their course than those who showed a negative response. In a more extensive retrospective review of all patients studied in this laboratory, this pattern has been found to be consistent, with positive reactions to autologous blasts occurring significantly more often in studies performed earlier in remission.\textsuperscript{21} It is felt that these results do not reflect loss of antigenicity during storage, but rather that the positive early responses may represent a recognition of the presence of persistent tumor. If this theory is true, those with positive blastogenic response late in their disease may turn out to have a worse prognosis than those with a negative response after definitive chemotherapy. Further observation will be required to substantiate this hypothesis.

In the current study there was no increase in percentage of positive studies seen in relation to time after chemotherapy, although such a relationship has been reported from this laboratory previously,\textsuperscript{4} and a pattern of recurring peaks of reactivity after individual 5-day courses of methotrexate has been seen.\textsuperscript{8}

**ACKNOWLEDGMENT**

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**REFERENCES**

ing factors" from human tumors capable of abrogating tumor cell destruction by specifically immune lymphocytes. Int J Cancer 9:274, 1972


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