High Frequency of Cytolytic T Lymphocytes Directed against a Tumor-specific Mutated Antigen Detectable with HLA Tetramers in the Blood of a Lung Carcinoma Patient with Long Survival

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ABSTRACT

We have identified an antigen recognized by autologous CTL on the lung carcinoma cells of a patient who enjoyed a favorable clinical evolution, being alive 10 years after partial resection of the primary tumor. The antigenic peptide is presented by HLA-A2 molecules and encoded by a mutated sequence in the gene coding for malic enzyme, an essential enzyme that converts malate to pyruvate. In the tumor cell line derived from the patient, only the mutated malic enzyme allele is expressed, because of a loss of heterozygosity in the region of chromosome 6 that contains this locus. Tetramers of soluble HLA-A2 molecules loaded with the antigenic peptide stained ~0.4% of the patient’s blood CD8 T cells. When these cells were stimulated in clonal conditions, 25% of them proliferated, and the resulting clones were lytic and specific for the mutated malic enzyme peptide. T-cell receptor analysis indicated that almost all of these antimaltic CTLs shared the same receptor. Antimaltic T cells were consistently found in blood samples collected from the patient between 1990 and 1999, at frequencies ranging from 0.1 to 0.4% of the CD8 cells. Their frequency appeared to double within 2 weeks after intradermal inoculation of lethally irradiated autologous tumor cells. These results indicate that nonmelanoma cancer patients may also have a high frequency of blood CTLs directed against a tumor-specific antigen.

INTRODUCTION

Over the last decade, efforts to characterize antigens that are specifically recognized on human tumors by autologous T lymphocytes led to the identification of a large number of genes that code for such antigens (1). These genes fall into five main groups: cancer-germ-line genes, which are expressed in tumors and not in normal cells except for male germ-line cells, which do not carry HLA molecules and, therefore, cannot present antigens to T cells; differentiation genes, which are expressed in tumors and not in normal cells except for male germ-line cells; genes that are mutated or overexpressed in tumor cells as viruses (1). These genes fall into five main groups: cancer-germ-line and male; genes that are mutated or overexpressed in tumor cells as viruses (1).

Antigens that are encoded by cancer-germ-line genes such as the MAGE-A genes ought to be absolutely tumor-specific and could be used safely to vaccinate cancer patients. In a clinical trial involving immunization with the MAGE-3.A1 peptide, encoded by gene MAGE-3 and presented by HLA-A1, 25 melanoma patients with measurable tumors received three monthly injections of peptide, without adjuvant (2, 3). Seven patients showed tumor regressions, three of which were complete. Partial tumor regressions were also observed in other trials, involving injections of dendritic cells incubated with MAGE-1 and MAGE-3 peptides presented by HLA-A1 or HLA-A2 (4, 5). These results suggest that therapeutic vaccination with tumor-specific antigens may be effective, although the majority of patients fail to respond. One of the several causes that could explain the low proportion of clinical responses is a poor ability of the vaccine to stimulate a specific T-cell response. Somewhat surprisingly, we could not detect peptide-specific T cells in the blood of the HLA-A1 patients who received the MAGE-3.A1 peptide, even in those patients who showed tumor regression (2). This could result either from a low immunogenicity of the vaccine or from the fact that such responses are not to be expected. For instance, CTLs that are truly tumor specific may not circulate in the blood, or those that are in the blood may be anergic and remain undetected after in vitro restimulation.

To obtain quantitative information on circulating tumor-specific CTLs, we resorted to the analysis of a few cancer patients who enjoyed unusually favorable clinical evolutions. One such patient is melanoma patient LB33, who is alive 12 years after the first appearance of metastases (6). One of the several antigens recognized by autologous CTLs on LB33 melanoma cells is presented by HLA-A28 molecules and encoded by a mutated sequence in gene MUM-3 (7). We showed with limiting dilution analysis and with tetramers that ~1% of blood CD8 cells of the patient were MUM-3-specific, and that these cells were responsive to restimulation in vitro. These results suggested that a strong CTL response, detectable in the blood with tetramers, was an achievable goal for therapeutic vaccination.

Admittedly, the case of patient LB33 could be a rare exception to the absence of CTLs directed against tumor-specific antigens in the blood. However, we report here a similar observation with a lung carcinoma patient. Patient LB37 presented in 1990 with a squamous-cell carcinoma of the lung that invaded the mediastinum and could be resected only partially. A cell line, LC-5, was derived from the tumor. The patient received adjuvant chemotherapy, and a complete tumor response was obtained. Subsequent to 1990, he was vaccinated with lethally irradiated autologous tumor cells. In 1993, enlarged mediastinal lymph nodes were shown to be invaded by tumor cells. The patient was treated with radiotherapy and then again with autologous vaccines. Remarkably, he has been without evidence of cancer for the last 7 years. We described previously the derivation of tumor-specific CTL clones from blood lymphocytes of this patient (8). Here we report on the identification of the antigen that is recognized by these CTLs, and we show that it is recognized by a high frequency of blood CD8 T cells.
MATERIALS AND METHODS

Patient LB37. Patient LB37 (HLA-A2, A26, B8, B51, Cw2, Cw7), a 55-year-old man, presented in January 1990 with a squamous cell carcinoma of the lung. Thoracotomy showed a T3N2 tumor, which could be partially resected. The patient received two courses of cisplatin, vindesin, and mitomycin C as an adjuvant chemotherapy, and a complete response was obtained. From October 1990 on, the patient was vaccinated with intradermal inoculations of lethally irradiated autologous tumor cells, namely LC-5 cells and five clones derived from a population of LC-5 cells that survived a mutagen treatment with 

ennon, were cotransfected with 1.5 CTAAACTA(T)18 VZ with V

DNA to cDNA with the Superscript Choice System (Life Technologies, Inc., Gaithersburg, MD), supplemented with 10% FCS (Life Technologies, Inc.) and maintained in Iscove’s medium (Life Technologies, Inc.) with 10% FCS and 20% FBS (Life Technologies, Inc., San Diego, CA).

The library was divided into 1900 pools of 80–100 cDNA clones. Each pool containing a HLA-A2 construct were cultured in DMEM (Life Technologies, Inc.) with 10% FCS. The TNF-α-sensitive WEHI-164cl3 cells were cultured in RPMI 1640 (Life Technologies, Inc.) with 5% FCS (9). The derivation and culture of CTL clones 110 and 1/7 were described previously (8).

Construction and Screening of the CDNA Library. Total RNA was extracted from LC-5 cells using the guanidine-isothiocyanate procedure (10). Polyadenylated RNA was enriched with an oligo(dT)-cellulose column (mRNA Purification kit, Pharmacia Biotech, Uppsala, Sweden) and converted to cDNA with the Superscript Choice System (Life Technologies, Inc., Gaithersburg, MD) using an oligo(dT) primer [5'-ATAAGAATTCGCGCCCTTAACTA(T)18 VZ with V = G or A or C or Z = G or A or T or C] containing a NotI site. The cDNA was ligated to HindIII-EcoRI adaptors (Stratagene, Heidelberg, Germany), phosphorylated, digested with NotI, and inserted into the HindIII and NotI sites of expression vector pCEP4 (Invitrogen, San Diego, CA). Escherichia coli DH5α were transformed by electroporation with the recombinant plasmids and selected with ampicillin (50 μg/ml). The library was divided into 1900 pools of 80–100 cDNA clones. Each pool was amplified for 4 h, and plasmid DNA was extracted using the QIAprep 8 plasmid kit (Qiagen, Hilden, Germany). Duplicate microcultures of 293-EBA cells, plated in flat-bottomed 96 microplates (3–4 x 10⁴/well) 24 h before transfection, were cotransfected with 1.5 μg of Lipofectamine (Life Technologies, Inc.), 100–200 ng of DNA from a pool of the cDNA library and 50 ng of plasmid pcDNA1/Amp containing a HLA-A*0201 genomic clone. Transfected cells were tested after 24 h for expression of the antigen in a CTL stimulation assay. Briefly, CTL clone 1/7 was added, and the TNF content of the supernatant was measured 24 h later by testing its cytotoxic effect on WEHI-164cl3 cells (9) with a MTT colorimetric assay (11). One pool of cDNA proved positive. It was subcloned, and cDNA clone 94 was found to transfer the expression of the antigen. cDNA 94 was 1886 bp long and contained at its 3′ end a polyadenylation signal and a polyadenylate tail.

Sequence Analysis. To demonstrate that the malic enzyme gene of the tumor cells contained a point mutation, RNA extracted from LC-5 and from LB37-EBV-B cells was converted to cDNA using an oligo(dT) primer. The cDNA served as templates for four independent PCR amplifications with primers OPCEO2 (5′-TGCGGAGGAGTATCATCCTC) and OPC586 (5′-GTTAAGGACGCAGCTCC). The PCR products were pooled and cloned into vector pCR3 with the Eukaryotic TA Cloning kit (Invitrogen). Six clones from the tumor cells and five clones from the EBV-B cells were sequenced with the BigDye Terminator Cycle Sequencing kit (Perkin-Elmer Applied Biosystems, Warrington, Great Britain), using primer OPC602. Products of the sequencing reactions were analyzed on an ABI 310 Sequencer (Perkin-Elmer). To prove that the mutation was present in the tumor cells in vivo, RNA was extracted from tumoral tissue obtained through a mediastinoscopy performed in 1993 for an enlarged mediastinal lymph node, and was converted to cDNA. Three independent PCR products, obtained with primers OPC632 (5′-GAAAATGAGGAGTATCATCCTC) and OPC633 (5′-ATCATGAGTCGACCTAGTTTGCC), were pooled and cloned in pCR3 as above. Seven independent clones were sequenced, and all of them corresponded to the mutated gene. Reverse-transcribed RNA from lung tumor samples was converted to cDNA and amplified by PCR, and the products were sequenced. For the microsatellite analysis, genomic DNA was extracted from LC-5 and from tumors using blood lymphocytes using the Qiagen Qiachip purification kit. PCR amplification was performed on 20 ng of DNA using pairs of primers specific for microsatellites of chromosome 6. The reaction mixture contained 1 μl-malate, 0.25 mM NADP⁺, 5 mM MgCl₂, 50 mM KCl, 5 mM KF, and 25 mM HEPES (pH 7.1).

Peptides. Peptides were synthesized on solid phase using F-moc for transfer of the mature protein domains (amino acids 1–276 of the mature protein) and characterized by mass spectrometry. All of the peptides were >80% pure, as determined by analytical high-performance liquid chromatography. Lyophilized peptides were dissolved at 10 mg/ml in DMSO, aliquoted, and frozen at −20°C. Binding of wild-type and mutated malic enzyme peptides was tested with a competition assay, essentially as described previously (13). Briefly, (Genzyme, Cambridge, MA), and a mixture of irradiated feeder cells consisting of lethally irradiated autologous tumor cells, namely LC-5 cells and five clones from the EBV-B cells were sequenced with the

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ferred into 2-ml wells and restimulated as above, but with a 10-fold higher number of feeder cells. Clones were maintained with weekly restimulations. Phenotypic analysis of PBMCs was performed using the following monoclonal antibodies conjugated to either FITC, PE, or PerCP: Leu2a (anti-CD8; PerCP; Becton Dickinson); and HI100 (anti-CD45RA; PE; PharMingen). Cells were washed, stained with tetramers for 15 min at ambient temperature and for an additional 15 min with antibodies at 4°C, washed, and fixed with paraformaldehyde. Isotype-matched antibodies and HLA-A2 tetramers with irrelevant peptides were used to verify the staining specificity, and as a guide for setting the markers to delineate positive and negative populations.

TCR Vα and Vβ usage of the CTL clones was assessed by reverse transcription-PCR and sequencing. cDNA served as a template for a PCR amplification using panels of Vα- or Vβ-specific forward primers and one reverse Cα or Cβ primer. The PCR products were purified and sequenced.

RESULTS

A Malic Enzyme cDNA Coding for an Antigen Recognized by CTL Clones on Autologous NSCLC Cells. CTL clones 110 and 1/7 were derived from blood lymphocytes collected from patient LB37 in 1994 and 1995, respectively, and stimulated with the autologous NSCLC clonal line LC-5 (8). Both clones lysed the tumor cells but did not lyse autologous PHA-activated T lymphocytes, autologous fibroblasts, or K562 cells (Fig. 1A). An anti-HLA-A2 monoclonal antibody inhibited the recognition of LC-5 cells by the two CTLs. CTL clone 1/7 lysed autologous EBV-transformed B cells, whereas CTL 110 did not. This suggested that the two CTLs recognized distinct antigens.

We identified a cDNA clone, named 94, encoding the antigen recognized by CTL 1/7 by screening for CTL recognition cells transfected with a cDNA library derived from LC-5 cells and with an HLA-A2 construct. cDNA 94, which could also transfer the expression of the antigen recognized by CTL clone 110 (Fig. 1B), corresponded to the gene encoding the cytoplasmic form of malic enzyme. This ubiquitous enzyme catalyzes the oxidative decarboxylation of malate to pyruvate with concomitant reduction of NADP+ to NADPH. cDNA 94 contained an open reading frame corresponding to residues 70–572 of malic enzyme, which contains 572 amino acids.

Presence of a Point Mutation in the cDNA. The malic enzyme sequence encoded by cDNA 94 matched exactly those present in data banks (HSU43944, L34035, and X77244) except for one amino acid. A cysteine to guanine substitution, at position 485 of cDNA 94, modifies the alanine residue (GCA), present at position 231 in residues 70–572 of malic enzyme, which contains 572 amino acids.

Identification of the Antigenic Peptide. The mutated glycine sequence encoded by cDNA 94 matched exactly those present in data banks (HSU43944, L34035, and X77244) except for one amino acid. A cysteine to guanine substitution, at position 485 of cDNA 94, modifies the alanine residue (GCA), present at position 231 in residues 70–572 of malic enzyme, which contains 572 amino acids.

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this CTL. We resorted to P815 cells transfected with an HLA-A2 gene. CTL 1/7 lysed P815-A2 that was sensitized with the mutated malic peptide, with a half-maximal effect at 1 nM (Fig. 3B). Similar results were obtained with CTL 110. But contrary to CTL 110, CTL 1/7 recognized the normal peptide, although half-maximal lysis required 100 nM peptide. This recognition of the normal peptide was confirmed when CTL 1/7 was stimulated to produce TNF by P815-A2 cells incubated with peptides. In this assay, the mutated peptide was 300-fold more efficient than the normal peptide.

We conclude that CTL 1/7 and 110 recognize the same mutated peptide from malic enzyme, but that CTL 1/7 recognizes the normal peptide also, although with a much lower affinity.

**Staining Antimalic T Cells with Soluble HLA/Peptide Complexes.** Soluble HLA-A2/peptide complexes were prepared with the mutated or the wild-type malic enzyme peptide, biotinylated, and multimerized with avidin conjugated to PE. CTL 110 was labeled with the “tetramer” containing the mutated malic peptide, but only weakly with that containing the normal peptide (Fig. 4A). CTL 1/7 was labeled with both tetramers, but the labeling was stronger with the tetramer containing the mutated peptide. These results are in agreement with the pattern of recognition of the mutated and normal peptides by the CTL clones (Fig. 3B).

Blood mononuclear cells collected from patient LB37 in October 1998 were labeled with an anti-CD8 antibody and tetramers containing the mutated peptide. Approximately 0.4% of CD8 T cells were
stained (Fig. 4B). To assess the specificity of this staining, the tetramer-positive cells were seeded at 1 cell/well and stimulated with PHA, IL-2, IL-4, IL-7, and feeder cells. After 3 weeks, populations of lymphocytes were present in 26% of the 160 wells in which the presence of a sorted cell had been confirmed visually 2 h after the cloning. These populations proved to be antimalic CTL clones: all of the cells were labeled with the tetramer containing the mutated malic enzyme peptide and with tetramers containing the normal peptide, albeit with a lower intensity. This pattern of labeling corresponded to that of CTL clone 1/7. All of the clones displayed a pattern of lysis that was also similar to that of clone 1/7, namely a strong lysis of LC-5 cells and a weaker lysis of autologous EBV-B cells. We conclude that the blood of patient LB37 contained a high frequency of antimalic CD8 T cells, which appeared functional in so far as they proliferated after an in vitro stimulation.

Analysis of the Antimalic T-cell Response of Patient LB37. We followed the frequency of CD8 cells labeled with the mutated malic tetramer in blood samples collected from patient LB37 between 1990 and 1999 (Fig. 5). These cells could be detected in all of the samples that were tested, at a frequency of 0.1–0.4% of the CD8 cells.

Before the injection of autologous tumor cells, 26% of the tetramer-positive cells were CD45RA− (Fig. 6). Two weeks after the vaccine was given, this proportion was 49%. No significant change was observed for the CD8 cells labeled with the tetramer containing the BMLF1.A2 peptide, most of which were CD45RA−. These results are consistent with the hypothesis that the autologous vaccine restimu-
lated some of the antimalic T cells, which proliferated and lost the expression of the CD45RA marker.

Diversity of the Antimalic CTL Response of Patient LB37. As expected, CTL clones 110 and 1/7 expressed different TCRs, using Vα14-Vβ4 and Vα12-Vβ5 gene segments, respectively (Fig. 7). The same Vα12-Vβ5 TCR was used by almost all (28/29) of the CTL clones that were tested and that were derived from tetramer-positive CD8 cells found in blood collected in 1990 or 1998, which indicated that the antimalic CTL response of patient LB37 is quasimonoclonal.

DISCUSSION

NSCLC, which includes squamous, adeno-, and large-cell carcinomas, represents 60% of lung cancers. NSCLC cell lines are difficult to derive, but a few of them could be used to stimulate autologous lymphocytes and to derive tumor-specific CTLs (8, 15). Two antigens recognized by such CTLs were identified previously: a peptide presented by HLA-A2 molecules and encoded by HER2/neu, which is overexpressed in many tumors (16), and a peptide on HLA-A28 and encoded by a mutated elongation factor 2 gene (17). We describe here the third NSCLC antigen that is defined with autologous CTLs. It is also generated by a point mutation present in the tumor.

The change of alanine to glycine caused by the mutation corresponds to the loss of a methyl group on the side chain of the amino acid at position 8 of the antigenic peptide. According to previous structures of peptides bound to HLA-A2 molecules, this side chain points toward the TCR (18). CTL clone 110 is perfectly specific for the mutated peptide, whereas CTL 1/7 also recognizes the normal peptide, although with a much lower affinity. This CTL recognizes EBV-B cells but not normal fibroblasts or activated T cells. A likely explanation for the lysis of autologous EBV-B cells by CTL 1/7 is that the high level of surface expression of HLA-A2 molecules on EBV-B cells leads to the presentation of a sufficient number of normal malic peptides for recognition by the CTLs. Fibroblasts or activated T lymphocytes would not be recognized because of their lower level of expression of HLA class I molecules. Another possibility is that the wild-type malic enzyme peptide can be processed by immunoproteasomes, present in EBV-transformed B cells, but not by standard proteasomes present in other types of normal cells that were tested. A few antigenic peptides were described that are generated by the immunoproteasome but not by the standard proteasome, which operates a cleavage within the epitope (19, 20).

Almost all of the antimalic CTLs that we could detect in the blood of patient LB37 have the same TCRs as CTL clone 1/7 and lyse, although at a low level, autologous EBV-B cells. Notwithstanding, no clinical signs of autoimmunity were observed in patient LB37, which suggested that the incomplete tumor-specificity of the response is without serious consequences. This finding is important for immunotherapy, because it indicates that antigens recognized to a certain extent on EBV-B cells should not be excluded a priori from a further identification procedure.

There are many similarities between the tumor-specific CTL responses of lung cancer patient LB37 and of melanoma patient LB33 (7). Both patients are long survivors (>10 years) after having metastatic tumors and having received injections of irradiated autologous tumor cells. Most of the tumor-specific CTLs derived from their blood recognize tumor-specific antigens resulting from point mutations. These CTLs were present before the patients received autologous vaccines, and then persisted at high numbers representing 0.1–1% of the blood CD8 cells. They are essentially monoclonal populations. They can be labeled with the relevant tetramers and respond to restimulation in vitro with antigen and growth factors. These corroborating results demonstrate that strong CTL responses against antigens that are absolutely tumor-specific can be detected in the blood of cancer patients using tetramers, and justify the use of this methodology to evaluate the immunization of cancer patients against tumor-specific antigens such as those encoded by the MAGE-A genes.

It is possible that antimalic CTL played a role in the clinical outcome of patient LB37. High numbers of tumor-specific CTLs were consistently found in the blood of patient LB37, between 1990 and 1999. Almost identical results, in terms of frequency and persistence, were found in melanoma patient LB33 (7). These CTL responses may be contributing to the very long survival of these patients. The antigen that is presented to these autologous CTLs on the LB37 tumor cells is strictly tumor-specific and is encoded by a gene for which there is a loss of heterozygosity in the tumor. These tumor cells most likely cannot escape from CTL attack by losing the expression of the antigenic peptide. They have only one copy of the chromosomal region encoding malic enzyme, and the loss of this copy would be lethal because either malic enzyme or other proteins encoded in this region of chromosome 6 are essential for survival. It should be noted that the gene encoding N-acetylglucosamine-mutase, an essential enzyme for glycoprotein synthesis, is just next to that of malic enzyme in the human genome. The absence of an appropriate tumor sample unfortunately prevented us from demonstrating that the same gene deletion in chromosome 6 was present in the tumor in vivo.

It is noteworthy that a high number of antimalic T cells were already present in the blood of patient LB37 before the first vaccination with autologous tumor cells. A similar observation has been made for the anti-MUM-3 CTLs of melanoma patient LB33. This patient had received large amounts of IL-2 prior to the first collection of blood in which anti-MUM-3 CTL were found, leaving room for doubt about the fact that the tumor alone induced this CTL response. Patient LB37, however, did not receive immunostimulatory treatments, and the antimalic response was, therefore, most probably induced by the tumor. This was not reported before, although melanoma patients were shown to have high frequencies of blood CTLs against Melan-A/MART-1 or tyrosinase peptides presented on HLA-A2 (21–23). But considering that these antigens are also present on melanocytes, and that normal individuals may also have anti-Melan-A/MART-1 CTLs in their blood, it is impossible to evaluate the role played by tumor cells in the induction of these CTLs in melanoma patients.

It is remarkable that essentially all of the antimalic CTLs that were present in the blood of patient LB37 express the same TCR. We also observed the dominance of one clone in the antimelanoma CTL response of patient LB33 (7). In addition, although patients LB33 and LB37 received multiple injections of autologous tumor cells, which express several antigens, their antitumor CTL response appears to be locked on one antigen recognized by one CTL clone. A simple explanation for the dominance of one antigen/CTL pair is that it results from the proliferation of the first antitumor CTL precursor that was stimulated, possibly by cells of the tumor. It is then maintained at
each vaccination because the increased frequency of this CTL favors its restimulation before that of other precursors of CTL, until the antigen is cleared. This model, which was proposed earlier (24), is not without consequence for vaccination. For example, if the vaccine is a recombinant protein or consists of a sequence encoding several antigenic peptides, it leads to the presentation of various antigens on the same cells. If, as it is the case for patients LB33 and LB37, the T-cell response is monoclonal, this dominance will persist throughout the vaccination protocol. A tumor variant with a loss of the corresponding antigen could then escape, although vaccinations are pursued with several other antigens.

ACKNOWLEDGMENTS

We thank Thérèse Aerts and Catherine Muller for expert technical assistance.

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*Cancer Res* 2001;61:3718-3724.

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