MIXED LYMPHOCYTE-TUMOR CELL REACTION BETWEEN REMISSION LYMPHOCYTES AND AUTOCHTHONOUS LEUKEMIA CELLS AND ITS RELATIONSHIP TO THE PROGNOSIS OF ACUTE LEUKEMIA*1

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The mixed lymphocyte-tumor cell reaction (MLTR) between peripheral lymphocytes and cryopreserved autochthonous leukemia cells was studied in 36 adult patients with acute leukemia after complete remission as a result of chemotherapy. In 20 patients the lymphocytes showed a significant blastogenic response to autochthonous leukemia cells. Eleven out of 18 patients with acute myeloblastic leukemia and 4 out of 5 with acute monocytic leukemia showed positive MLTR, whereas 5 out of 13 with acute lymphoblastic leukemia gave a positive reaction. Cryopreserved leukemia cells of the patients with not only positive but also negative MLTR were able to stimulate allogeneic lymphocytes, except in one case.

The relationship between MLTR and the prognosis of leukemia was analyzed in 30 patients who were alive more than 6 months after diagnosis. No apparent correlation was observed between MLTR and the survival time from diagnosis.

Key words: Mixed lymphocyte-tumor cell reaction (MLTR) — Human acute leukemia — Prognosis of acute leukemia — Lymphocyte leukemia

Immunotherapy has aroused great interest for the treatment of acute leukemia since the first report of its effectiveness by Mathé et al.14 If immunotherapy is to be applied to human leukemia, however, it is essential to confirm that the leukemia cell possesses a tumor-specific antigen provoking an immune response. There have been a number of reports that lymphocytes from patients with acute leukemia in complete remission were stimulated by cryopreserved leukemia cells.1, 3, 5, 11, 16, 18, 20 This mixed lymphocyte-tumor cell reaction (MLTR) was assumed to demonstrate the existence of tumor-specific or tumor-associated antigens on the surface of the leukemia cells.

Since 1974 we have conducted MLTR in 36 adult patients with acute leukemia and observed that the lymphocytes of some patients were stimulated by cryopreserved autochthonous leukemia cells. We could not, however, find any correlation between MLTR and the prognosis of the patients.

MATERIALS AND METHODS

Patients Thirty-six adult patients with acute leukemia in complete remission were included in this study. There were 18 patients with acute myeloblastic leukemia (AML), 5 with acute monocytic leukemia (AMoL) and 13 with acute lymphoblastic leukemia (ALL). Remission had been induced by a combination of daunorubicin, cytosine arabinoside, 6-mercaptopurine riboside and prednisolone (DCMP)15 for AML and AMoL, and by a combination of vincristine and prednisolone (DVP) for ALL. After 2 to 3 courses of consolidation therapy with the same regimens, maintenance therapy was given with DCMP or

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a combination of vincristine, 6-mercaptopurine, cyclophosphamide and prednisolone for AML and AMoL, and with DVP or a combination of methotrexate and 6-mercaptopurine for ALL.

**Cryopreservation of Leukemia Cells** Leukemia cells were obtained when the patients were first admitted. When the leukemia cells consisted of more than 80% of peripheral leukocytes, they were separated from the leukocyte-rich plasma of heparinized peripheral blood which had been left standing for 20 to 30 min. When the leukemia cells consisted of less than 80% of peripheral leukocytes, they were obtained from the bone marrow by centrifuging the marrow aspirate at 400g for 30 min on Ficoll-Conray gradients. The cells thus obtained, of which more than 90% were leukemia cells, were suspended in RPMI-1640 medium with 10% fetal calf serum and 10% dimethyl sulfoxide. They were cooled and frozen gradually at 4º for 1 hr, -20º for 2 hr and -80º overnight, and preserved in liquid nitrogen (-196º). Upon use they were rapidly thawed in a water-bath at 37º and diluted very slowly with phosphate-buffered saline (-) with 2% fetal calf serum, then washed three times with the same solution. The median viability of the studied leukemia cells was 89%.

**Mixed Lymphocyte-Tumor Cell Reaction (MLTR)** After complete remission had been achieved and consolidated by chemotherapy, the peripheral lymphocytes were isolated on a Ficoll-Conray layer by centrifugation at 400g for 30 min. Fifteen × 10⁶ lymphocytes were taken into 0.2 ml of RPMI-1640 medium supplemented with 20% fresh human AB serum, 25mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 100 unit/ml penicillin G and 50 µg/ml streptomycin in each well of a microplate (Microtest II, Falcon Plastics, Oxnard, Calif.), and the same number of or twice as many autochthonous leukemia cells were added. Both 4,000 R irradiated and non-irradiated leukemia cells were used as stimulator cells. Appropriate controls (as shown in Table I) were prepared and the biological activities of the studied lymphocytes were confirmed by stimulation with phytohemagglutinin P (PHA) (Difco Lab., Detroit, Mich.), pokeweed mitogen (PWM) (Gibco, Grand Island, N.Y.) or concanavalin A (Con A) (×3, Miles-Yeda, Elkhart, Ind.). All cultures were prepared in quadruplicate, and were incubated at 37º in 5% CO₂ in air for 7 days, or for 5 days in some experiments. To measure the rate of DNA synthesis of the cultured lymphocytes, 0.2 µCi of ³H-thymidine (³H-TdR) (New England Nuclear, specific activity 11.5 Ci/mmol) was added to each culture 7 hr prior to harvest. After harvesting with a Multiple Automated Sample Harvester II (Microbiological Assoc., Bethesda, Md.), filter discs were placed and dried in counting vials to which 5 ml of toluene containing 0.5% PPO and 0.01% POPOP was added. The incorporated radioactivity was counted in a liquid scintillation counter (LS-330; Beckman Instru., Fullerton, Calif.). The counts were expressed as mean cpm/10⁶ lymphocytes on 1 hr incubation with the isotope. The stimulation index (SI) was defined a follows:

\[
SI = \frac{\text{cpm of lymphocytes + irradiated leukemia cells}}{\text{cpm of lymphocytes + irradiated lymphocytes}}
\]

or

\[
SI = \frac{\text{cpm of lymphocytes + non-irradiated leukemia cells}}{\text{cpm of irradiated lymphocytes + non-irradiated leukemia cells}}
\]

for the test using irradiated leukemia cells as stimulators, or

**RESULTS**

MLTR MLTR between lymphocytes and cryopreserved autochthonous leukemia cells was tested in 36 patients with acute leukemia after complete remission had been achieved...
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by chemotherapy. The experiments were performed when the patients had recovered from the effect of chemotherapy, usually 2 weeks after the end of a course of chemotherapy, and were repeated at least twice (usually 4 to 5 times) at intervals of 1 to 5 weeks. A representative result is shown in Table I. In 20 out of 36 patients the lymphocytes showed a significant blastogenic response to autochthonous leukemia cells in at least 2 experiments, as shown in Table II. Sixteen out of 20 cases with positive MLTR showed a significant response to both irradiated and non-irradiated autochthonous leukemia cells, whereas 4 (2 AML, 2 ALL) showed a significant response only to non-irradiated leukemia cells. In the latter cases, the significance was determined by means of the *-test after subtraction of the *H-TdR uptake of the appropriate control as described in "Materials and Methods." In many cases, lymphocytes obtained from patients in the earlier period of remission responded to non-irradiated leukemia cells more strongly than to irradiated leukemia cells, but lymphocytes in the latter period showed almost the same response to both non-irradiated and irradiated leukemia cells. In some experiments, MLTR was tested by both 7- and 5-day cultures. The reactions were almost the same, although the reaction of the 7-day culture tended to be slightly stronger than that of the 5-day culture. As regards the morphological classification of leukemia, 11 out of 18 AML and 4 out of 5 AMoL showed

Table I. MLTR in a Case of AML in Complete Remission

<table>
<thead>
<tr>
<th>Mixed cultured cells*</th>
<th>Nov. 28, 1975</th>
<th>Dec. 24, 1975</th>
</tr>
</thead>
<tbody>
<tr>
<td>L+Lx</td>
<td>809±43</td>
<td>1,351±164</td>
</tr>
<tr>
<td>L+LCx</td>
<td>899±246</td>
<td>22,465±1,796 (16.6)*</td>
</tr>
<tr>
<td>L+2LCx</td>
<td>487±62</td>
<td>20,982±778</td>
</tr>
<tr>
<td>L+LC</td>
<td>12,187±2,500 (5.4)**</td>
<td>21,618±428 (11.3)*</td>
</tr>
<tr>
<td>L+2LC</td>
<td>13,969±1,448</td>
<td>19,228±2,358</td>
</tr>
<tr>
<td>Lx+LC</td>
<td>7,796±556</td>
<td>6,398±2,057</td>
</tr>
<tr>
<td>LC+LCx</td>
<td>3,707±213</td>
<td>7,883±1,375</td>
</tr>
<tr>
<td>L+LCx</td>
<td>281±42</td>
<td>310±22</td>
</tr>
<tr>
<td>L+PHA</td>
<td>5,446±1,143</td>
<td>9,210±1,580</td>
</tr>
<tr>
<td>L+PWM</td>
<td>9,167±1,330</td>
<td>17,528±1,923</td>
</tr>
</tbody>
</table>

* L: Lymphocytes isolated from peripheral blood. LC: Leukemia cells cryopreserved at −196°C. 
* x: Irradiated (4,000 R) with a linear accelerator (Toshiba LMR-13). 
* Mean cpm of quadruplicate cultures ± standard deviation 
* * P<0.01, ** P<0.05

Table II. Mixed Lymphocyte-Tumor Cell Reaction (MLTR) between Remission Lymphocytes and Cryopreserved Autochthonous Leukemia Cells

<table>
<thead>
<tr>
<th>Type of leukemia</th>
<th>No. of cases</th>
<th>MLTR</th>
<th>Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>AML</td>
<td>18</td>
<td>11 (2)</td>
<td>7</td>
</tr>
<tr>
<td>AMoL</td>
<td>5</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>ALL</td>
<td>13</td>
<td>5 (2)</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td>36</td>
<td>20 (4)</td>
<td>16</td>
</tr>
</tbody>
</table>

Numbers in parentheses indicate the numbers of cases which showed positive MLTR only against non-irradiated leukemia cells.
positive MLTR, whereas only 5 out of 13 ALL showed a positive response. Two out of 13 ALL were B cell type and the others were null cell type, as defined by E- and EAC-rosette assay. Both B cell leukemias showed negative MLTR. The stimulation index of positive MLTR ranged from 2.5 to 16.6 with a mean value of 8.1.

**Stimulation of Allogeneic Lymphocytes by Cryopreserved Leukemia Cells** (Table III) Irradiated cryopreserved leukemia cells were cultured with allogeneic lymphocytes from healthy individuals in 29 cases. Not only the leukemia cells of 14 patients with positive MLTR but also the cells of 15 patients with negative MLTR stimulated allogeneic lymphocytes significantly; the only exception was one case of AML. Therefore, almost all leukemia cells were found to hold HLA-D after cryopreservation for more than 2 months.

**Relationship between MLTR and Prognosis of Acute Leukemia** Fig. 1 shows the relationship between MLTR, expressed as the stimulation index, and the survival time from diagnosis in 30 patients. Six patients were excluded from this analysis because they had not survived for more than 6 months since diagnosis at the time of preparation of this manuscript. Although 9 out of 30 analyzed patients are still alive, and further observations may be required, no apparent correlation was observed between MLTR and the prognosis of the patients. Six out of 19 patients with positive MLTR and 3 out of 11 with negative MLTR were alive. The median survival of the patients with positive MLTR was 14 months, ranging from 8 to 26 months, and that of the patients with negative MLTR was also 14 months, ranging from 7 to 28 months. There was also no correlation between MLTR and the length of the complete remission period of the patients. When MLTR was expressed in terms of net counts of $^3$H-TdR incorporated instead of stimulation index, there was still no correlation between MLTR and the prognosis. Although the numbers of morphological types of leukemia are rather small for individual analysis, no apparent correlation was observed between MLTR and the prognosis in each type of leukemia.

### Table III. Response of Allogeneic Lymphocytes against Cryopreserved Leukemic Cells

<table>
<thead>
<tr>
<th>MLTR</th>
<th>No. of cases</th>
<th>Response of allogeneic lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Positive</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>Negative</td>
<td>15</td>
<td>14</td>
</tr>
</tbody>
</table>

Fig. 1. Relationship between MLTR and the survival time of the patients from diagnosis

Cases above the dotted line correspond to positive MLTR. O AML and AMoL, alive; • AML and AMoL, dead; △ ALL, alive; ▲ ALL, dead.

**DISCUSSION**

Remission lymphocytes from some patients with acute leukemia were stimulated by cryopreserved autochthonous leukemia cells, as several investigators have already observed. It is a matter of controversy whether the positive in vitro response of lymphocytes to autochthonous leukemia cells indicates the existence of tumorspecific or tumor-associated antigens on the
surface of the leukemia cells. Originally it was assumed that this MLTR detected the antigens because there was no difference of major histocompatibility antigens between the stimulator and the responder. It was also reported that MLTR was able to detect a tumor-specific antigen in a syngeneic animal tumor system.\(^9\) Opelz et al.\(^{17}\) and Kuntz et al.,\(^{10}\) however, have recently reported that the T cell-enriched fraction obtained from normal healthy individuals responded to the B cell-enriched fraction from the same donors and underwent blastoid transformation in \textit{in vitro} mixed cultures. These observations have raised doubts regarding the original interpretation that positive MLTR indicates the presence of leukemia-specific or leukemia-associated antigens on the surface of the leukemia cells. In fact several reports indicate that there are B cell antigens or Ia-like antigens not only on acute lymphoblastic leukemia cells (except for the T cell type), but also on acute myelogenous leukemia cells, most of which were detected by the use of xenogeneic antisera against B cells.\(^2,6,14\) Although we have also confirmed that the fractionated T cells were stimulated by the fractionated B cells (unpublished data), the possibility is not entirely excluded that this autologous stimulation is a procedural artifact, since T cells and B cells were fractionated on the basis of rosette formation with sheep erythrocytes in these experiments. Hence, the lymphocytes are exposed to the xenogeneic antigen, which does evoke an immunological reaction in human lymphocytes (unpublished data). Therefore, the above interpretation may not be entirely consistent. Moreover, positive MLTR is observed more frequently in patients with AML or AMoL than in patients with ALL.\(^7,11\) In our study, it was positive in 65% of AML and AMoL cases, but only in 38.5% of ALL cases. Although there are several reports indicating that AML or AMoL cells express B cell antigens,\(^2,6,13,14\) there is no evidence that they express the antigens more consistently than ALL cells. Two out of 13 ALL cases in our study were of B cell type, as defined by E-rosette and EAC-rosette assay. Neither of them showed positive MLTR. All ALL cases which showed positive MLTR were of null cell type. It was reported that serologically detectable B cell antigens were present on all types of leukemic lymphoblasts, including those which formed E-rosettes and null cell type lymphoblasts that showed negative MLTR.\(^{13}\) Thus, the serologically defined B cell antigens on the leukemia cells do not seem to be solely responsible for MLTR. This important problem should be studied further to determine whether MLTR does detect tumor-specific or tumor-associated antigens.

Gutterman \textit{et al.}\(^7\) reported a positive correlation between MLTR and the prognosis of adults with acute leukemia. Leventhal \textit{et al.}\(^{12}\) also reported a positive correlation, but the number of patients studied was too small to permit any statistical conclusion, as the authors stated in their paper. Our present study, however, was unable to confirm the observations of Gutterman \textit{et al.} The discrepancy may be explained by the fact that their study was performed while the patients were under remission induction therapy, whereas our study was conducted after the patients had achieved complete remission and when their immunological competence had recovered almost to the normal level. Since it is widely accepted that there is a good correlation between general immunocompetence and prognosis in acute leukemia, and that immunocompetent patients have a higher remission rate,\(^8\) positive MLTR before remission may merely indicate that the lymphocytes from immunocompetent patients, in whom complete remission is subsequently more likely to be achieved, responded to autochthonous leukemia cells in the cases of Gutterman \textit{et al.} In our cases, all patients were in complete remission when the study
was conducted and the immunocompetence was at almost the same level in all cases. The immunological relationship between leukemia cells and patients is not the sole factor affecting the prognosis of acute leukemia. There are also other important factors such as the susceptibility of leukemia cells to anti-leukemia drugs, the morphological types of leukemia and complications during the course of the disease. However, it ran counter to our expectations that MLTR showed no correlation with the prognosis of the patients in our study. Nevertheless, this interpretation may be valid, since it has been demonstrated that in mixed lymphocyte cultures among human allogeneic lymphocytes the cells proliferating in response to an allogeneic stimulus belong to a different T cell subpopulation from the cells which become cytotoxic to stimulator cells. The proliferative response of lymphocytes would depend on the difference of lymphocyte-defined histocompatibility antigens (i.e. HLA-D), whereas once the sensitization process has been achieved, cytotoxicity would be related to the difference of serologically defined antigens (i.e. HLA-A and HLA-B). Through MLTR, therefore, we might have observed only the recognition phase of tumor immunity, and not the effector phase of tumor cell killing by the cytotoxic cells. To validate this hypothesis, we are now conducting a study to identify cytotoxic lymphocytes specific to autochthonous leukemia cells in patients with acute leukemia, and will look for a correlation between the presence of specific cytotoxic cells and the prognosis of the patients.

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REFERENCES