Identification of a Tumor-specific Shared Antigen Derived From an Eph Receptor and Presented to CD4 T Cells on HLA Class II Molecules

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ABSTRACT

We obtained a lytic CD4 T-cell clone that recognized an antigen presented by HLA-DRB1*1101 on the tumor cells of a melanoma patient who enjoyed an unusually favorable clinical evolution. The antigen appeared to be shared between several melanoma cell lines. To identify the encoding gene, we used a new method, based on the cotransfection into human embryonal kidney cell line 293 of a cDNA library from the tumor together with a cDNA clone encoding the class II transactivator, which induces the expression of HLA class II molecules. The product of the gene coding for the antigenic peptide is EphA3, a member of the Eph family of tyrosine kinase receptors, which mediate the repulsion of neural cells by cells carrying the ligand Ephrins on their surface. EphA3 is expressed at a high level in the retina and fetal brain, at a lower level in several normal tissues, and not at all in hematopoietic cells, the only cells that constitutively express HLA class II molecules. It is overexpressed in several types of tumors, including melanoma, lung carcinoma, and sarcoma. On the basis of this pattern of expression, EphA3 may be a source of tumor-specific antigens recognized on tumor cells that express HLA class II molecules. Anti-EphA3 T cells may have participated in a tumor rejection response in the patient, because the cells of metastases collected several years later than the metastasis used to characterize the antigen had lost expression of HLA-DR or EphA3, therefore escaping recognition by these lymphocytes.

INTRODUCTION

A large number of tumor antigens have been identified that are recognized by CTLs derived from blood lymphocytes or tumor-infiltrating lymphocytes stimulated with autologous tumor cells. Most of these antigens are presented by HLA class I molecules to CD8 lymphocytes. But tumor-specific CD4 T cells have also been obtained in vitro by stimulation of T cells with autologous tumor cells. In some instances, by using CD4 T-cell clones that specifically recognized the autologous tumor cells, it was possible to isolate genes coding for the target antigens. Some of these genes, such as tyrosinase or gp100, are expressed in normal melanocytes and in melanoma cells (1–4). Other genes were mutated or rearranged. One antigenic peptide was encoded by a mutated triosephosphate isomerase (5). Another was derived from a mutated CDC27 protein that had a cytotoxic localization, whereas the normal protein is found in the nucleus (6). This led to the generation of a peptide presented to CD4+ T cells through the MHC class II pathway. A third was encoded by a fusion gene consisting of a low density lipid receptor gene and the GDP-l-fucose-d-galactoside 2-L-fucosyltransferase gene (7).

We have pursued our analysis of the antitumor T-cell response of melanoma patient LB33, because this patient enjoys a favorable evolution of her disease that is associated with a very strong and sustained antitumor CTL response (8). Two tumor cell lines, MEL.A and MEL.B, were derived from metastases resected in 1988 and 1993, respectively. The patient developed a very strong CTL response against the MEL.A cells (9). From blood lymphocytes collected in 1990, we derived a panel of anti-MEL.A CTL clones that recognize at least seven distinct antigens presented by various HLA class I molecules. Four of these antigens were shown to result from point mutations in genes with ubiquitously expression (10–12). One of them, presented on HLA-A28 molecules, is recognized by >1% of the autologous blood CD8 T lymphocytes, as measured with complexes of soluble HLA-A28 molecules loaded with the antigenic peptide (12). This is the highest frequency of truly tumor-specific CTLs that has been found in a cancer patient. These CTLs could be restimulated in vitro and lysed the autologous tumor cells. The MEL.B cells resist lysis by the anti-MEL.A CTLs because they have lost expression of HLA class I molecules, except HLA-A24, which did not present antigens to anti-MEL.A CTLs. This strongly suggests that MEL.B cells were selected in vivo by the strong anti-MEL.A CTL response.

Thus far, all of the antitumor CTLs that we have obtained by stimulating blood lymphocytes of patient LB33 with the MEL.A cells recognized antigens that are absolutely tumor specific. In addition, one of these CTLs is present in the blood in great number. This strong tumor-specific CTL response may very well have participated in the remarkably favorable clinical course of the patient since 1990, characterized by the appearance of single metastases that were treatable by surgery in 1993, 1994, and 1999. In view of this, it was interesting to analyze the involvement, if any, of HLA class II-restricted T lymphocytes in the anti-MEL.A immune response. We report here the identification of MEL.A cells of a tumor-specific shared antigen presented to autologous lytic T cells on HLA-DR11 molecules.

MATERIALS AND METHODS

Cell Lines. The clinical course of melanoma patient LB33 (HLA-A24, A28, B13, B44, Cw6, and Cw7) and the obtention of the various LB33-MEL clonal cell lines and antigen-loss variants were described (8, 9). Melanoma cell lines LB33-MEL.A and MEL.B were derived from a cutaneous and an intestinal metastasis resected from patient LB33 in 1988 and 1993, respectively. Clonal cell lines MEL.A-1 and MEL.B-1 were derived from MEL.A and MEL.B, respectively, by limiting dilution. HLA-DR-negative and HLA-DR-positive MEL.B-1 cells were sorted by flow cytometry, and MEL.B-1.1 and MEL.B-1.2 clonal cell lines were derived from each population by limiting dilution. The MEL.D cell line was derived from a lymph node metastasis resected from patient LB33 in 1999. An individual point mutation in the ubiquitously expressed gene MUM-1, responsible for the expression of antigen LB33-B on the tumor (10), was found in DNA extracted from MEL.A, MEL.B, and MEL.D, confirming that the three cell lines derive from the same tumor. LB33 tumor cell lines and melanoma cell lines LB4-MEL, LB34-MEL, and MZ2-MEL were cultured in Iscove’s medium (Life Technologies, Inc., Grand Island, NY).
EphA3-ENCODED TUMOR ANTIGEN PRESENTED BY HLA-DR

Island, NY) supplemented with 10% FCS (Life Technologies), 116 mg/l t-arginine, 36 mg/l t-asparagine, and 216 mg/l t-glutamine (AAG). 293-E DNA cells (Invitrogen, San Diego, CA) were maintained in DMEM (Life Technologies) with 10% FCS. WEHI-16413 cells (13) were cultured in RPMI 1640 (Life Technologies) with 5% FCS. LG2-EBV were cultured in Iscove’s medium containing 10% FCS. M-07e cells were kept in Iscove’s medium supplemented with AAG, 10% FCS, and 50 ng/ml of recombinant human IL-3 (Sandoz).

**HLA Class II Expression and Cloning of cDNA Encoding HLA-DR.**

For the analysis of class II expression of HLA class II molecules, tumor cells were incubated with monoclonal antibodies Leu-10 (anti-HLA-DQ), L243 (anti-HLA-DR), and B7/2 (anti-HLA-AP) from Becton Dickinson for 20 min at 4°C in 134 mM NaCl, 5 mM KCl, 0.4 mM MgSO4, 0.3 mM MgCl2, 5 mm NaHCO3, 1 mM EDTA, 500 units/ml penicillin, 1 μg/ml streptomycin, and 1% FCS and buffered with phosphate (1 mM; pH 7.4). The cells were washed, incubated further with FITC-conjugated antimouse immunoglobulin for 20 min at 4°C, fixed with 0.5% paraformaldehyde, and analyzed by flow cytometry.

cDNA clones encoding the HLA-DRB1*1101 and DRB3*0202 chains of patient LB33 were obtained as follows. RNA prepared from LB33-EBV-B cells was converted to cDNA with Moloney murine leukemia virus reverse transcriptase (Boehringer Mannheim) using an oligo-dt primer. The cDNA was used as a template for a PCR amplification with primers PCX3DR (5'-CCGCGATCCGATGGTGTTGCTG) and PCX4DR (5'-GAGATTCTCAGCTGGATAGATGTTTGGT). The PCR product was purified using the QiAquick PCR purification kit (Qiagen, Hillen, Germany), digested with BamHI and EcoRI, and ligated into expression vector pcDNA3 (Invitrogen). The constructs were transfected by electroporation into Escherichia coli DH5a, and plasmid DNA extracted from several independent colonies was sequenced. The DRA*0101 cDNA clone was obtained from an MZ2-MEL cDNA library cloned into pcDNA1/Am. An li (invariant chain) RT-PCR product obtained from RNA of LB3-EBV-B cells with oligonucleotides B3G (5'-AGCGGATCCCTGCGGAAGATCAGAAG) and BG4 (5'-CAAGATTCTCAGCTGGATAGAGAAGAGAAGAGAAGAAGAGAAGAAGAAGAAGAAG) was blunted using the Klenow fragment of DNA polymerase I (New England Biolabs, Beverly, MA), inserted into the EcoRV site of pcDNA3/Am, and completely sequenced.

**T-Cell Clone.**

Blood mononuclear cells collected from patient LB33 in 1990 were isolated by Lymphoprep (Nycomed, Oslo, Norway) density-gradient centrifugation and cryopreserved. They were stimulated three times at 24 h, [3H]thymidine was added, and thymidine incorporation was measured on April 13, 2017. © 2000 American Association for Cancer Research. on LB33-EBV-B cells preincubated 2 h at 37°C in the presence of the different peptides in a M-07e proliferation assay, as described above. HLA-DR molecules were immunopurified from the EBV-transformed B cell line SWEIG as described by Gorga et al. (17). A competitive binding assay to DRB1*1101 molecules was performed in 10 μM phosphate, 150 mM NaCl, 1 mM DM, 10% FCS, and 0.03% thimerosal, and 2 mM DTE (ph 5) buffer, as described previously (18). Briefly, an appropriate amount of HLA class II molecules was incubated for 24 h with 20 nm biotinylated HA 306–318 peptide (PKYVKQNTLKLAT). Various concentrations of competitor peptides. The DR-peptide complexes were transferred into microwells coated with the anti-HLA-DR monoclonal antibody L243 and incubated for 2 h. Unbound peptides were washed off the plates, and peptide-DR complexes were revealed with streptavidine phosphatase (Amersham, Buckinghamshire, United Kingdom) and 4-methylumbelliferol phosphate as substrate (Sigma Chemical Co., St. Louis, MO). Fluorescence was measured at 450 nm upon excitation at 365 nm on a Fluorolite 1000 fluorimeter (Dynex, Issy les Moulineaux, France). The relative affinity of each competitor peptide was evaluated by the concentration that prevented 50% of binding of the biotinylated peptide (IC50). Each experiment was validated with the IC50 of nonbiotinylated HA 306–318 peptide, which did not vary by more than a factor of three from one experiment to another.

**Construction and Screening of the cDNA Library.**

Total RNA was extracted from MEL-A-1 cells by the guanidinium isothiocyanate procedure. Poly(A)+ RNA enriched with an oligo(dT)-cellulose column (Pharmacia, Biotech, Uppsala, Sweden) was converted to cDNA with the Superscript Choice System (Life Technologies, Inc., Gaithersburg, MD) using an oligo(dT) primer [5’-TAAGAATGCGGCCGCTAAACTA(T)18VZ (V = G, A, or C; Z = G, A, or T, or C) containing a NorI site at its 5’ end. The cDNA was ligated to HindIII-EcoRI adaptors (Stratagene, Heidelberg, Germany), phosphorylated, digested with NorI, and inserted in the HindIII and NotI sites of expression vector pCEP4 (Invitrogen). E. coli DH5a were transformed by electroporation with the recombinant plasmid and selected with ampicillin (50 μg/ml). The library was divided into 528 pools of ~100 cDNA clones. Each pool was amplified for 4 h, and plasmid DNA was extracted using the QIAprep 8 ml per kit (Qiagen). Duplicate microcultures of 293-E DNA cells, plated in flat-bottomed 96 microwells (3.5 × 104/well) 24 h before transfection, were transfected with each complex and labeled with 1.5 μl of LipofectAMINE reagent (Life Technologies), 100 ng of plasmid DNA of each pool of the cDNA library, 12 ng of plasmid pcDNA3 containing the HLA-DRB1*1101 cDNA, 12 ng of plasmid pcDNA1/Amp containing an li cDNA, and 24 ng of plasmid EBO-76pl containing a CIITA cDNA (19). After 24 h, clone 19 (5000 cells/well) was added to each microculture in 100 μl of Iscove’s medium supplemented with AAGM, 1% human plasma, and IL-2 (25 units/ml). After another 24 h, 75 μl of supernatant were collected, and cytokine production was measured with the M-07e cells.

**Sequences and Construction of Minigenes.**

cDNA clone 279 contained the published EphA3 sequence (M83941). Four nucleotide changes were found: two conservative substitutions (G instead of A at position 2184 of clone 279, changing S into T in the translated protein; and a T modification at position 2995, changing R into W. In cDNA clone 60, which did not vary by more than a factor of three from one experiment to another.

Sequences and Construction of Minigenes. cDNA clone 279 contained the published EphA3 sequence (M83941). Four nucleotide changes were found: two conservative substitutions (G instead of A at position 2184 of clone 279, T instead of C at position 3027 of clone 279); a T→A modification at position 2956 of clone 279, changing S into T in the translated protein; and a C→T modification at position 2995, changing R into W. In cDNA clone 60, the open reading frame ended at nucleotide 1705. Nucleotide 1681 presumably corresponded to the end of exon 7, as deduced from a comparison with the genomic sequence of the chicken EphB2 receptor [GenBank M62325 (20)], and the reading frame extended for another 23 nucleotides into a putative intronic sequence.

Minigenes were constructed as follows. We first cloned the signal sequence of EphA3; cDNA clone 279 was used as a template for a PCR amplification with primers OPC 895 (5’-CCGGAATTCTGAATCCAGTAGATTGACG- GC) and OPC 895 (5’-CCGGAATTCTGAATCCAGTAGATTGACG- GC) in the following conditions: 5 min at 94°C, followed by 30 cycles consisting of 1 min at 94°C, 2 min at 64°C, and 3 min at 72°C. The PCR

1 The abbreviations used are: IL, interleukin; RT-PCR, reverse transcription-PCR; GM-CSF, granulocyte/macrophage-colony stimulating factor; TNF, tumor necrosis factor; CIITA, class II transactivator.
product was purified using QIAquick PCR purification kit (Qiagen), digested with BamHI and EcoRI, and ligated into pcDNA to obtain pcDNA3-EphA3-signal. Subsequently, fragments of cDNA clone 279, corresponding to truncated portions of the extracellular part of the receptor, were amplified by PCR using three different sense primers, OPC 899 (5′-CCGGAAATCTCGAGACAACATGGATTGTCAGCTCTC-3′) and OPC 806 (5′-TGTGGTAGCCGGAATTCTGTACCCCAAACTCC-3′), or OPC 896 (5′-CCGGAAATCTCGAGACCGCCAATTAGCCG-3′) with the same antisense primer OPC 897 (5′-ATACTTACGCGCCGAGCAGACGCAATTGGACGCCCAGATGT-3′), where TAG is a stop codon in frame with the main open reading frame. The three PCR products were cloned into the EcoRI and NotI sites of pcDNA3-EphA3-signal. The resulting constructs cDNA clones were transfected, using LipofectAMINE, into 293T cells, yielding two cDNA clones. One clone encoded the EphA3 protein with insertion of two amino acids (E-F) after the first 10 residues of the mature protein.

**PCR Assay for EphA3 Expression.** Total RNA extraction and reverse transcription of RNA were performed as described previously (21). For the analysis of EphA3 expression in tumor and normal tissues samples, PCR primers were OPC 818 (5′-AGCAACATGGATTGTCAGCTCTC-3′) and OPC 941 (5′-CCGGGAATTCTGTACCCCAAACTCC-3′), where TAG is a stop codon in frame with the main open reading frame. The three PCR products were cloned into the EcoRI and NotI sites of pcDNA3-EphA3-signal. The resulting constructs cDNA clones were transfected, using LipofectAMINE, into 293T cells, yielding two cDNA clones. One clone encoded the EphA3 protein with insertion of two amino acids (E-F) after the first 10 residues of the mature protein.

**RESULTS**

Obtention of a CD4 T-Cell Clone Recognizing MEL.A-1. Clonal melanoma line MEL.A-1 was derived from a metastasis resected from patient LB33 in 1988. The cells were incubated for 2 days in media with and without IFN-γ and labeled with monoclonal antibodies specific for HLA-DP, DQ, or DR molecules (Fig. 1). In the absence of IFN-γ, ~15% of the cells expressed HLA-DR but not DP or DQ molecules. After incubation with IFN-γ, all of the cells carried HLA-DP and DR molecules at levels comparable with that found on autologous EBV-transformed B cells. HLA-DQ molecules were not detected on the tumor cells.

Blood mononuclear cells collected from patient LB33 in 1990 were stimulated with irradiated, IFN-γ-treated MEL.A-1 cells in the presence of IL-2 and IL-4. On day 7, the lymphocytes were restimulated with the tumor cells. CD4 cells were purified on day 10, restimulated with tumor cells on day 14, and cloned by limiting dilution on day 21. We obtained CD4 T-cell clone 19 that lysed MEL.A-1 cells but not the autologous EBV-B cells. HLA-DQ molecules were not detected on the tumor cells.

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Recognition of MEL.A-1 cells by clone 19 was inhibited by an anti-HLA-DR monoclonal antibody (Fig. 2B). Patient LB33 was typed HLA-DR11 by serology. The melanoma cells of another DR11 patient, LB4, were also recognized by clone 19, indicating that antigen Z was shared between at least some melanomas (Fig. 2B). The
Presentation of Antigens on HLA Class II Molecules by 293 Cells after Transfection with CIITA. In previous efforts to identify genes coding for antigens presented by class I molecules, we have cotransfected 293-EBNA cells with a cDNA library from the tumor and a cDNA encoding the presenting HLA. Considering that the protein containing the LB33-Z antigenic peptide was naturally processed through the class II pathway in MEL-A-1, we expected a recombinant protein to do the same in cells transfected with the corresponding full-length cDNA. However, 293-EBNA cells express neither of the genes coding for HLA-Dra, DRβ, the li invariant chain, HLA-DMA, or -DMB, which are required for the processing of antigens presented by class II molecules. The expression of these genes is controlled by the class II transactivator CIITA, and it was shown that class II-negative cells acquired the capacity to present antigens on class II molecules after transfection with CIITA (19, 22). We confirmed that 293-EBNA cells transfected with a CIITA cDNA expressed the Dra, DRβ (DRB1*1501) DMA, DMB, and li genes and carried HLA-DR molecules on their surface.

These results suggested that 293-EBNA cells could be made to carry an antigen presented by class II molecules upon cotransfection of cDNA clones coding for the antigen, for CIITA, and for the appropriate DRβ chain. To verify this, we conducted a reconstruction experiment with a defined antigen. We made use of CD4 T-cell clone 37, which recognizes a MAGE-A3 peptide presented by HLA-DR11 molecules (23). It had been shown that HLA-DR13 melanoma cells that did not express gene MAGE-A3 were recognized by clone 37 after transfection with a cDNA coding for an li-MAGE-A3 chimeric protein (23). Therefore, we cotransfected 293-EBNA cells with various amounts of the li-MAGE-A3 cDNA and with a constant amount of the CIITA and DRB1*1301 cDNA. After 24 h, the transfected cells were tested for the expression of the MAGE-A3.DR13 antigen by adding clone 37 and measuring the production of GM-CSF (Fig. 3). The CD4 clone recognized the transfectedants. No recognition was observed when CIITA was not cotransfected (data not shown). Surprisingly, the additional cotransfection of a cDNA clone coding for a full-length li dramatically improved the results (Fig. 3). When 293-EBNA cells were cotransfected with DR13, CIITA, and li, and with 100 ng of mixtures of pCEP4 and pCEP4 containing the li-MAGE-A3 cDNA, a clear recognition of the transfectedants by the CD4 clone could be obtained with only 0.1 ng of pCEP4-li-MAGE-A3, corresponding to 1/1000 of the total amount of pCEP4. These results suggested that if this CD4 clone was used to screen 293-EBNA cells transfected with a cDNA library from a MAGE-A3-positive tumor, the library could be divided in pools of 1000 cDNA clones.

Identification of cDNA Clones Coding for Antigen LB33-Z. A cDNA library prepared with mRNA extracted from MEL-A-1 cells was cloned into pCEP4 and divided into 528 pools of 100 clones. About 100 ng of DNA extracted from each pool were cotransfected with the DRB1*1101, CIITA, and li cDNA into 293-EBNA. After 24 h, the transfectedants that expressed antigen LB33-Z were detected by their capacity to stimulate the production of GM-CSF by clone 19. Two pools of cDNA proved positive. They were subcloned, and cDNA clones 60 and 279 were isolated (Fig. 4A). Their sequences corresponded to that of the human tyrosine kinase receptor HEK (GenBank M83941), a member of the Eph family of receptors that was originally cloned from the human pre-B cell leukemia cell line KLu (24). This receptor was recently renamed EphA3 by the Eph Nomenclature Committee (25).

cDNA 279 was 5804 bp long and contained an open reading frame encoding the full-length EphA3 receptor (Fig. 4B). cDNA 60 was 2546 bp long and contained a poly(A) tail, a polyadenylation signal, and an open reading frame that was identical to that of cDNA 279 but ended before the transmembrane region. It is likely to code for a secreted form of the receptor, similar to those described for other Eph gene products (20, 26, 27).

Identification of the Antigenic Peptide. 293-EBNA cells were cotransfected with CIITA, li, DRB1*1101, and with constructs coding for membrane-bound truncated proteins corresponding to the extracellular portion of EphA3. The transfectedants were tested for recognition by CD4 clone 19, and the results indicated that the antigenic peptide was encoded between nucleotides 1064 and 1237 of cDNA 279 (Fig. 4B).

Two peptides in this region contain the HLA-DRB1*1101 binding motif, i.e., W or Y or F at position 1, R/K/H at position 6, and A/G/S/P at position 9 (28). One peptide of 16 amino acids, DVTFNIICKCG-WNIK, contained this motif and bound to purified DR11 molecules with an affinity similar to that of the reference influenza peptide HA306–318 (Fig. 5A). It sensitized autologous EBV-B cells to recognition by clone 19, with a half-maximal effect at 2 μM (Fig. 5B). Considering that peptides presented by HLA class I molecules are usually recognized by CTL when present at a concentration of about 100 nM, we explored the possibility that the antigenic EphA3 peptide was shorter than 16 residues. Several peptides were tested, but none of them was recognized better than the original peptide. The smallest peptide that was recognized, FNIICKKCG, was only nine amino acids long and contained the putative DR11 anchor residues (Fig. 5B).

To verify the binding motif, we used peptide DVTFNIICKCG, which bound to DR11 molecules (Fig. 5A). Variant peptides incorporating substitutions of the putative anchor residues F at relative position 1, and K at position 6, bound with 100-fold less affinity,
whereas binding was not affected by changing the isoleucine residues at positions 3 or 4 (Fig. 5A). These results indicated that the peptide bound to DR11 molecules with a canonical motif, including a F residue most probably anchored into pocket 1. As expected from these binding data, peptides without the anchoring residues F or G were not recognized by the CD4 clone (Fig. 5B).

Expression of Gene EphA3. Expression in normal tissues was studied by RT-PCR amplification (Fig. 6). High levels of expression were found in fetal brain and retina. Samples of adult brain, colon, liver, bladder, and prostate expressed EphA3 at levels between 10 and 30% of that found in MEL-A-1 cells. Samples of skin, muscle, lung, kidney, adrenals, ovary, testis, heart, liver, and breast expressed between 3 and 10% of that level. In several tissues, no expression of EphA3 was detected. It is important to note that tissues expected to contain a significant proportion of hematopoietic cells that carry HLA class II molecules, such as bone marrow, blood mononuclear cells, or...
thymus, were negative. EBV-transformed B lymphocytes, or CTL clones, which express HLA class II molecules, were also found negative. We also failed to detect expression of EphA3 in immature dendritic cells derived from adherent blood mononuclear cells cultured with GM-CSF and IL-4 for 7 days (29) or in mature dendritic cells obtained by incubating the immature cells for 2 days with TNF-α, IL-1, IL-6, and prostaglandin E2 (30).

Significant proportions of tumors, such as 44% (11 of 25) of small cell lung cancer, 24% (10 of 41) of non-small cell lung cancer, 58% (17 of 29) of sarcomas, or 31% (12 of 38) of renal cell carcinomas, expressed EphA3 at a level that corresponded to 10% of that found in MEL.A-1 (Fig. 7). It is noteworthy that this level of expression was higher than that found in the corresponding normal tissues; some lung cancer samples, for example, expressed EphA3 100 times more than normal lung tissue.

Melanocytes did not appear to express EphA3, whereas 20% of the melanoma samples expressed the gene at a high level, corresponding to 30% of that found in MEL.A-1. No significant difference was observed between primary or metastatic melanoma samples. EphA3 was also expressed at a high level by 76% of the melanoma cell lines, a proportion significantly higher than that of metastatic melanoma samples, from which these lines were derived. For eight lines, we observed that the level of EphA3 expression was at least 30 times higher than that found in the corresponding tumor sample. This did not result from much lower proportions of tumor cells in the samples, because the levels of expression of the actin and tyrosinase genes were comparable. These results suggested that only a fraction of the melanoma cells expressed EphA3 at a high level in vivo, and that these cells were selected during the establishment of the cell lines.

In Vivo Selection of LB33 Melanoma Cells That Do Not Carry the EphA3 Antigen. Considering that the melanoma cells MEL.B-1, derived from a metastasis resected from patient LB33 in 1993, were shown previously to resist lysis by most of the autologous CTL clones that recognized MEL.A-1 (9), we explored the possibility that they also avoided recognition by the anti-EphA3 CD4 T lymphocytes. MEL.B-1 has been derived from the cell line MEL.B by limiting dilution (9), but only 60% of the cells were labeled with the anti-

![Fig. 6. Expression of the EphA3 gene analyzed with RT-PCR on normal tissues. Expression was tested by reverse transcription of total RNA and PCR amplification with primers OPC818 and OPC806 shown in Fig. 4. The 1179-bp product is not observed when genomic DNA is tested. A semiquantitative measurement was obtained by combining a limiting number of PCR cycles, comparing the result with a standard curve of RNA from MEL.A-1 cells, and making a correction for the integrity of the RNA by taking into account the expression level of the β-actin gene. Each point represents the level of EphA3 gene expression found in a sample of normal tissue. The results are expressed relative to the level of expression of the EphA3 gene measured in the MEL.A-1 cells. Broken line, the limit of detection.](image)

![Fig. 7. Expression of EphA3 by tumor tissues. Expression was tested on RNA extracted from tumor samples or from tumor cell lines. Methodology and quantification are as indicated in Fig. 6.](image)
HLA-DR monoclonal antibody, suggesting that it was not a clone. The cells expressed EphA3 but, surprisingly, were not recognized by CD4 clone 19 (Fig. 8).

The MEL.B-1 cells were subcloned, and two groups of clones were obtained, represented by clones MEL.B-1.1 and MEL.B-1.2. MEL.B-1.1 expressed EphA3 but not HLA-DR, whereas MEL.B-1.2 expressed HLA-DR but not EphA3. As expected, neither MEL.B-1.1 nor MEL.B-1.2 were recognized by clone 19. These results explain why the MEL.B-1 cell population was not recognized by the anti-EphA3 T-cell clone.

Patient LB33 relapsed in 1999 with a unique lymph node metastasis that was resected and from which a new cell line, MEL.D, was derived. MEL.D was not recognized by clone 19, because it did not express EphA3. Altogether, these results suggest that melanoma cells derived from patient LB33 in 1988 carried the EphA3 antigen, whereas tumor cells derived in 1993 and 1999 did not. It is possible that, in vivo, the anti-EphA3 CD4 T lymphocytes participated in the selection of tumor cells that escaped recognition by these T cells.

**DISCUSSION**

We identified the EphA3 antigen by cotransfecting into 293-EBNA cells a cDNA library from the tumor and cDNA clones coding for CIITA and for the relevant HLA class II chain. This genetic approach should be generally applicable to clone other genes coding for antigens presented by MHC class II molecules. Although we verified that CIITA induced the expression of Ii in 293-EBNA cells and endowed them with the capacity to present antigens on HLA class II molecules, we observed that the additional cotransfection of an Ii cDNA improved antigen presentation. This proved true for antigens encoded either by the Ii-MAGE-A3 or by the EphA3 cDNA clones (data not shown). A free pool of Ii has been observed in class II-positive cells (31, 32), suggesting that an excess of Ii in the endoplasmic reticulum may be important for class II function. This may explain our results.

The name Eph was given to a putative receptor cloned from a human erythropoietin-producing hepatocarcinoma cell line (33, 34). Eph was then found to belong to a family of 14 receptors, divided into two classes on the basis of sequence homologies in their extracellular domains. Eight EphA receptors interact with a group of five glycosylphosphatidylinositol-linked membrane proteins known collectively as ephrin-A. Six EphB receptors interact with three transmembrane proteins known as ephrin-B. Ephrins act as repulsive factors of receptor-bearing cells. Eph receptors and ephrins are involved in a range of developmental processes related to embryonic patterning. One well-studied example is the specification of the retinotectal topography in the chicken. Cek4, the chicken homologue of
EphA3, is expressed in retinal neurons with an increasing nasal to temporal gradient. In the tectum, the two ligands, ephrins A2 and A5, are distributed with an increasing anterior-posterior gradient (35–38). Temporal retinal axons, which express Cek4 at a high level, grow to the anterior tectum because they are repulsed from the posterior tectum, where the expression of the two ligands is high.

EphA3 is frequently expressed in tumors. In melanomas, it seems to be a tumor-specific expression, because the two melanocyte RNA preparations that were tested were negative. In other types of tumors, such as sarcomas or lung carcinomas, EphA3 appears to be overexpressed compared with the corresponding normal tissues. Finally, in brain tumors, for example, levels of EphA3 expression are comparable with that of normal tissue. Several reports suggest that a dysregulated expression of Eph receptors or ephrins plays a role in tumorigenesis. Several EphB receptors and B ephrins were shown to be coexpressed in small cell lung cancer samples and cell lines, suggesting that ephrin B-EphB autocrine loops contributed to tumor growth (39). The EphB4 receptor and its ligand, ephrin-B2, are implicated in the hormone-dependent morphogenesis of the mammary gland, and abnormal patterns of expression were observed in breast tumors (40). EphA2 and ephrin B2 were reported to be expressed at higher levels in metastatic melanoma samples than in normal melanocytes (41). Interestingly, melanoma biopsies did not contain large amounts of the EphA2 transcript, as tested with in situ hybridization and immunohistochemistry, whereas >90% of melanoma cell lines clearly expressed EphA2 (42). This difference between melanocytes and melanomas is very similar to what we observed for EphA3.

A recent analysis of the EphA3 gene promoter in leukemia cells lines and in blood samples containing high proportions of leukemic cells indicated a correlation between EphA3 expression and demethylation of CpG dinucleotides surrounding the transcription initiation site (43). However, this correlation was not seen with normal tissues; EphA3 was expressed at low levels, and the gene was not methylated. In accordance with these results, we observed that the treatment of fibroblasts or phytohemagglutinin A-stimulated blood mononuclear cells with demethylating agent 5-aza-2’-deoxycytidine did not activate the EphA3 gene, whereas it did activate gene MAGE-A1, which is activated after demethylation of its promoter (44). This treatment also failed to induce the expression of EphA3 in melanoma, sarcoma, renal carcinoma, and choriocarcinoma cell lines that did not express EphA3. These results suggest that neoplastic transformation results in EphA3 regulation by DNA methylation in leukemias but not in solid tumors. Because CD4 clone 19 did not recognize autologous EBV-transformed B cells, it appeared to specifically recognize tumor cells. To understand the basis of this specificity, we carefully analyzed the expression of EphA3 by normal tissues. Several Eph receptors are known to be expressed in adult tissues, predominantly in the brain (45). In accordance with these results, we observed expression of the EphA3 gene in various human tissues, although at levels that are 30–1000 times lower than in fetal brain. The only adult tissue where we found a high level of expression of EphA3 is retina. It is important to note that we did not detect expression of EphA3 in tissues that express HLA class II molecules, such as bone marrow, blood mononuclear cells, or thymus. Cell lines derived from these tissues, such as CTL clones or EBV-transformed B cells, were also negative, as well as monocyte-derived mature or immature dendritic cells. We cannot exclude the possibility that some normal cells present the EphA3 antigenic peptide on HLA class II molecules. The presence of class II molecules appears to be restricted to immune cells of hematopoietic origin, which do not express EphA3, with the exception of endothelial cells. Expression of HLA class II molecules was shown on microvascular endothelial cells but not on endothelial cells of large vessels (46). This normal HLA class II expression was lost when the endothelial cells were cultured, suggesting that it is maintained by circulating factors. In vitro, endothelial cells can be induced to express class II molecules by IFN-γ or by contact with natural killer cells. Natural killer cells induce HLA class II expression not only through the production of IFN-γ but also through an adhesion-dependent and IFN-γ-independent mechanism, which does not depend on the induction of CIITA in the endothelial cells (47, 48). Whether class II-positive endothelial cells express EphA3 is not known.

The combined patterns of expression of HLA class II molecules and of EphA3 suggest that the antigen described here is a truly tumor-specific antigen. Interestingly, tumoral specificity does not result solely from the pattern of expression of the antigen encoding gene, as is the case for the tumor-specific antigens presented on HLA class I molecules, but from the mutually exclusive patterns of expression of the HLA class II and the antigen encoding genes. The presence of anti-EphA3 CD4+ T cells in patient LB33, who has no symptoms of autoimmunity, and the apparent loss of expression of the EphA3 antigen during the progression of the disease suggest that EphA3 could be a safe and efficient source of antigens for the specific immunotherapy of class II-positive tumors such as melanomas.

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Identification of a Tumor-specific Shared Antigen Derived From an Eph Receptor and Presented to CD4 T Cells on HLA Class II Molecules

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