Evidence for in Situ Amplification of Cytotoxic T-Lymphocytes with Antitumor Activity in a Human Regressive Melanoma

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ABSTRACT

We have derived from lymphocytes infiltrating a human regressive melanoma lesion a series of T-cell receptor α/β-dependent, HLA-B14-restricted cytotoxic T-lymphocyte clones reacting against the autologous tumor. Analysis of the T-cell receptor gene expression revealed that all the clones represented a unique cell expressing a Vβ13.1/β1.1 gene segment. T-cell receptor transcripts expressed in the cloned cells were compared to those present in the uncultured tumor tissue. This analysis demonstrated that the specific cytotoxic T-lymphocyte clones characterized in vitro was actually selected and amplified in vivo at the lesion site. These results provide strong evidence that effector T-cells have contributed to tumor regression.

INTRODUCTION

The contribution of the immune system to host defense against tumors has been an extensively debated question over the last decades (1, 2). In the human, there is still little if any direct evidence to support the immunosurveillance concept in particular for solid tumors. Malignant melanoma has represented one of the important models to assess the putative mechanisms of antitumor immunity. Numerous studies have shown that one can generate TIL2-derived T-cell lines or clones with major histocompatibility complex-restricted cytotoxic activity against autologous tumor cells in this disease (3–5). The relevance of these experiments is, however, questionable because there is usually no relation established between the in vitro cultured cells and the actual in vivo immune response. In addition, the clones studied are often derived from patients developing multiple metastases (3, 6, 7), where potential immunosurveillance mechanisms have evidently failed. To circumvent both problems, we have recently established an experimental system based on the direct in situ and subsequent in vitro study of “spontaneously” regressive malignant melanoma lesions.

We have analyzed previously a case of melanoma displaying the pathological and clinical criteria of tumor regression. It was shown that the regressive lesion was infiltrated by T-lymphocytes with a pathological and clinical criteria of tumor regression. It was shown that the specific cytotoxic T-lymphocyte clones characterized in vitro was actually selected and amplified in vivo at the lesion site. These results provide strong evidence that effector T-cells have contributed to tumor regression.

T-Cell Receptors and Amplification of TIL

We have derived from lymphocytes infiltrating a human regressive melanoma lesion a series of T-cell receptor α/β-dependent, HLA-B14-restricted cytotoxic T-lymphocyte clones reacting against the autologous tumor. Analysis of the T-cell receptor gene expression revealed that all the clones represented a unique cell expressing a Vβ13.1/β1.1 gene segment. T-cell receptor transcripts expressed in the cloned cells were compared to those present in the uncultured tumor tissue. This analysis demonstrated that the specific cytotoxic T-lymphocyte clones characterized in vitro was actually selected and amplified in vivo at the lesion site. These results provide strong evidence that effector T-cells have contributed to tumor regression.

Additional tumor cell lines maintained in culture include a human allogeneic melanoma cell line (K562), a nonadherent human myeloid leukemia line, and natural killer target. LAZ388 and LCL are EBV-transformed lymphoblastoid (EBV-B) cell lines established from a healthy donor (LAZ388) and from the melanoma patient (LCL), respectively.

mAbs Serological Reagents, and Phenotypic Analysis of TIL Cell Lines and T-Cell Clones

BMA 031, kindly provided by Dr. R. Kurrle (Behring Company, Marburg, Germany), reacts with a monomorphic determinant of TCR-α/β receptor (9). Anti-TCR-β1 mAb (T Cell Science, Cambridge, MA) recognizes a constant epitope of the human TCR-β chain (10). OKT3, OKT4, and OKT8 (Ortho Diagnostics Systems, Inc., Westwood, MA) react with the CD3, CD4, and CD8 proteins, respectively. W6/32 (11) and 9^Wood (12) mAbs recognize nonpolymorphic determinants of HLA class I and class II gene products, respectively. Anti-NK Tα mAbs recognizes an infrequent TCR-α/β clonotypic determinant (13). XC3, 3B8 (14), and anti-LFA1α react with the CD2, CD56, and CD11a antigens, respectively. EP-2 mAb, kindly provided by Dr. S. Ferrone (Department of Microbiology and Immunology, New York Medical College, Valhalla, NY), detects the major histocompatibility complex-restricted cytotoxic activity against autologous tumor cells in this disease (3–5). The relevance of these experiments is, however, questionable because there is usually no relation established between the in vitro cultured cells and the actual in vivo immune response. In addition, the clones studied are often derived from patients developing multiple metastases (3, 6, 7), where potential immunosurveillance mechanisms have evidently failed. To circumvent both problems, we have recently established an experimental system based on the direct in situ and subsequent in vitro study of “spontaneously” regressive malignant melanoma lesions.

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Phenotypic analysis of TIL cell lines and T-cell clones was carried out by indirect immunofluorescence using an Elite flow cytometer (Coulter Electronics, Inc., Hialeah, FL) as described previously (13).

**Cytotoxicity Assays.** The cytotoxic activity of TIL cell lines and T-cell clones was measured by a conventional 4-h 51Cr release assay using triplicate cultures in V-bottomed plates. E:T ratios were 25:1, 5:1, 1:1, 0.2:1, and 0.04:1 on 2000 target cells/well. The percentage of specific cytotoxicity was calculated conventionally; SDs were <5%. Functional effects of the antibodies and antiserum, either on effector cells (OKT3, OKT4, OKT8, XC3, BMA031, anti-LFA1-α, 3B8, NKTA) or on target cells (W6/32, 9–49, EP-2, NKTA, anti-HLA-A, -HLA-A2, -HLA-B12, -HLA-B14, -HLA-B35), were tested by incubating each of them for 2 h at 37°C before the assay at the predetermined saturating concentration.

The percentage of inhibition of lysis was calculated as

\[ 1 - \frac{\% \ 	ext{of specific lysis by mAb/antisera-treated wells}}{\% \ 	ext{of specific lysis in control wells}} \times 100\% \]

**Proliferation of T-Cell Clones to Autologous Tumor Cells.** Proliferative responses of the T-cell clone were examined by incubating 5 × 10^4 responder cells with 2.5 × 10^4 mitomycin C-treated autologous or allogeneic tumor or EBV-B cells for 2 days in 96-well round-bottomed plates in RPMI 1640 supplemented with 10% AB serum alone or in combination with 5 units/ml of IL-2. The cells were pulsed with 1 µCi of [3H]thymidine 16 h before harvest. [3H]Thymidine incorporation was measured in a Beckman liquid scintillation counter.

**Synthesis of the First Strand of cDNA.** Total RNA was prepared from the tumor fragment, autologous PBL, the TIL-derived cell line, and one T-cell clone using a single-step guanidium isothiocyanate-phenol chloroform extraction method (16). First strand cDNA was synthesized with oligo(dT) priming and reverse transcriptase.

**Molecular Analysis of TCR V Gene Segments Using PCR.** The procedure used to analyze TCR Vα and Vß gene segment expression has been described previously (17). Briefly, these TCR transcript cDNAs were amplified in a 30-cycle PCR, using the 29 different Vα and 24 Vß 5'-oligonucleotide primers (Val-w29/Vßl-w24), each of them paired with the corresponding Color Cα-specific 3' primer. Positive α and β controls consisted of constant region amplifications (180 and 190 base pairs, respectively) and negative controls were performed without cDNAs (17). The amplified products were detected by Southern blot analysis using a Cα or Cß oligonucleotide probe. Autoradiographs were scanned by a computer-assisted imaging system (Bio-Profil, Vil-bry-Lourmat, France) providing an absolute value for each autoradiographic spot. Each Vα or Vß spot was expressed as a percentage of the sum of all Vα or Vß signals detected on the autoradiogram, respectively.

**Cloning and Sequencing of Vß13 Transcripts.** The technique used for cloning and sequencing of Vß transcripts has been described previously (8). Briefly, the primer for the cloning of Vß13 sequences were 5'-CACTGCG-GTGACCCAGGATATGA-3' in the V region and 5'-ACCAGCTCAGCTC-GGCGGGOTTGCG-3' in the Cß region, the latter containing a SacII restriction site. The amplifications were performed in 2 rounds of 30 cycles. After ethanol precipitation, the amplified material was digested with SacII, separated on a 2% agarose gel, and purified by adsorption on glass beads (Gene Clean; Bio 101, Inc., La Jolla, CA). The material was ligated into a SacII-EcoRV pBS-K+ cloning vector (Strategene, La Jolla, CA) and used to transform XL-1 blue Escherichia coli strains (Strategene). The white colonies were screened by the dot blot technique and a Cß oligonucleotide probe. Plasmid DNA was extracted from positive colonies and sequenced by the dideoxy-chain termination method (Sequenase 2.0; United States Biochemicals, Cleveland, OH).

**RESULTS**

**Derivation and Characterization of TIL Cell Lines.** TILs, freshly isolated from the previously in situ studied tumor specimen, were expanded with IL-2/TCGF for 3 weeks and then plated at 5000 cells/well in the presence of irradiated autologous EBV-B cells plus allogeneic PBL as feeder cells.

This TIL-derived T-cell line increased by 120-fold at 2 weeks in the presence of IL-2/TCGF and displayed high levels of cytotoxicity against autologous tumor cells (data not shown). In contrast, no lysis of allogeneic melanoma, autologous and allogeneic EBV-B cell lines, or K562 was observed.

**Molecular Analysis of TCR Vα and Vß Gene Segments Using PCR.** The expression of TCR Vα and Vß gene segments in the cultured TIL line (day 13) was assessed here and compared to that observed previously in situ, where an increased expression of the Vß13, Vß14, and Vß16 gene segments was demonstrated (8). As shown in Fig. 1A, marked alterations in the expression of the Va gene segments were observed in the TIL-derived cell line. Some of them were absent or strongly decreased such as Va3, Va6, Va14, or Va20 gene segments, while others such as Va5 and Va15 were markedly upregulated. With respect to TCR β chains, the Vß13 and Vß22 subfamilies were increased in the TIL cell line (Fig. 1B). Conversely, Vß16, previously shown to be the most overexpressed Vß gene segment in situ (8), had strongly decreased during in vitro culture (Fig. 1B). Note that another TIL-derived T-cell line, where autologous tumor cells had been used for in vitro stimulation, has been analyzed. Results regarding the expression of TCR Vα and Vß gene segments were similar (data not shown).

**Generation of CTL Clones Directed against Autologous Melanoma.** Cells from the TIL line were cloned in the presence of irradiated autologous EBV-B cells, allogeneic PBL, and IL-2/TCGF. More than 100 T-cell clones were obtained. Twenty-five were examined by flow cytometry and all were found to display a CD3+, CD4+, CD8+, TCRα/β*, TCRγ/δ* phenotype. They were subsequently analyzed by PCR amplification with a series of previously described oligonucleotides (17). Of the 25 clones, 23 were found to express a Vß13 transcript and two α chain signals involving the Va3 and Va25 gene segments. Note that it is now well established that a unique T-cell may express two distinct TCR Vα chain transcripts (18–20).

Sequence of the Vß13 gene segment from the TIL cell line and one of the T-cell clones, designated 5G, were compared. cDNAs from the TIL-derived cell line and that of the T-cell clone 5G were found to represent a unique transcript including the Vß13,1 gene segment rearranged to Jß1.1 with a WGGD encoding junctional sequence (Table 1).

It thus became evident that a unique T-cell had been greatly amplified in culture and the representative T-cell clone 5G was selected for functional studies. It was tested for cytotoxicity against autologous and allogeneic melanoma cells, autologous and allogeneic EBV-B cells, autologous and allogeneic PHA blasts, and K562 in conventional 51Cr release assays.

As shown in Fig. 2, 5G cells displayed high levels of cytotoxicity (>60% specific lysis at an E:T ratio of 5:1) against the autologous melanoma cells but failed to lyse autologous EBV-B cells, autologous PHA blasts (data not shown), and all the allogeneic targets (melanoma cells, EBV-B cells, PHA blasts) (see Fig. 2).

Note that a specific cytotoxic activity was also found against the fresh autologous melanoma cells (data not shown).

**Inhibition of Cytolysis with Monoclonal Antibodies.** A panel of monoclonal antibodies was tested for the inhibition of specific cytolytic activity against the cultured autologous melanoma target cells (see Fig. 3A). Anti-C3D, anti-TCRα/β, anti-CD11a, and to a lesser degree anti-C2D mAb inhibited CTL activity. Anti-CD4 mAb was also strongly inhibitory, while anti-CD4 mAb had no effect. We tested the ability of either anti-HLA class I mAb (W6/32) or anti-HLA class II mAb (9–49) to block the cytotoxic activity at the target cell level. Pretreatment of autologous melanoma cells with mAb W6/32 inhibited the lytic interactions while mAb 9–49 and other control reagents had no effect (Fig. 3A).
Inhibition of Cytotoxicity by Human Anti-HLA-B14 Sera. HLA typing of the patients' cells showed that they are HLA-A3, -A10, -B14, -B35, -CW4, -CW8. To characterize more precisely the restriction element recognized by the CTL clone 5G, we used a series of relevant antisera from pregnant women in blocking experiments. It was found that HLA-B14 is likely to be involved in the recognition of the autologous tumor cells. Indeed, as shown in Fig. 3B, the corresponding antiserum inhibited the CTL activity up to 80% in 4-h 51Cr release assays at an E:T ratio of 1:1, while those directed against other specificities had little if any effect.

Proliferation of the CTL Clone 5G in Response to Autologous Melanoma Cells. Proliferative response of the 5G CTL clone was tested in 24-h coculture in the presence of either autologous or allogeneic melanoma cells and autologous or allogeneic EBV-transformed B-cells. As shown in Table 2, clone 5G proliferated following stimulation by the autologous melanoma cells, whereas other stimulator

Table 1 Analysis of TCR Vβ13 chain transcripts

<table>
<thead>
<tr>
<th>Cell</th>
<th>No. of sequences</th>
<th>No. of occurrences</th>
<th>Vβ</th>
<th>(N)D/R(N)</th>
<th>Jβ</th>
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<tr>
<td>Tumor</td>
<td>20</td>
<td>6</td>
<td>CASS WGGD</td>
<td>TEAFF (Jβ1.1)</td>
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<tr>
<td></td>
<td>7</td>
<td></td>
<td>CASS FRSAPP</td>
<td>NTEAFF (Jβ1.1)</td>
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<tr>
<td></td>
<td>1</td>
<td></td>
<td>CASS YRI</td>
<td>TEAFF (Jβ2.7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td></td>
<td>CASS RLAVG</td>
<td>NTEAFF (Jβ1.1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td></td>
<td>CASS YGRDG</td>
<td>YEQYF (Jβ2.7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td></td>
<td>CASS ERR</td>
<td>NTEAFF (Jβ1.1)</td>
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<tr>
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<td>CASS NPLR</td>
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<td>CASS YS</td>
<td>YEQYF (Jβ2.7)</td>
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<tr>
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<td>1</td>
<td></td>
<td>CASS LDGGG</td>
<td>TEAFF (Jβ1.1)</td>
<td></td>
</tr>
<tr>
<td>TIL line</td>
<td>19</td>
<td>19</td>
<td>CASS WGGD</td>
<td>TEAFF (Jβ1.1)</td>
<td></td>
</tr>
<tr>
<td>Clone 5G</td>
<td></td>
<td></td>
<td>CASS WGGD</td>
<td>TEAFF (Jβ1.1)</td>
<td></td>
</tr>
</tbody>
</table>
cells had no effect. Addition of low doses of IL-2 (5 units/ml) augmented substantially the proliferation of the CTLs in response to the irradiated autologous melanoma cells.

Together, the results demonstrate that the Vβ13+ T-cell clone is a specific CTL recognizing the autologous melanoma in a conventional HLA class I-restricted, TCR-dependent fashion.

**Evidence for in Situ Clonal Expansion of the Vβ13+ CTL.** The relevance of the observation that 5G cells are able to kill autologous melanoma cells was assessed by testing whether this clone was actually present and potentially overrepresented at the tumor site.

Analysis of the Vβ13 transcripts in the tumor in situ revealed that the cDNA clone, encoding the same WGGD junctional sequence as CTL clone 5G, represented 30% (6 of 20 sequences) of the Vβ13 transcripts in the tumor (Table 1). Note that another Vβ13 cDNA clone, encoding an FRSAPP junctional sequence, found in 7 of 20 sequences (33%) in the tumor (Table 1), was not identified in the cell cultures.

In contrast, Vβ13+ TCR β chain transcripts from autologous PBL were polyclonal; i.e., none of the 20 sequences studied was repeated (data not shown). Moreover, the predominant cDNA clone encoding the WGGD amino acid junctional sequence was not found among the 20 Vβ13 transcripts of the autologous PBL (data not shown).

**DISCUSSION**

In a previous study, we have shown through direct sequencing of TCR transcripts that unique T-cells were selected and amplified at the tumor site in a spontaneously regressing melanoma lesion (8). Here we have generated T-cell lines derived from lymphocytes infiltrating a malignant melanoma. These findings are in line with the view that in

exclusively may be misleading. In this regard, we have established an original experimental system which allows us to compare *in vitro* and *in situ* data.

The Vβ13+ T-lymphocyte clones characterized here display a conventional CTL activity against autologous melanoma cells. Indeed, cytolyis was found to be CD3/TCR and CD8 dependent, HLA class I restricted, without cross-reactivity to any additional target cells (Figs. 2 and 3). In addition, the cloned lymphocytes are able to proliferate specifically in the presence of autologous tumor cells (Table 2).

Blocking experiments with antisera to the relevant HLA molecules provided evidence that HLA-B14 is the restriction element recognized by the Vβ13+ CTLs (Fig. 3). Most of the melanoma-specific CTL clones presently described have been shown to use HLA-A alleles for tumor recognition, in particular HLA-A1/A2, known to be represented in the population with a high frequency (21–25). In contrast, the role of HLA-B molecules as restriction elements has been suggested only following immunoselection experiments (26) or after pretreatment of tumor cells with γ-interferon (27). Our results demonstrate that HLA-B alleles are indeed used operationally to present antigens in malignant melanoma. These findings are in line with the view that
vivo antitumor responses are complex involving different HLA loci for presentation of either the same or distinct tumor-associated antigenic peptides.

The major contribution of this study is the demonstration that the specific Vß13+ melanoma-reactive CTL clone, generated in vitro, is actually selected and amplified at the tumor site even though this particular cell may not be the most represented in vivo (i.e., in light of in vitro selection). The fact that it was characterized here as a specific CTL while cancer regression was unequivocally documented both clinically and histologically in this patient strongly supports the view that the adaptive immune response has represented an active mechanism of defense against the tumor.

Further studies in this experimental system should provide useful information on the nature of the immune response. Attempts to expand and characterize additional T-cell populations, known to be represented in situ, may help to assess the diversity of the antitumor reaction. This approach should also lead to characterize tumor-associated antigenic peptides such as for, example, those recently described in the MAGE series (28) in a situation of actual clinical relevance.

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REFERENCES

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