Specific cytolysis of fresh tumor cells by an autologous killer T cell line derived from an adult T cell leukemia/lymphoma patient.

M. Kannagi, K. Sugamura, K. Kinoshita, H. Uchino and Y. Hinuma

*J Immunol* 1984; 133:1037-1041;  http://www.jimmunol.org/content/133/2/1037
SPECIFIC CYTOLYSIS OF FRESH TUMOR CELLS BY AN AUTOLOGOUS KILLER T CELL LINE DERIVED FROM AN ADULT T CELL LEUKEMIA/LYMPHOMA PATIENT

MARI KANNAGI,* KAZUO SUGAMURA,2* KEN-ICHIRO KINOSHITA,† HARUTO UCHINO,4 AND YORIO HINUMA*

From the *Institute for Virus Research and the †First Division, Department of Internal Medicine, Faculty of Medicine, Kyoto University, Kyoto 606; and the §Department of Hematology, Atomic Disease Institute, Nagasaki University School of Medicine, Nagasaki 852, Japan

Cytotoxic T cells (Tc) derived from one patient with adult T cell leukemia/lymphoma (ATL) killed fresh autologous lymphoma cells in vitro. The Tc were induced from peripheral blood leukocytes (PBL) of this patient during remission by multiple in vitro stimulations with an autologous ATLV-bearing cell line (ILT) that was previously established by cloning of PBL in the presence of interleukin 2 (IL 2). PBL from eight other ATL patients were stimulated in the same manner, and responder cells from a patient in remission also showed cytotoxicity specific for ATLV virus (ATLV)-bearing cells. Fresh lymphoma cells were obtained in relapse and were used as target cells for the autologous Tc induced. They became susceptible to the Tc within 4 hr of incubation, and their susceptibility increased with incubation time for at least 12 hr. ATLV antigens on the cell surface of these lymphoma cells, however, were not detected by radioimmunoassay during these incubation periods, but were detectable after 16 hr of incubation. In addition, cytotoxicity against lymphoma cells was completely inhibited by autologous ILT cells used as "cold" target competitor cells. These findings indicate that the target antigen of the Tc was expressed on both autologous ILT cells and lymphoma cells, and it may be different from ATLV antigens detected by serologic methods. In addition, the data suggested allogeneic restriction of the Tc in that the preferentially killed autologous ATLV-bearing cells share several HLA antigens.

A human retrovirus, adult T cell leukemia virus (ATLV)2/human T cell leukemia-lymphoma virus, was found to be associated with human adult T cell leukemia/lymphoma (ATL), which had been reported as a new entity with endemic characteristics as well as unique clinical and hematologic features (1). Proviral DNA of ATLV was detected in leukemic cells of all ATL patients tested, which strongly supports the concept that ATLV is a causative agent of ATL (2). On the other hand, certain residents in endemic areas are known to be healthy ATLV carriers, whose peripheral blood contains a small number of lymphocytes infected with ATLV (3, 4). In fact, development of ATL was observed among these healthy virus carriers (5). However, it took at least 10 yr to develop ATL after ATLV infection. Thus, there seems to be multiple steps involved in the process of oncogenesis of ATLV in vivo. To date, impairment of the host immunosurveillance system has been indicated to influence development of various tumors in humans as well as in animals (6–8). In this respect, analysis of the immunity of ATL patients against ATL tumor cells or ATLV would aid in resolving the mechanisms of oncogenesis of ATLV. It is known that ATL patients and healthy ATLV carriers have serum antibodies against ATLV as well as cytoplasmic antigens reacting with these antibodies, called ATLV-associated antigens (ATLA), that have been shown to consist mainly of ATLV antigens (9). It is not known, however, whether humoral antibodies to ATLV antigens contribute to immunosurveillance of ATL tumor cells in vivo, in that ATLA were not expressed on fresh ATL cells (10). In a previous study, we established an experimental system for the generation of cytotoxic T cells (Tc) specific for ATLV-bearing cells from healthy individuals by multiple stimulations with autologous ATLV-bearing cell line cells (11). In the present study, we induced Tc specific for ATLV-bearing cells from ATL patients by using a similar experimental protocol, and determined whether the induced Tc killed fresh autologous lymphoma cells.

MATERIALS AND METHODS

Blood donors. Nine patients diagnosed as ATL were used for blood donors. Six were in partial remission and the remaining three were not in remission when we obtained peripheral blood from them. The criteria used for determining partial remission were no clinical symptoms and pathologic cells being less than 2% of the total peripheral white blood cells. A description of the donors is provided in Table 1.

Induction of Tc. Peripheral blood leukocytes (PBL) from donors were separated on a Ficoll-Conray gradient. A small fraction was used for obtaining an interleukin 2 (IL 2)-dependent ATLV-bearing T cell line (ILT), and the remainder was cryopreserved. ILT were obtained with cloning or mass cultivation of PBL in medium contain-
Tc SPECIFIC FOR FRESH AUTOLOGOUS TUMOR CELLS FROM ATL PATIENT

Clinical and hematologic findings of nine patients with ATL when their PBL were used as responders

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age/Sex</th>
<th>Anti-ATLA Titer in Serum*</th>
<th>Leukemia at Onset</th>
<th>Chemotherapy</th>
<th>Condition*</th>
<th>Survival (mo)</th>
<th>Peripheral White Blood Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hm</td>
<td>68/F</td>
<td>160</td>
<td>Yes</td>
<td>Yes</td>
<td>PR &gt;17</td>
<td>4,800</td>
<td>3</td>
</tr>
<tr>
<td>My</td>
<td>50/M</td>
<td>120</td>
<td>Yes</td>
<td>Yes</td>
<td>PR &gt;22</td>
<td>4,000</td>
<td>5</td>
</tr>
<tr>
<td>Kit</td>
<td>63/M</td>
<td>20</td>
<td>Yes</td>
<td>Yes</td>
<td>PR &gt;15</td>
<td>6,900</td>
<td>3</td>
</tr>
<tr>
<td>Tag</td>
<td>69/F</td>
<td>40</td>
<td>Ne</td>
<td>Yes</td>
<td>PR &gt;16</td>
<td>4,050</td>
<td>2</td>
</tr>
<tr>
<td>Yl</td>
<td>46/M</td>
<td>40</td>
<td>No</td>
<td>Yes</td>
<td>PR &gt;14</td>
<td>2,500</td>
<td>0</td>
</tr>
<tr>
<td>Tk</td>
<td>31/M</td>
<td>20</td>
<td>No</td>
<td>Yes</td>
<td>PR &gt;5</td>
<td>2,400</td>
<td>0</td>
</tr>
<tr>
<td>Sy</td>
<td>61/F</td>
<td>40</td>
<td>Yes</td>
<td>No</td>
<td>PR &gt;4</td>
<td>28,000</td>
<td>35</td>
</tr>
<tr>
<td>Stm</td>
<td>52/F</td>
<td>20</td>
<td>Yes</td>
<td>No</td>
<td>PR &gt;8</td>
<td>20,000</td>
<td>48</td>
</tr>
<tr>
<td>Kas</td>
<td>42/M</td>
<td>20</td>
<td>Yes</td>
<td>Yes</td>
<td>PR &gt;13</td>
<td>17,500</td>
<td>49</td>
</tr>
</tbody>
</table>

* Detected by indirect immunofluorescence staining method.
** PR, partial remission; NR, no remission.

ATL cell, abnormal lymphocyte that has nuclear abnormality.

Patient My died from another cause without relapse of ATL.

RESULTS

Tc responses in autologous stimulation cultures. The kinetics of the generation of Tc in PBL cultures of nine ATL patients stimulated with autologous ILT cells were examined for autologous ILT-bearing ILT and natural killer-sensitive K-562 target cells. As shown in Table I, significant cytotoxicity was not observed in most patients before in vitro stimulation, but weak cytotoxicity for K-562 was shown in patients Hm and Tk. After the first stimulation, significant cytotoxicity against autologous ILT cells was observed in patient Hm and cytotoxicity against K-562 was also increased in several patients in remission, including Hm. After the third stimulation on the 15th day in culture, cytotoxicity against autologous ILT cells in patient Hm was additionally enhanced, whereas cytotoxicity against K-562 was undetectable.

The lytic activity of effector cells from patient Hm was sustained after the fourth stimulation. The effector cells were positive for Leu-1 and Leu-2a antigens, markers for Tc. The Tc of patient Hm were further maintained in medium containing IL-2. Responder cells from patient My also showed significant cytotoxicity against autologous ILT cells by 30 days in culture after four stimulations. In patients not in remission, the generation of cytotoxic effector cells was not demonstrated for autologous ILT or K-562 target cells during stimulation cultures. Responder

<table>
<thead>
<tr>
<th>Patient</th>
<th>Anti-ATLA Titer in Serum*</th>
<th>Leukemia at Onset</th>
<th>Chemotherapy</th>
<th>Condition*</th>
<th>Survival (mo)</th>
<th>Peripheral White Blood Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hm</td>
<td>68/F</td>
<td>Yes</td>
<td>Yes</td>
<td>PR &gt;17</td>
<td>4,800</td>
<td>3</td>
</tr>
<tr>
<td>My</td>
<td>50/M</td>
<td>Yes</td>
<td>Yes</td>
<td>PR &gt;22</td>
<td>4,000</td>
<td>5</td>
</tr>
<tr>
<td>Kit</td>
<td>63/M</td>
<td>Yes</td>
<td>Yes</td>
<td>PR &gt;15</td>
<td>6,900</td>
<td>3</td>
</tr>
<tr>
<td>Tag</td>
<td>69/F</td>
<td>Yes</td>
<td>No</td>
<td>PR &gt;16</td>
<td>4,050</td>
<td>2</td>
</tr>
<tr>
<td>Yl</td>
<td>46/M</td>
<td>Yes</td>
<td>Yes</td>
<td>PR &gt;14</td>
<td>2,500</td>
<td>0</td>
</tr>
<tr>
<td>Tk</td>
<td>31/M</td>
<td>Yes</td>
<td>Yes</td>
<td>PR &gt;5</td>
<td>2,400</td>
<td>0</td>
</tr>
<tr>
<td>Sy</td>
<td>61/F</td>
<td>Yes</td>
<td>No</td>
<td>PR &gt;4</td>
<td>28,000</td>
<td>35</td>
</tr>
<tr>
<td>Stm</td>
<td>52/F</td>
<td>Yes</td>
<td>No</td>
<td>PR &gt;8</td>
<td>20,000</td>
<td>48</td>
</tr>
<tr>
<td>Kas</td>
<td>42/M</td>
<td>Yes</td>
<td>Yes</td>
<td>PR &gt;13</td>
<td>17,500</td>
<td>49</td>
</tr>
</tbody>
</table>

* Anti-ATLA-negative sera at the same concentration as anti-ATLA-positive sera were used as controls. The Bi of ATLANegative cell lines MOLT-4 and CCRF-CEM always ranged from 3 to 2.

Preparation of fresh lymphoma cells. One ATL patient, Hm, relapsed with systemic lymphomas. The axillary lymph node was resected, diced into small pieces, and passed through stainless steel mesh screens. Isolated lymphoma cells were separated on a Ficoll-Conray gradient and were maintained at 4°C for about 8 hr as a suspension in RPMI 1640 medium supplemented with 10% FCS until used for additional experiments. They were cultured in RPMI 1640 + 20% FCS + 10% IL 2 at 37°C in 5% CO2 in humidified air, and were tested for kinetics of expression of ATLV antigens and susceptibility to autologous Tc.
cells from four patients (Yt, Tk, Sy, and Sim), which were unresponsive to autologous ILT cell stimulation, expressed ATLA after 1 mo in culture.

**Specificity of effector cells.** The Tc derived from patient Hm(Tc-Hm) were examined for cytotoxic specificity and HLA restriction (Table III). Tc-Hm showed significant cytotoxicity against allogeneic ATL-bearing cells, ILT-Mor, as well as autologous ILT cells. They also showed lower but significant cytotoxicity against TCL-Mor cells derived from the same donor as ILT-Mor. TCL-Mor and ILT-Mor cells shared HLA-Aw33, Bw44, DR4, and DR6Y antigens with Tc-Hm cells. However, they were not cytotoxic for other allogeneic ATL-bearing cells that shared HLA-Aw24 and DR4 (ILT-Ter and TCL-Ter), or only HLA-Aw24 (ILT-Yan, ILT-Su, and TCL-As). Tc-Hm did not kill ATLV-negative cells such as K-562, MOLT-4, CCRF-CEM, and HPB-ALL, nor did they kill autologous PBL stimulated with PHA, which contain 3% leukemic cells as defined histologically.

**Susceptibility of autologous lymphoma cells to Tc-Hm.** Patient Hm had a relapse of ATL, with swelling of systemic lymph nodes without leukemia, after 9 mo of remission. Her axilla lymph node was resected and histologically diagnosed as "diffuse pleomorphic type," which is characteristic of ATL (16). Isolated lymphoma cells possessed Leu-1 and Leu-3a antigens, but not ATLA, before in vitro incubation. They were examined for kinetics of susceptibility to autologous cryopreserved Tc-Hm and the expression of ATLA-antigens (Fig. 1). Autologous Tc-Hm did not kill the lymphoma cells without in vitro incubation before cytotoxicity assays. However, when the lymphoma cells were preincubated for at least 4 hr, significant lysis by Tc-Hm resulted. Their cytotoxic levels increased linearly up to 12 hr of incubation, then reached a plateau over the next 24 hr of incubation. Contrarily, the fresh lymphoma cells without preincubation were significantly killed by allogeneic killer cells derived from a mixed lymphocyte culture (Fig. 1). Cytolysis of the lymphoma cells by the allogeneic killer cells was observed at almost the same level during in vitro incubation for at least 24 hr.

On the other hand, after 10 hr in incubation, ATLAs were detected in a small population of the lymphoma cells, and 95% of the cells expressed ATLA by 24 hr in

**DISCUSSION**

We previously demonstrated that ATLV-specific Tc from healthy ATLV carriers were induced by multiple stimulations of PBL with autologous ILT cells, and that they killed fresh allogeneic leukemia cells from an ATL patient (11). The present study demonstrated that Tc-Hm were also induced by a similar experimental protocol from PBL of an ATL patient in remission, and they killed fresh incubation. The kinetics of ATLV antigen expression were also examined by using a monoclonal antibody (GIN-14) reacting with p19 of ATLV on acetone-fixed samples, and the result was similar to ATLA. ATLSA, antigens reacting with anti-ATLA-positive sera on the cell surface, were minimal for 12 hr in incubation but were detected at 16 hr by radioimmunoassay. The BI for ATLSA of the lymphoma cells gradually increased up to 23 BI at 36 hr in culture, which was much lower than 164 BI for ATLSA of autologous ILT-Hm cells used as stimulus cells. Thus, the cytotoxicity of Tc-Hm against autologous lymphoma cells was observed earlier than the detection of ATLA or ATLSA.

**Cytotoxicity blocking by "cold" target competitor cells.** A cytotoxicity inhibition test with unlabeled target cells was performed to examine whether the effector cells of Tc-Hm directed to autologous lymphoma cells were the same as those directed to autologous ILT-Hm cells derived from PBL and used as stimulus cells. Figure 2 shows that cytotoxicity against the lymphoma cells was completely inhibited by "cold" target cells, both ILT-Hm and lymphoma cells, whereas control CCRF-CEM cells had little effect on the cytotoxicity. These results indicate that the lymphoma cells and ILT-Hm cells expressed the same target antigen of Tc-Hm.
autologous lymphoma cells obtained during relapse. ILT-Hm cells used as stimulator cells were ATLV-positive T cells established during remission by the cloning of PBL in medium containing IL 2. Mitsuaya et al. (17) also reported that Tc were induced from PBL of a patient with cutaneous T cell lymphoma in in vitro stimulation cultures with an autologous human T cell leukemia-lymphoma virus (HTLV)-positive cell line derived from PBL with IL 2-containing medium (18), and the Tc killed the cell line. They stated that the HTLV-positive cell line cells established were "tumor cells." However, it is difficult to determine whether these virus-carrying cells obtained from the PBL of patients originated from actual tumor cells, because it is possible that virus-infected nontumor cells could proliferate in the presence of IL 2. Therefore, our present data are the first evidence for the generation of Tc specific for fresh autologous tumor cells from an ATL patient. This suggests that Tc play an important role in anti-tumor immunity in vivo.

Two patients from whom ATLV-specific Tc were induced were both in remission when their PBL were used as responder cells. However, PBL from other ATL patients did not respond to autologous ILT cells to generate Tc. The reduced were both in remission when their PBL were used. Our present data are the first evidence for the generation of Tc specific for fresh autologous tumor cells during relapse. ILT-Hm cells used as stimulator cells were ATLV-positive T cells established during remission by the cloning of PBL in medium containing IL 2. Mitsuaya et al. (17) also reported that Tc were induced from PBL of a patient with cutaneous T cell lymphoma in in vitro stimulation cultures with an autologous human T cell leukemia-lymphoma virus (HTLV)-positive cell line derived from PBL with IL 2-containing medium (18), and the Tc killed the cell line. They stated that the HTLV-positive cell line cells established were "tumor cells." However, it is difficult to determine whether these virus-carrying cells obtained from the PBL of patients originated from actual tumor cells, because it is possible that virus-infected nontumor cells could proliferate in the presence of IL 2. Therefore, our present data are the first evidence for the generation of Tc specific for fresh autologous tumor cells from an ATL patient. This suggests that Tc play an important role in anti-tumor immunity in vivo.

Two patients from whom ATLV-specific Tc were induced were both in remission when their PBL were used as responder cells. However, PBL from other ATL patients did not respond to autologous ILT cells to generate Tc. The unsuccessful induction of Tc in these patients might be due to a relative reduction of cytotoxic precursor or memory T cells, or immunosuppression that is widely found in tumor-bearing hosts (6, 19). Recently an immunosuppressive state was also suggested in ATL patients (20–22). Responder cells from at least four patients expressed ATLV antigens after 1 mo in stimulation cultures. This indicates that ATLV antigens were induced in ATLV-genome-positive cells in the original PBL or they were infected in vitro with virus of ILT cells used as stimulators and were expanded in medium containing IL 2. These observations might result from the noninducibility for ATLV-specific Tc.

The lymphoma cells of patient Hm were killed by Tc-Hm within 4 hr of in vitro incubation, whereas ATLA and ATLSA of the lymphoma cells were detected after 10 and 16 hr of cultivation, respectively. Even if the reaction time of 4 hr in cytotoxicity assays was considered, this time lag strongly suggests the possibility that the target antigen recognized by Tc-Hm is different from ATLV antigens detected by serologic methods. This concept is supported by our previous observation that Tc from a healthy ATL carrier killed not only autologous ILT cells but also allogeneic ATL leukemic cells negative for ATLV antigens (11). In addition, autologous serum with anti-ATLV antibodies did not block the cytotoxicity of Tc-Hm for ATLV-positive target cells (data not shown). Therefore it is unlikely that the difference in time for antigen recognition by the Tc vs humoral antibodies (ATLA) is a quantitative difference of the same antigen.

The lymphoma cells without in vitro incubation did not express ATLA or ATLSA, nor were they susceptible to Tc-Hm cytotoxicity. However, alloreactive killer cells showed significant cytotoxicity for the lymphoma cells without preincubation. This observation suggests that the delay in susceptibility to Tc-Hm of the lymphoma cells would not be due to nonspecific resistance to killer cells but rather to the requirement for in vitro induction of the specific target antigen recognized by Tc-Hm. We reported a similar observation that fresh ATL cells did not express ATLV antigens until cultured in vitro, although the time of cultivation required for expression of antigens varied among individuals (10). The absence of both ATLV antigens and the target antigen of Tc might result from immune selection or inhibition of antigen expression in vivo by humoral and cell-mediated immunity. Development of nonimmunogenic tumor cell variants has been well documented in animal models, and it has been suggested they escape from the host immune system (23, 24). In the present study, however, the lymphoma cells obtained during relapse became susceptible for Tc so early in culture that they most likely were not stable antigen loss variants, because they were able to express the target antigen. Therefore, it is still possible that the tumor cells also may express the target antigen in vivo in certain condition, and Tc specific for ATL tumor cells such as Tc-Hm would effectively operate on them.

Finally, HLA restriction of the cytotoxicity of Tc-Hm was suggested from the observation that Tc-Hm were also cytotoxic for allogeneic ATLV-bearing cells ILT-Mor and TCL-Mor. These two target cell lines were derived from the same donor and shared several HLA antigens with Tc-Hm cells. We previously reported that the cytotoxicity of ATLV-specific Tc derived from one healthy donor was restricted to HLA-A2 antigen (11). Tc-Hm in the present study, however, did not have HLA-A2 and were cytotoxic for ATLV-bearing cells sharing Aw33, Bw44, DR4, and D6Y, but not for those sharing Aw24 and DR4. Therefore, the cytotoxicity of Tc-Hm seemed to be restricted to Aw33, Bw44, or D6Y, although we did not determine which HLA antigen among them participated in the restricted recognition of Tc-Hm. In addition, we could not detect alien HLA expression on our ATLV-bearing T cell lines, as recently reported by Mann et al. (18).

Acknowledgments. We thank Dr. Toshihumi Kondo, Uwajima Municipal Hospital, Uwajima, and Dr. Yoshiteru Konaka, Kitano Hospital Medical Research Institute, Osaka, for supplying specimens from patients. We also
thank Drs. Kazuo Okochi and Hiroyuki Sato, Central Clinical Laboratory, Kyushu University Hospital, Fukuoka, for HLA serotyping of cell lines, and Dr. James R. Blakeslee Jr., the Ohio State University, for reviewing this manuscript.

REFERENCES