Cloning of Human Lymphocytes Reactive with Autologous Leukemia Cells

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ABSTRACT

The primed lymphocyte typing test has been used to detect leukemia-associated antigens, but interpretation has been difficult because of significant levels of reactivity with normal cells. Elimination of unwanted reactivities could be accomplished by (a) use of the patient's own lymphocytes as responders to the leukemia cells and (b) cloning of the responding cells. Cloning of antigen-activated human lymphocytes can be accomplished through the use of T-lymphocyte growth factor, which permits the long-term growth of antigen-activated lymphocytes. In the study reported here, the remission lymphocytes of a patient with acute myelogenous leukemia were sensitized in culture to the patient's own leukemic myeloblasts and then grown from wells containing one or a few replicating units. Sufficient cells of three clones were grown for further testing of specificity: one responded only to the sensitizing myeloblast but not to normal cells tested; one responded to the sensitizing myeloblasts and one allogeneic myeloblast but not to normal cells; and one responded to none of the cells tested, although it proliferated vigorously with growth factor alone. These results demonstrate the feasibility of cloning human lymphocytes putatively responsive to leukemia-associated antigens in order to improve their discriminatory capacity in the primed lymphocyte typing test. The response pattern observed was that expected of a clone responding to a leukemia-associated antigen.

INTRODUCTION

Specific immunotherapy represents a potentially important form of adjuvant therapy in AML. A large proportion of patients destined to die of this disease develop an undetectable tumor burden early in their courses of treatment, and this is an ideal time for the use of specific antileukemia immunotherapy. Success of this approach requires, however, vigorous demonstration and definition of leukemia-associated antigens. This may be attempted with antisera (10), particularly if the sera are monoclonal (17), but cellular reagents are preferred for antigen detection in this context, since the bulk of evidence now suggests that antitumor effects are mediated by cellular mechanisms (7).

For study of leukemia-associated antigens with cellular reagents, Reinsmoen et al. (16) introduced the PLT test, originally designed to identify HLA-D antigens of the human major histocompatibility complex (22). In this assay, the proliferative response of lymphocytes primed in vitro for 9 to 14 days is accelerated upon restimulation by cells sharing HLA-D determinants with the priming cell. Utilizing lymphocytes from healthy HLA-identical siblings primed against the myeloblasts from patients with AML, Reinsmoen et al. found, in 2 cases, secondary responses to the respective leukemic blasts. However, it has been reported that HLA-identical siblings frequently possess minor stimulating determinants detectable in the PLT test (23). The discrimination between normal cell surface determinants such as these and putative leukemia-associated antigens is technically difficult.

A recently developed methodology has made it feasible to maintain and expand, in long-term culture, activated human T lymphocytes known to mediate antigen recognition and cytotoxic effects (2, 3, 5, 8, 11, 19, 21, 25). The continuous proliferation of these antigen-specific cells is absolutely dependent on the presence of T-cell growth factor found in the crude supernatant of mitogen-stimulated human lymphocytes (HCM) (11). Zarling and Bach (27) were able to grow an AML patient's remission lymphocytes sensitized (a) to pooled allogeneic normal human cells or (b) to autologous leukemia cells plus allogeneic normal cells for 19 days in HCM, after which high levels of cytotoxicity were detected against autologous leukemia cells but not against autologous remission bone marrow cells. Lymphocytes primed to HLA-D-region antigens could also be successfully expanded to large numbers in HCM with retention of specificity (3, 8, 21), and Bach et al. (1) have been able, using HCM, to isolate clones of such cells with restricted proliferative or cytotoxic capacity.

In order to improve the discrimination between autostimulatory normal cell surface determinants and leukemia-associated antigens, we used the patient's own remission lymphocytes as responders in the PLT assay, after we cloned them in the presence of HCM. Using this technique, we were able to grow, for many months, clones of a leukemic patient's remission lymphocytes which possessed the capacity to proliferate upon restimulation by autologous leukemia blasts but not by normal cells.

MATERIALS AND METHODS

Preparation of Lymphocytes. Lymphocytes or leukemic blasts were prepared from heparinized peripheral blood by Ficoll-Hypaque flotation. Mononuclear cells from the gradient interphase were washed, suspended in cryoprotective medium, and frozen with a programmed controlled-rate freezer, after which they were stored in the vapor phase of liquid nitrogen.

Priming of Lymphocytes. Frozen cells were thawed by dropwise addition of fresh cultured medium after melting of the last crystal. Fresh or freshly thawed lymphocytes, (2.5 to 7.5 × 10⁶) were primed by addition of an equal number of irradiated
stimulator cells. Culture medium consisted of Roswell Park Memorial Institute Tissue Culture Medium 1640 supplemented with 25 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, 200 mM L-glutamine, penicillin-streptomycin mixture, and 10 to 20% pooled, screened, heparinized human AB plasma. Cultures were incubated for 7 to 14 days in humidified 5% CO2 in air.

PLT Test. Twenty-five thousand primed responders were mixed with 50,000 irradiated test stimulators in V-bottomed microtiter plates with 200-μl wells. Plates were cultured for 3 days, and each well was pulsed during the final 16 hr of culture with 1 μCi of [3H]thymidine (2 Ci/mmol). Plates were harvested in air.

Memorial Institute Tissue Culture Medium 1640 supplemented with 10% fetal calf serum and 25% HCM. They were originally seeded at 1.0 to 1.5 x 10⁵/ml and cut back to the original cell density every 3 to 4 days, with replenishment of medium and HCM. After several cutbacks, the cells were frozen. Later, the cells were thawed and grown in medium with HCM for 3 days. They were then dispensed into V-bottomed plates at densities of 10, 30, 50, 100, 200, 300, 1000, and 3000/well, 24 wells/density, in 0.2 ml of medium with fetal calf serum and 25% HCM. Twice weekly, one-half of the medium was replaced with fresh medium containing 10% fetal calf serum and 50% HCM. Cells were distributed into additional wells as needed to avoid exceeding a density of 200,000 cells/well. For specificity testing, cells from respective wells were pooled on Day 11 of cloning and washed with HCM-free medium. Cells were plated as responders at 10,000 cells/well with 25,000 irradiated stimulators in 0.2 ml of medium with 10% fetal calf serum and further processed as in the PLT test.

Preparation of HCM. HCM was prepared by a modification⁴ of the method described by Strausser and Rosenburg (25). Peripheral blood lymphocytes from prescreened donors were passed over nylon wool columns. The nonadherent mononuclear cells were cultured at 10⁵/ml in modified McCoy’s Medium 5A with added glutamine, antibiotics, 2% autologous plasma, and 0.08% phytohemagglutinin (Phytohemagglutinin P; Difco) for 48 hr, after which the cells were removed by centrifugation and the supernatants were frozen for subsequent use as HCM.

RESULTS

The remission cells from the AML patient, JJ, were cultured for 9 to 14 days with autologous myeloblasts; they were then tested for a proliferative response after restimulation by the same cells (Table 1, Experiments 1 to 3). A significant recall response was observed when restimulation by autologous myeloblasts (JJbL) was compared with restimulation by autologous lymphocytes (JJ) or medium controls. Cross-reactive proliferation induced by the leukemic cells (BLbL) of the unrelated AML patient, BL, was observed repeatedly. AML Patient BL also responded to his own leukemic cells (Table 1, Experiment 4). Interpretation of the reaction to leukemic cells was complicated by the occurrence of significant responses to the peripheral blood lymphocytes of normal allogeneic donors such as CG, in Experiment 2 or HG, in Experiment 3. Remission lymphocytes primed to autologous leukemic cells should recognize “new” determinants such as leukemia-associated antigens; however, proliferative responses to normal and presumably nonleukemic autologous determinants have been described (6, 14). The likelihood that such determinants might be involved in addition to or instead of leukemia-associated antigens was increased, since we observed responses to normal allogeneic cells.

In order to distinguish among the possibilities, we cloned the primed responding cells. Remission cells from the AML patient, JJ, were sensitized to autologous myeloblasts in a primary culture; then, the primed cells were further cultured in HCM. These cells responded, with substantially increased ([3H]thymidine incorporation, to a 3-day restimulation by autologous leukemic blasts (data not shown). The HCM-dependent primed cells were cloned in the presence of HCM by the limiting dilution technique. Growth was evaluated by counting aliquots from each well on Day 38 of cloning. From the Poisson distribution, it was calculated that there was an average of 1 replicating unit/2000 cells plated; moreover, in the case of 3000 cells, i.e., the highest number plated per well, the probability of 1 population arising from more than 4 clones was less than 2%. Most likely, each population arose from 1 or 2 clones.

Cells from 3 wells, plated at 300, 3000, and 3000/well, were grown over a period of 111 days to a number which made possible their functional testing. The specificity of the clones was probed against a panel of irradiated autologous and allogeneic normal and leukemic cells which were capable of stimulating appropriately primed lymphocytes (data not shown). As seen in Table 2, clone L#29-G8 showed a significant response to autologous leukemic blasts as compared to remission lymphocytes. Clone L#29-G2 specifically reacted with both autologous myeloblasts and allogeneic myeloblasts from BL but not with remission lymphocytes or allogeneic lymphocytes tested. Clone L#29-C5 was unresponsive to any stimulating cell but, as expected, proliferated upon addition of HCM. None of the

### Table 1

<table>
<thead>
<tr>
<th>Stimulators</th>
<th>Experiment 1: JJ vs. JJbL</th>
<th>Experiment 2: JJ vs. JJbL</th>
<th>Experiment 3: JJ vs. JJbL</th>
<th>Experiment 4: BL vs. BLbL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>117 ± 12^a</td>
<td>349 ± 27</td>
<td>273 ± 30</td>
<td>24 ± 7</td>
</tr>
<tr>
<td>JJ</td>
<td>231 ± 27</td>
<td>259^b</td>
<td>409 ± 32</td>
<td>2170 ± 131</td>
</tr>
<tr>
<td>JJbL</td>
<td>616 ± 263</td>
<td>3935 ± 504</td>
<td>511 ± 28</td>
<td>1078 ± 216</td>
</tr>
<tr>
<td>BLbL</td>
<td>135 ± 21</td>
<td>1979 ± 207</td>
<td>618 ± 20</td>
<td></td>
</tr>
<tr>
<td>CG</td>
<td>Subscript x, irradiation at 2,000 rads, HG and CG, normal volunteers.</td>
<td>Mean ± SE.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^a JJ and BL, patients with leukemia; JJbL and BLbL, presentation blasts; Subscript x, irradiation at 2,000 rads, HG and CG, normal volunteers.  
^b Mean ± SE.  
^c Result of a single well.

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clones showed increased [3H]thymidine incorporation in response to phytohemagglutinin.

DISCUSSION

Although the cloned cells proliferated sufficiently to allow initial testing of specificity, their slow growth was a major technical limitation. During the more than 100 days required to produce enough cells for testing, disastrous incubator malfunction and fungal contamination occurred in these experiments. Acceleration of the growth rate to rates achievable in murine systems (18) would minimize the likelihood of such technical setbacks. In part, the slow growth may be a consequence of the use of suboptimal HCM. Screening for more active producers and concentration or purification of the supernatants used as HCM might be expected to improve its growth-supporting properties. It has been reported that phytohemagglutinin in the HCM has inhibitory properties (8), and removal of this material by affinity chromatography may be expected to improve growth. The slow growth and low uptake of [3H]thymidine may also be indications that some of these clones are functional differentiated cells with a relatively limited capacity for proliferation. In support of this was the observation that the clone which showed no reactivity to antigen (L#29-C5) may not matter whether the antigen detected is truly leukemia specific if display of the antigen has predictive value for the clinical course of patients. This potential correlation is easily pursued after expansion of the "cloned" cells to sufficient numbers.

The ultimate proof that antigens detected by such clones are leukemia specific will be difficult. The possibility of a differentiation antigen expressed only on a minute percentage of normal bone marrow stem cells may be the most important alternative, since effective immunotherapy directed at such antigens might cure leukemia at the cost of a life-threatening, selective aplasia.

These cloned populations were not tested for cytotoxic potential because of the limited numbers of cells available. In an analogous murine system, as many as one-half of the proliferating clones were also cytotoxic (20). Clones with cytotoxic activity may be developed for use in passively administered specific immunotherapy. Whether antigen specificity persists forever and whether the clones are devoid of inducible reactivity to other antigens remain to be proved. In addition, it is not known whether such cells have an antitumor effect in vivo. If

Table 2

<table>
<thead>
<tr>
<th>Responders</th>
<th>Experiment 1</th>
<th></th>
<th>Experiment 2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cloned cells from JJ vs. JJbl</td>
<td></td>
<td>Cloned cells from JJ vs. JJbl</td>
<td></td>
</tr>
<tr>
<td>Stimulators</td>
<td>L#29-G8</td>
<td>L#29-G2</td>
<td>Medium</td>
<td>Medium</td>
</tr>
<tr>
<td>Medium</td>
<td>11 ± 0.4*a</td>
<td>10 ± 4</td>
<td>18 ± 3</td>
<td>14 ± 3</td>
</tr>
<tr>
<td>JJ</td>
<td>43 ± 13</td>
<td>60 ± 12</td>
<td>92 ± 14</td>
<td>98 ± 14</td>
</tr>
<tr>
<td>JJbl</td>
<td>26 ± 4</td>
<td>136 ± 19</td>
<td>139 ± 7</td>
<td>139 ± 7</td>
</tr>
<tr>
<td>BL</td>
<td>14 ± 1</td>
<td>63 ± 5</td>
<td>57 ± 0</td>
<td>57 ± 0</td>
</tr>
<tr>
<td>BLbl</td>
<td>19 ± 3</td>
<td>61 ± 3</td>
<td>106 ± 17</td>
<td>106 ± 17</td>
</tr>
<tr>
<td>DK</td>
<td>21 ± 3</td>
<td>23 ± 5</td>
<td>15 ± 1</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>DKbl</td>
<td>19 ± 3</td>
<td>19 ± 1</td>
<td>95 ± 7</td>
<td>95 ± 7</td>
</tr>
<tr>
<td>CG</td>
<td>16 ± 3</td>
<td>30 ± 3</td>
<td>50 ± 12</td>
<td>50 ± 12</td>
</tr>
<tr>
<td>Phytohemagglutinin P</td>
<td>20 ± 2</td>
<td>20 ± 6</td>
<td>107 ± 6</td>
<td>107 ± 6</td>
</tr>
</tbody>
</table>

*a JJ, BL, and DK, leukemia patients' remission lymphocytes; JJbl, BLbl, and DKbl, presentation leukemia cells; CG, normal volunteer; Subscript x, irradiation at 2,000 rads.

*b Mean ± S.E.

adequate testing of this possibility with the clones described here.

Several investigators have previously reported proliferative or cytotoxic responses to leukemic myeloblasts (4, 9, 15, 16, 24, 26–28). These reports support the hypothesis that the tumor possesses leukemia-specific antigens which may form the basis for specific immunotherapy. However, the specificity of the reported responses was only relative, and the capacity of the sensitized lymphocytes to interact with normal cells posed a barrier both to unequivocal demonstration of tumor-specific antigens and to immunotherapeutic use of the sensitized cells with an acceptably low risk of graft-versus-host complications.

The cloning methodology presents a means of averting both of these dilemmas. Clones of autoreactive cells such as those described here may be useful for classification of leukemia. It may not matter whether the antigen detected is truly leukemia specific if display of the antigen has predictive value for the clinical course of patients. This potential correlation is easily pursued after expansion of the "cloned" cells to sufficient numbers.

The results of these experiments suggest the existence of antigen(s) preferentially expressed on the leukemic myeloblasts of JJ and, presumably, also on those of BL. This antigen may be a true leukemia-specific antigen. A variety of additional controls are required to exclude the idea that this represents an altered major or minor histocompatibility antigen or an allelic differentiation antigen of normal myeloblasts. The known autostimulatory properties of mononuclear populations enriched for B-lymphocytes (14) raises the serious concern that the leukemic population is enriched for the same presumably normal property. Limitations of patient blood supply prevented
so, they represent a promising new approach to antitumor therapy.

ACKNOWLEDGMENTS

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REFERENCES


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