Isolation of Human Lymphocyte Antigens Class I-restricted Cytotoxic T Lymphocytes against Autologous Myeloma Cells

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ABSTRACT
Peripheral blood T cells from a patient with multiple myeloma in complete remission were selected in vitro against an autologous myeloma cell line (SBN-1), using a protocol designed for the selection of relatively rare precursor cytotoxic T cells (pCTL). Delayed addition (2 weeks) of interleukin 2 induced T-cell proliferation, and a bulk culture (T-cell line) was obtained 2 days later. This T-cell line displayed cytotoxicity against SBN-1. A CD8+ CD4- cytotoxic T-cell clone (CT5) was then obtained that recognized SBN-1 but not autologous EBV+ B-lymphoblastoid cells, autologous T PHA-blasts, or Daudi, Raji, K562, and 11 allogeneic myeloma cell lines. Moreover, CT5 cytotoxic activity against SBN-1 was blocked by monoclonal antibodies recognizing human lymphocyte antigen class I molecules. This seems to be the first demonstration of myeloma-specific pCTL in peripheral blood T cells of patients with multiple myeloma.

INTRODUCTION
MGUS1 and MM are characterized by the persistence of a plasma cell clone secreting a monoclonal Ig (1, 2). MGUS is common in the elderly (1% above 60 years), but only around 20% of individuals with MGUS develop MM (1, 2). T cells play a crucial role in antigenic response by controlling B-cell growth and differentiation (for a review, see Ref. 3). Thus, the emergence of MGUS, which is correlated with age and immune deficiency, may result from less efficient T-cell functions and/or chronic antigenic stimulation (1, 2). An alteration of T-cell immunity seems likely because MGUS is more frequent in HIV+ individuals (up to 4.6%) and AIDS and Kaposi patients (13 and 89%, respectively; Refs. 1 and 4). In patients with MM, T cells have proved abnormally sensitive to apoptosis (5), and the T-cell repertoire is highly altered inasmuch as some Vβ families are no longer represented, whereas others show clonal expansions (6). The specificity of these clonal expansions has not been investigated, although it has been determined that they are not directed against myeloma cells (6). MGUS and MM are characterized by the secretion of a monoclonal Ig expressing a private idiotype that could constitute a specific tumor antigen (Ag) for malignant plasma cells. There is no evidence that normal or malignant plasma cells express complete surface Ig or the idiotype in HLA-restricted fashion, although Yi et al. (7) have shown that idiotype-reactive T-cell subsets exist in patients with MGUS and MM and that the presentation of the idiotype by B cells and macrophages is HLA-II-restricted. The capacity of idiotype-specific T cells to recognize the idiotype directly on malignant plasma cells has not been investigated, whereas cytotoxic antimyeloma T cells have already been described (7). Myeloma cells were found to express polymorphic epithelial mucin (MUC1), and cytotoxic T cells (CTL) were produced by two of six patients with MM (8, 9). Cytotoxicity against MUC1-expressing MM cell lines has been shown to be HLA-unrestricted. MUC1 antigen, which is expressed on normal and tumoral epithelial cells (e.g., in breast carcinomas), is recognized directly by CTL, and preferentially in underglycosylated form (8, 9).

This report concerns the isolation of autologous cytotoxic T cells that killed myeloma cells specifically in an HLA-I-restricted reaction. Our data suggest that one or more myeloma antigens exist as potential targets for T-cell-mediated cytotoxicity.

MATERIALS AND METHODS
MM Patient (G. B.) and Autologous Cell Lines. A myeloma cell line was established from the malignant pleural effusion of a patient (G. B.) with nonsecretory MM after 6 weeks of culture in RPMI 1640 supplemented with 10% FCS, 1 ng/ml IL-6, and 10% pleural effusion (10). The cell line (SBN-1) was IL-6-dependent and had the same phenotype as the freshly explanted myeloma cells, i.e., CD19-CD28-CD38+ CD40+CD56-CD138+ (data not shown), which is characteristic of human myeloma cells (11, 12). The patient was in complete remission after melphalan-prednisone treatment but died from severe myelodysplasia 2 years after establishment of the MM cell line. Identical Ig rearrangements of SBN-1 and myeloma cells, as determined by Southern blot analysis, con-
confirmed that SBN-1 emerged from the freshly explanted myeloma cells (data not shown). The patient’s B lymphoblastoid cell line was obtained by coculturing his PBMC with EBV-containing supernatant from the virus-producing B95.8 cell line in the presence of 1 μg/ml PHA. SBN-1 and B-EBV cell lines were cultured in RPMI 1640 (Life Technologies, Inc., Cergy-Pontoise, France) supplemented with 10% FCS and 2 × 10⁻⁵ M 2-mercaptoethanol. For SBN-1, the medium was supplemented with 1 ng/ml IL-6.

**Other MM Cell Lines.** The IL-6-dependent MM cell lines XG-1, XG-2, XG-5, and XG-6 were established by Zhang et al. (12), and ANBL-6 was a kind gift of Dr. D. Jelinek (Mayo Clinic, Rochester, MN). The exogenous IL-6-independent MM cell lines RPMI-8226 and U266 were from the American Type Culture Collection, and LP1, L363, OPM-2, and NCI-H929 were from DSM (Braunschweig, Germany). All of the cell lines were cultured in RPMI 1640 supplemented with 10% FCS and 2 × 10⁻⁵ M 2-mercaptoethanol with (for cell lines XG1, XG2, XG5, XG6, and ANBL-6) or without IL-6 (cell lines RPMI-8226, U266, LP1, L363, OPM2, and NCI-H929).

**Reagents.** mAbs anti-CD8, -CD54, -CD70, -CD80, and -TCRoβ were from Immunotech (Marseille, France); anti-CD45RO from Dako (Glostrup, Denmark); anti-CD86 from Diaclon (Besançon, France); and anti-CD58 from the Vth Human Leukocyte Typing Workshop. Hybridomas W6/32 (anti-HLA-1) and BB7.2 (anti-HLA-A2) were from the American Type Culture Collection, and B1.23.2 (anti-HLA-B/Cw; Ref. 13) was a gift from Dr. F. A. Lemonnier (Institut National de la Santé et de la Recherche Médicale U152, Paris, France). Filtered ascitic fluids of W6/32, B1.23.2, and BB7.2 were used in cytotoxicity blocking experiments.

**Generation and Expansion of SBN-1-specific T Cells.** For the generation of SBN-1-specific T cells, the protocol used was similar to that described by Smith et al. (14) for EBV-specific cytotoxic T cells. Fresh C (5 × 10⁶ cells) of the patient G. B. were cocultured with 35 Gy-irradiated SBN-1 (ratio 40:1) in 10% pooled human sera without cytokine. After 10 days, surviving cells were isolated by Ficoll-Hypaque centrifugation and restimulated with 35 Gy-irradiated SBN-1 (ratio 4:1). Four days later, 150 units/ml IL-2 were added to the culture, and 15 × 10⁶ T lymphocytes (T-cell line) were obtained after another two days.

**Cloning, Restimulation of Clones, and Cytotoxic Assays.** Cloning and cytotoxic assays were performed as described previously (15). Briefly, cloning was performed in 96-well U-bottomed plates (0.3 cell/well) in the presence of irradiated allogeneic PBMC (10⁶ cells/plate) and EBV cell lines (10⁵ cells/plate) in 10% pooled human sera in the presence of 150 units/ml IL-2 and 1 μg/ml PHA. Clones were restimulated monthly in the presence of irradiated allogeneic PBMC (10⁶ cells/plate) and EBV-B cell lines (10⁵ cells/plate) in 10% pooled human sera in the presence of 150 units/ml IL-2 and 1 μg/ml PHA.

Cytotoxic assays were performed in 96-well V-bottomed plates with 3000 ⁵¹ Cr-labeled target cells in 200 μl (4 h). The effector:target ratio ranged from 50 to 0.1 as determined by serial dilution of effector cells. The percentage of specific cytotoxicity was determined as follows:

\[
\text{Percent specific cytotoxicity} = \left( \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Total release} - \text{spontaneous release}} \right) \times 100
\]

Spontaneous release was approximately 10%. Assays were performed in duplicate or triplicate wells, as indicated in the figure legends.

**RESULTS**

**Specific Recognition of Autologous MM Cells by the CT5 Clone.** After 16 days of mixed lymphocyte tumor culture between SBN-1 and the patient’s autologous PBMCN, the T-cell line obtained showed significant cytotoxic activity against SBN-1 (Fig. 1). More than 30 T-cell clones were then derived from the cell line by limiting dilution culture and were screened for their ability to kill autologous myeloma cells. One clone, CT5, displayed cytotoxicity against SBN-1 and was kept for additional studies (Fig. 1). CT5 was found to be a TCRoβ⁺ CD8⁺ clone.

As shown in Fig. 2, CT5 was able to kill autologous myeloma cells but was not cytotoxic against autologous EBV⁺ B cells (generated from peripheral blood of G. B.) or autologous T cells (from PHA blasts). Moreover, CT5 failed to kill Daudi, Raji or K562 (the regular target cells for NK or LAK cytotoxicity). Our data suggest that CT5 recognized a determinant present only on myeloma cells. HLA typing and DNA polymor-
Analysis of SBN-1, the autologous EBV-B cell line and the autologous T-cell line confirmed that all of the cell lines derived from patient G. B. (data not shown).

Recognition of Autologous MM Cells by the CT5 Clone is HLA Class I-restricted.

To investigate whether this determinant was shared with other myeloma cells, CT5 cytotoxic activity was assessed against a large panel of allogeneic MM cell lines. HLA class I typing of all of the MM cell lines is shown in Table 1. As indicated in Fig. 3, none of these lines was recognized by the clone, suggesting that myeloma cell recognition by CT5 is self-restricted. To confirm this hypothesis, SBN-1 recognition by CT5 was assessed in the presence of W6/32, a pan class I mAb, or mAb B1.23.2, that recognizes HLA-B and -Cw, and several HLA-A determinants such as HLA-A32, or BB7.2, a HLA-A2-specific mAb. Results of a representative experiment are shown in Fig. 4. SBN-1 killing was blocked by W6/32 and B1.23.2 mAbs, thus demonstrating that SBN-1 recognition was HLA-restricted. Because of a lack of HLA-allele-specific mAbs and the limitation of available MM cell lines, the exact restricting element for clone CT5 could not be determined. On the basis of the data presented, only recognition restricted to HLA-A0201 could be excluded (inasmuch as mAb BB7.2 did not block SBN-1 recognition by CT5). Nevertheless, our data on the whole support the conclusion that the CT5 clone recognizes a peptide presented by myeloma cells but not by B lymphocytes in the context of a self class I molecule.

Table 1  HLA class I typing of MM cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>HLA-A</th>
<th>HLA-B</th>
<th>HLA-Cw</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBN-1</td>
<td>0201/3201</td>
<td>1402/57</td>
<td>0602/0802</td>
</tr>
<tr>
<td>XG1</td>
<td>02/29</td>
<td>40/44</td>
<td>0202/1601</td>
</tr>
<tr>
<td>XG2</td>
<td>01/29</td>
<td>08/44</td>
<td>07/1601</td>
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<tr>
<td>XG5</td>
<td>02</td>
<td>44/49</td>
<td>0501/07</td>
</tr>
<tr>
<td>XG6</td>
<td>02</td>
<td>15/57</td>
<td>0602/0303</td>
</tr>
<tr>
<td>ANBL-6</td>
<td>02/03</td>
<td>51</td>
<td>1202/1203</td>
</tr>
<tr>
<td>L363</td>
<td>02/31</td>
<td>07/4001</td>
<td>03/07</td>
</tr>
<tr>
<td>LP1</td>
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<td>07/18</td>
<td>07</td>
</tr>
<tr>
<td>NCI-H929</td>
<td>26/30</td>
<td>18/35</td>
<td>04/07</td>
</tr>
<tr>
<td>OPM2</td>
<td>24</td>
<td>07/15</td>
<td>04 or 1801/07</td>
</tr>
<tr>
<td>RPMI 8226</td>
<td>6002/30</td>
<td>4802 or 72/15</td>
<td>0202/0304</td>
</tr>
<tr>
<td>U266</td>
<td>02/03</td>
<td>07/40</td>
<td>0304/0701</td>
</tr>
</tbody>
</table>

DISCUSSION

This study considered whether specific cytotoxic T cells against autologous MM cells were present in a patient with MM in complete remission. Isolated T cells displayed cytotoxic activity against the autologous MM cell line but not against other autologous cells such as T PHA-blasts or EBV-transformed B cells. Moreover, CT5 reactivity was HLA class I-restricted, which indicates that a peptide specific for myeloma cells was recognized in the context of a self HLA class I molecule. Because no MM cell lines were HLA-matched with SBN-1, it was not possible to determine whether the peptide recognized by CT5 on SBN-1 was shared with other MM cell lines. The relevance of the CT5 clone in vivo was unclear. Was it primed in vitro or did it belong to a memory subset that already recognized myeloma cells in vivo? On the one hand, the CT5 clone phenotype (CD45RO1 CD701), to which memory cells belong (16), could have been acquired during in vitro stimulation. On the other hand, SBN-1, like most MM cell lines (but unlike other B-cell lines), does not express CD80 and CD86 (17), which are considered to be essential for the activation of naive but not memory T cells (18). Because the patient died in remission with no indication of myeloma relapse, CT5 may have belonged to a memory subset of cytotoxic T cells that were reactive in vivo. Interestingly, it has been shown that CD80 expression in melanoma is not essential for recognition by cytotoxic cells, unlike that of CD54 and CD58 (19), which are both expressed at a high level by SBN-1 as well as by most MM cell lines (Ref. 12 and data not shown).

To our knowledge, the present work provides the first evidence for the existence of HLA-restricted T cells cytotoxic against autologous myeloma cells in patients with MM. Previous reports have shown that MUC1-specific HLA-unrestricted cytotoxic T cells can kill MM cell lines. However, the recognition of MUC1, which is expressed on most MM cell lines (Ref. 8), was excluded by our data because CT5 recognized only SBN-1, and this recognition was HLA-restricted. Different reports have shown that idiotype-reactive T cells exist in patients with MM, but presently there is no evidence that these idiotype-specific T cells are able to recognize and kill myeloma cells. In

\[^4\text{C. Pellat-Deceunynck, unpublished data.}\]

Fig. 2  Cytotoxicity assays were performed as indicated in Fig. 1 (4 h; 3000 target cells/well). The effector:target ratio ranged from 50 to 0.1 by serial dilution of effector cells. Each value represents the mean of two separate experiments ± SD.
an in vivo assay, Kwak et al. (20) showed that myeloma idio-
type-specific immunity can be transferred from an actively 
imunized marrow donor. After allogeneic bone-marrow trans-
plantation, the recipient was cured of MM, although nothing 
indicated that myeloma idiootype-specific T cells were responsi-
brable because graft-versus-host disease developed. Recently, Yi et 
al. (21) reported that myeloma cells can present antigen derived 
from tetanus toxoid to autologous T cells, which indicates that 
malignant plasma cells can present exogenous immunoreactive 
antigens.

MM remains an incurable disease, although intensive 
chemotherapy associated with autologous bone-marrow trans-
plantation has led to a significant increase in the percentage 
of complete remissions and the overall survival of patients (22). 
The relapses observed systematically after transient complete 
remission are probably due to minimal residual disease. The 
development of specific cytotoxic T cells could be a major 
means of eradicating such residual disease. However, the estab-
ishment of this adoptive immunotherapy depends on whether 
myeloma cells can be successfully recognized by autologous 
cytotoxic T cells and on identifying the nature of the antigen(s) 
involved. Our report provides an answer to the first question and 
strongly suggests that the antigen recognized may be “restrict-
ed” to myeloma cells.


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