Vincent G. Brichard[1], Jean Herman[1], Aline Van Pel[1], Claude Wildmann[1], Béatrice Gaugler[1], Thomas Wölfel[2], Thierry Boon[1] and Bernard Lethé[1]

[1] Ludwig Institute for Cancer Research and Cellular Genetics Unit, Université Catholique de Louvain, Brussels, Belgium
[2] I. Medizinische Klinik und Poliklinik, Johannes Gutenberg-Universität, Mainz, Germany

A tyrosinase nonapeptide presented by HLA-B44 is recognized on a human melanoma by autologous cytolytic T lymphocytes

The human tyrosinase gene has been reported previously to code for two distinct antigens recognized on HLA-A2 melanoma cells by autologous cytolytic T lymphocytes (CTL). By stimulating lymphocytes of melanoma patient MZ2 with a subclone of the tumor cell line of this patient, we obtained a CTL clone that lysed this subclone but did not lyse other subclones of the same melanoma cell line. The sensitive melanoma subclone was found to express a much higher level of tyrosinase than the others, suggesting that the antigen recognized by the CTL might be encoded by tyrosinase. Transfection of a tyrosinase cDNA demonstrated that the CTL clone indeed recognized a tyrosinase product presented by HLA-B*4403. The relevant antigenic peptide corresponds to residues 192–200 of the tyrosinase protein. Lymphoblastoid cells of the B*4402 subtype were not recognized by the CTL following incubation with the peptide. Nevertheless, by stimulating *in vitro* lymphocytes of a healthy HLA-B*4402 donor with autologous adherent cells pulsed with the same peptide, we obtained a CTL clone which recognized tumor cells expressing tyrosinase and HLA-B*4402. As HLA-B44 is expressed in 24% of Caucasians, the tyrosinase-B44 antigen may constitute a useful target for specific immunotherapy of melanoma.

1 Introduction

Cultures of irradiated tumor cells mixed with blood lymphocytes of the same patient can produce responder lymphocyte populations that display cytolytic activity against the tumor cells [1–3]. By limiting dilution of these responder cell populations, it is possible to obtain cytolytic T lymphocyte (CTL) clones that lyse the tumor cells but do not lyse autologous EBV-transformed lymphoblastoid cells, autologous fibroblasts or natural killer targets such as K562 [2, 4, 5]. A number of genes coding for tumor antigens recognized by autologous CTL clones on melanoma cells have been identified. A first group of genes, which belong to three gene families named MAGE, BAGE and GAGE, is expressed in a significant proportion of tumors of various histological types [6–12]. These genes are not expressed in normal tissues except testis. A second group of genes codes for differentiation proteins that are specific for the melanocytic lineage. They are expressed both in normal melanocytes and in melanoma cells. This group of genes comprises tyrosinase, which codes for two antigens recognized by CTL on almost all HLA-A2 melanomas [13, 14], Melan-$A^{MART\text{-}1}$, $gp100^{Pmel17}$ and $gp75^{TRP\text{-}1}$ [15–19].

An extensive study of CTL clones of patient MZ2 that are directed against autologous melanoma cell line MZ2-MEL has been performed [5]. The lytic activity of a number of these CTL clones was examined on several clonal sublines derived from the initial tumor cell line, which was not clonal. The results led to a subdivision of the panel of CTL into three groups [20].

A first group recognized antigens expressed by all the sublines tested. Selections with these CTL clones *in vitro* produced MZ2-MEL cell variants that were resistant to some of these CTL, but not to others, and this led to the conclusion that the MZ2-MEL sublines expressed at least four distinct antigens. Two of these antigens are presented by HLA-A1 and are encoded by genes MAGE-1 and MAGE-3, respectively [21, 22]. Another antigen that is recognized on HLA-Cw6 is encoded by two members of the GAGE gene family [8].

A second group of CTL recognized an antigen which was present on the initial tumor cell line but not on a subline that had undergone more than 150 culture transfers. This subline had lost the expression of HLA-A29, B44 and Cw16. Two of the CTL clones that failed to recognize were found to be directed against antigens presented by HLA-Cw16. One antigen is encoded by MAGE-1 [23]. The other is encoded by a gene of the BAGE family [7].

A third group of CTL was obtained after stimulation with subclone MZ2-MEL.43, which had been isolated after mutagen treatment of clonal line MZ2-MEL3.0. These CTL recognized an antigen present on subclone MZ2-MEL.43, but neither on MZ2-MEL3.0 nor on other sublines. We then derived a large number of subclones from the original tumor cell line MZ2-MEL. The antigen was detected on approximately 5% of these subclones. We report here that this antigen, named MZ2-C, is encoded by the tyrosinase gene.

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Correspondence: Thierry Boon, Ludwig Institute for Cancer research, Brussels Branch, 74 avenue Hippocrate – UCL 74.59, B1200 Brussels, Belgium (Fax: +32-2-762 94 05)

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2 Materials and methods

2.1 Cell lines

The melanoma cell line MZ2-MEL was derived from an abdominal metastasis of patient MZ2. Subclone MZ2-MEL.3.0 was obtained by limiting dilution. Subline MZ2-

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MEL.43 was derived by limiting dilution from MZ2-MEL.3.0 cells that had survived to a mutagen treatment [5, 20]. The origins of the MZ2-MEL sublines and of cell lines LB33-MEL.A and SK29-MEL were previously described [20, 23–25]. Melanoma cell line LG2-MEL was a gift from Dr. G. Degiovanni (Université de Liège, Liège, Belgium). All melanoma cell lines were grown in Iscove's medium supplemented with 10% FCS. COS cells were grown in DME medium containing 10% FCS. EBV-transformed cell lines were obtained by transformation of peripheral blood lymphocytes with EBV and cultured in Iscove's medium supplemented with 10% FCS. BI2-EBV is a gift from Dr. Rickinson (Birmingham, GB).

Autologous CTL clone 22/31 was derived from peripheral blood lymphocytes of patient MZ2 and grown in conditions similar to those previously described [20]. CTL IVSB was derived from patient SK29(AV) and grown as described [25]. Autologous CTL clone 329B/5 was derived by stimulation *in vitro* as follows. Adherent cells from PBL of healthy donor LB-1161 (HLA-B*4402 positive) were grown for 1 week in RPMI medium supplemented with 10% FCS, IL-4 (50 U/ml) and GM-CSF (100 ng/ml). These cells were pulsed on 24-well plates (Nunc, Roskilde, Denmark) with peptide SEIWRDIDF (50 μM) for 4 h in the presence of β2-microglobulin (2.5 μg/ml, Sigma). After irradiation of the adherent cells, 2×10^6 $CD8^+$ sorted T lymphocytes were added in a final volume of 2 ml Iscove medium supplemented with 10% human serum, 1000 U/ml IL-6 and 5 ng/ml IL-12. The responder cells were stimulated on days 7 and 14 with LB1161 adherent cells pulsed with the peptide as above in medium supplemented with 10 U/ml IL-2 and 5 ng/ml IL-7 (British Biotechnology). On day 21, responder lymphocytes were cloned by limiting dilutions in microwells containing 10^4 irradiated LB33-MEL.A pulsed with peptide SEIWRDIDF (1 μM) and 2×10^4 irradiated lymphoblastoid cell line LG2-EBV cells as feeder cells. The microcultures were stimulated every 7 days by the same procedure. After 5 weeks, the CTL clone 329B/5 was grown on 24-well plates and stimulated weekly with 2×10^5 irradiated LB33-MEL.A cells pulsed with the peptide and 10^6 irradiated LG2-EBV cells in culture supplemented with IL-2 (50 U/ml) and IL-4 (5 U/ml).

2.2 CTL stimulation assay

Tumor cell lines and COS-7 transfectants were tested for their ability to stimulate the production of TNF by CTL, as described [26]. Briefly, 2000 CTL were added in 100 μl Iscove's medium supplemented with 10% human serum and 25 U/ml IL-2 in microwells containing 15 000–20 000 target cells. After 24 h, the supernatant was collected and its TNF content determined by testing its cytotoxicity for WEHI-164 clone-13 cells [27] in a colorimetric assay as described [28].

2.3 Transfection of COS-7 cells

Transfection experiments were performed by the DEAE-dextran-chloroquine method as described [13, 29]. Plasmid pcDNAI/Amp (100 ng; Invitrogen Corporation, San Diego, CA) containing the tyrosinase or a subgenic fragment was cotransfected with 100 ng of expression vector containing HLA-coding sequences. The HLA-A2.1 gene was cloned from a cosmid library prepared from DNA of lymphocytes of patient SK29(AV) [25]. The HLA-A1 gene was derived from another patient and provided by Dr. Girdlestone [30]. cDNA encoding HLA-B44, -A29, -B37, and -Cw16 were cloned from a cDNA library constructed with poly(A)$^+$ RNA extracted from MZ2-MEL.43 cells. COS cells were incubated for 24 h at 37 °C and a CTL stimulation assay was performed.

2.4 Cloning of subgenic fragments of the tyrosinase gene

Tyrosinase cDNA clone 123.B2 was obtained from a cDNA library of SK29-MEL [13]. Fragments were generated by PCR amplification and incorporated into the EcoRV site of pcDNAI/Amp. As primers, we used: oligonucleotides VB34 (5′-CCGAATTCGCCATGTCTGAAATCTGG-3′) and VB29 (5′-AATCTAGACGCCTAGCTACAGACAATCTGCCA-3′) to generate fragment 574–831; oligonucleotides VB58 (5′-CGGGATCCGCCGCCATGCCAGAGAAGGAC-3′) and VB60 (5′-GCTCTAGAGCCTTATGCTTCATGGGC-3′) to generate fragment 385–612.

2.5 HLA-B44 subtyping

The melanoma and EBV cell lines were characterized for the HLA-B44 subtypes as follows: genomic DNA (0.5 μg) of the sample was amplified with oligonucleotides BX3S1 (5′-GGGTCCAGGGTCTCACATCA-3′) and BX3R1 (5′-CCAGGTATCTGCGGAGCG-3′) for 10 cycles (denaturation 30 s at 94 °C, annealing 30 s at 65 °C and extension 1 min at 72 °C) followed by 25 cycles (30 s at 94 °C, 30 s at 60 °C, 1 min at 72 °C). The fragment corresponding to a part of exon 3 was digested with restriction enzymes (RsaI, PvuII and BsaAI) to discriminate between subtypes -B*4402, 03, 04 and 05.

2.6 Peptide synthesis and peptide recognition assays

Peptides were synthesized on solid phase using Fmoc for transient N-terminal protection and characterized by amino acid analysis. Standard ^{51}Cr-release assays were used to determine lysis of target cells [31].

Target cells ($1 \times 10^6 - 2 \times 10^6$) were labeled with 100 μCi of $Na^{51}CrO_4$ in 30 μl FCS. For assays with CTL 329B/5, the cells were labeled in the presence of anti-MHC class I mAb W6/32. After 1-h incubation, the cells were washed twice, then incubated in the presence of various concentrations of the peptide in a total volume of 100 μl in conical 96-well microtiter plates (Greiner, Nürtingen, Germany) for 30 min. CTL were added in 100 μl. Cr release was measured after incubation at 37 °C for 4 h.

2.7 Quantitative measurements of tyrosinase expression

Quantitative RT-PCR was essentially performed as described [11]. Briefly, isolation of total RNA was performed as reported [32]. cDNA was obtained by reverse

transcription performed on 2 μg of total RNA with an oligo(dT) primer. cDNA obtained from the clone SK29-MEL.1 was included pure and diluted in each assay of quantitative PCR and used as standard. cDNA corresponding to 100 ng of total RNA was amplified for 22 cycles by PCR. Trace amounts of [α-^{32}P]dCTP (0.2 μCi) were added and accurate quantitation was obtained using the phosphor-imaging technology (Phosphor-Imager, Molecular Dynamics, Sunnyvale, CA). Primers used for the amplification are VB17 (5′-GGATAGCGGATGCCTCTCAAAG-3′) located in exon 3 and VB18 (5′-CCCAAGGAGCCATGACCAGAT-3′) located in exon 5. The cycle conditions were: 94 °C 1 min, 65 °C 2 min and 72 °C 3 min. Expression of the various samples was reported as percentages of that found in SK29-MEL.1 after correction for RNA integrity by taking into account the expression level of the β-actin gene [11].

3 Results

3.1 High expression of the tyrosinase gene in the MZ2-MEL subline bearing antigen MZ2-C

We have observed that the tyrosinase gene is expressed in all melanoma tumor samples, whereas about 30 % of the melanoma cell lines are negative, suggesting that loss of expression of the tyrosinase gene can occur *in vitro* [13]. Quantitative PCR measurements of the expression of the tyrosinase gene were made on several sublines derived from melanoma cell line MZ2-MEL. The level of expression of these sublines was low, ranging from 1 % to 8 % of that observed with the tyrosinase-positive cell line SK29-MEL, except for subline MZ2-MEL.43 which expressed 120 % (Table 1). This observation caught our attention because MZ2-MEL.43 was the only subline that expressed antigen MZ2-C recognized by autologous CTL clone 22/31 which lysed this subline but failed to lyse any other MZ2-MEL subline (Fig. 1).

Table 1. Expression of the tyrosinase gene in MZ2-MEL sublines[a)]

Clone	Expression of tyrosinase (percent relative to SK29-MEL)
MZ2-MEL.43	119
MZ2-MEL 3.0	8
MZ2-MEL 3.1	< 1
MZ2-MEL 2.2	2
MZ2-MEL 2.2.5	< 1
MZ2-MEL 61	1

a) cDNA synthesis and quantitative PCR amplification were performed as described in Sect. 2.7. The results were normalized for the expression of β-actin. They are expressed as the percentage of the level of expression of melanoma line SK29-MEL. The origin of the MZ2-MEL sublines is described in Sect. 2.1.

3.2 Antigen MZ2-C is encoded by the tyrosinase gene and presented by HLA-B44

To test the possibility that anti-MZ2-C CTL 22/31 recognized a tyrosinase-encoded peptide, we cotransfected into COS-7 cells a tyrosinase cDNA cloned in expression vector pcDNAI/Amp together with a construct containing the coding sequences of either HLA-A1, A29, B37, B44 or Cw16, all being HLA alleles of patient MZ2. One day after the transfection, COS cells were incubated with CTL 22/31. Antigen recognition by the CTL was assessed by measuring the amount of TNF released after 24 h. A very significant amount of TNF was produced by CTL 22/31 stimulated with COS cells transfected with the tyrosinase sequence and with HLA-B44 (Fig. 2). No stimulation was observed with COS cells transfected with HLA-B44 alone or with the combination of tyrosinase and one of the other HLA clones.

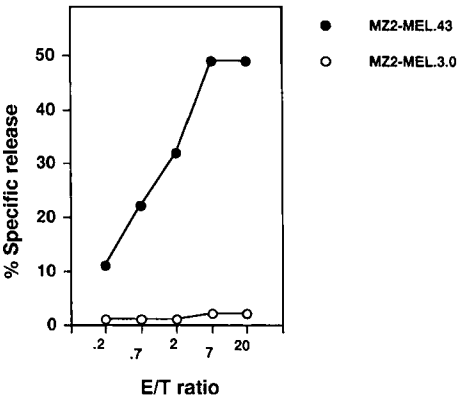

Figure 1. Lysis of MZ2-MEL.43 by CTL clone 22/31. The cytolytic activity was measured in a 4-h ^{51}Cr-release assay. MZ2-MEL.3.0 is a subclone of the original tumor cell line MZ2-MEL. MZ2-MEL.43 is a clonal subline derived from a mutagenized MZ2-MEL.3.0 culture.

3.3 Identification of the antigenic peptide

We resorted to a genetic approach to localize the tyrosinase sequence coding for antigen MZ2-C. Various fragments of the tyrosinase cDNA were obtained by digestion

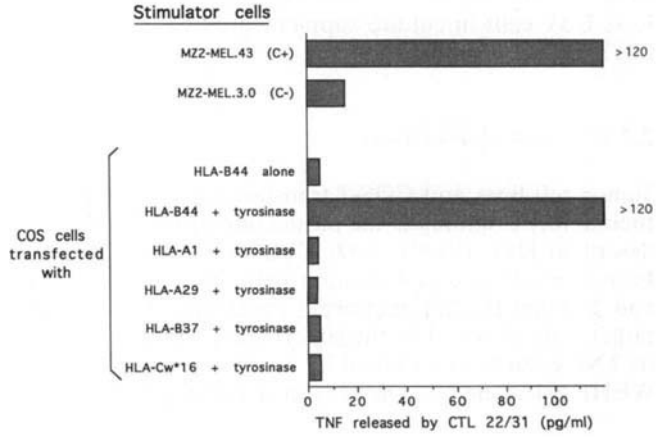

Figure 2. Expression of antigen MZ2-C by COS cells cotransfected with the tyrosinase and HLA-B44 coding sequences. COS-7 cells were cotransfected with expression vectors containing the tyrosinase sequence and the coding sequence of different class I alleles expressed by melanoma MZ2-MEL. One day later, 2000 cells of CTL 22/31 were added to 30000 transfected cells. After 24 h, the amount of TNF in the supernatant was measured by its toxicity on WEHI-13 cells.

Eur. J. Immunol. 1996. 26: 224–230

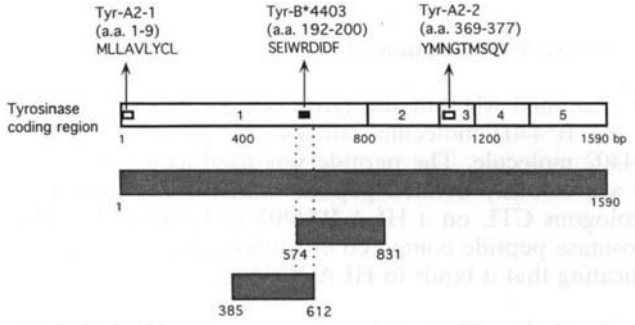

Figure 3. Location of the tyrosinase sequence coding for antigen MZ2-C. The coding part of the cDNA is shown with the exon boundaries. The limits of the cDNA fragments cloned into pcDNAI/Amp and transfected into COS cells together with HLA-B*4403 are numbered from the start of this coding region. The amount of TNF released by CTL 22/31 stimulated with the transfected COS cells is indicated.

with restriction enzymes or by PCR amplification. These fragments were cloned into expression vector pcDNAI/Amp and transfected into COS-7 cells together with HLA-B44. A PCR fragment ranging from nucleotides 574 to 831 conferred the expression of antigen C (Fig. 3). Another PCR fragment ending at position 612 was recognized by CTL 22/31. We concluded that the antigenic peptide was encoded by the sequence located between position 574 and 612. The SEIWRDIDFAHEA peptide encoded by this sequence was synthesized. It sensitized the allogeneic lymphoblastoid cell line LG2-EBV, which expresses HLA-B44, to lysis by CTL 22/31. Shorter peptides were synthesized and tested. The optimal peptide was found to be nonamer SEIWRDIDF (position 192–200) (Fig. 4). Recognition by CTL 22/31 was abolished by removal of the N-terminal Ser (Fig. 4) and also by the replacement of the C-terminal Phe by an Ala (data not shown).

Our experiments were performed using the tyrosinase cDNA clone 123.B2 that we had previously isolated from melanoma cell line SK29-MEL. This cDNA differs in three positions from two other cDNA clones coding for tyrosinase reported by Bouchard et al. [33] and Kwon et al. [34]. One difference is located in the codon coding for the N-terminal Ser residue of nonapeptide SEIWRDIDF. The alternative residue is a Tyr, another uncharged polar amino acid. The allele coding for the Tyr residue is present in ~ 50 % of Caucasians [35, 36]. To test whether this polymorphism could affect recognition by the CTL, we tested nonapeptide YEIWRDIDF. It also sensitized the LG2-EBV target cells to CTL lysis. The peptide concentration required to obtain 50 % of the maximal lysis was close to that required for nonapeptide SEIWRDIDF (data not shown).

3.4 Presentation to CTL 22/31 is restricted to the HLA-B*4403 subtype

Even though our results demonstrated that CTL 22/31 recognized a tyrosinase peptide presented by a HLA-B44 molecule, it was not stimulated by melanoma line SK29-MEL which expressed tyrosinase and was derived from a HLA-B44 patient (Fig. 5) We could exclude that this was due to polymorphism of the tyrosinase gene because the tyrosinase cDNA clone that was used in the experiments described above had been isolated from SK29-MEL.

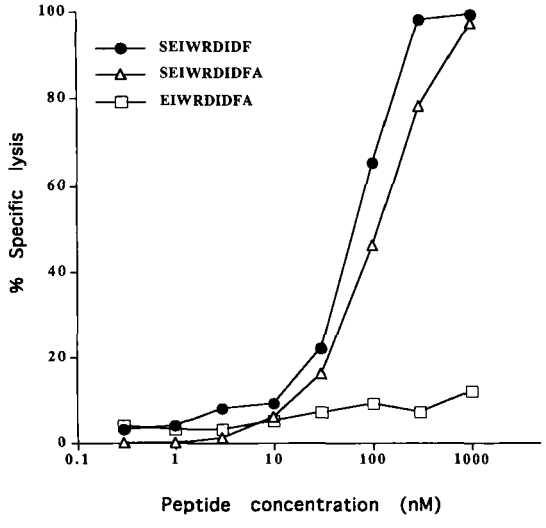

Figure 4. Lysis of target cells incubated with tyrosinase peptides. Lymphoblastoid cells LG2-EBV were Cr-labeled for 50 min. After washing, the cells were incubated for 30 min with the peptides at various concentrations. CTL clone 22/31 was added at an effector to target ratio of 50 and Cr release was measured after 4 h.

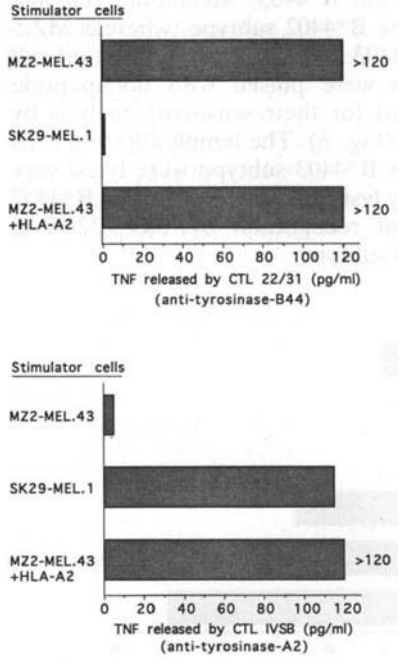

Figure 5. Recognition by CTL 22/31 and CTL IVSB of target cells expressing simultaneously HLA-A2 and B44. MZ2-MEL.43 expresses HLA-B44 only, SK29-MEL.1 is positive for both HLA-A2 and B44. MZ2-MEL.43 + HLA-A2:a MZ2-MEL.43 cell transfected with HLA-A*0201.

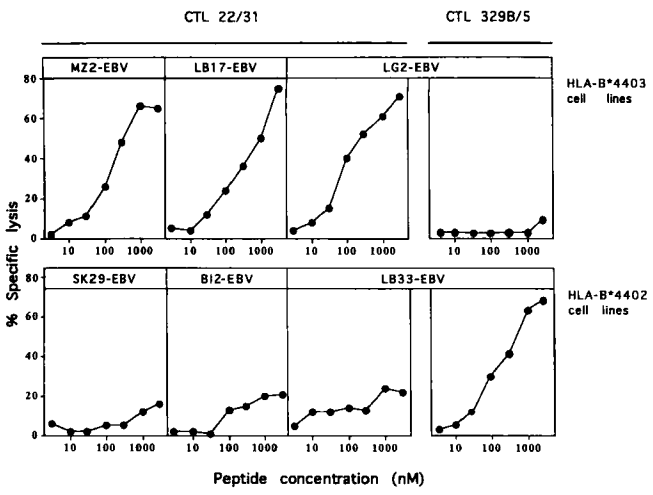

Figure 6. Lysis of lymphoblastoid cell lines expressing the B*4403 or B*4402 subtypes pulsed with the tyrosinase-B44 peptide. Cells were Cr-labeled for 50 min. After washing, they were incubated with peptide SEIWRDIDF for 30 min. CTL clones were added at an effector to target ratio of 30 for CTL 22/31 and 8 for CTL 329B/5. Cr release was measured after 4 h.

We examined the possibility that the presence on SK29-MEL cells of HLA-A2 molecules, which present two tyrosinase peptides, might prevent the other tyrosinase peptide from being presented by HLA-B44 molecules. This was found not to be the case: MZ2-MEL.43 cells transfected with the HLA-A2 gene were still recognized by CTL 22/31 (Fig. 5).

We then tested whether a difference in HLA subtype could account for the absence of recognition of melanoma cell line SK29-MEL. Two major subtypes have been reported for HLA-B44: B*4402 and B*4403. Melanoma cell line SK29-MEL expresses the B*4402 subtype, whereas MZ2-MEL expresses the B*4403 subtype. Lymphoblastoid cell lines of both subtypes were pulsed with nonapeptide SEIWRDIDF and tested for their sensitivity to lysis by anti-MZ2-C CTL 22/31 (Fig. 6). The lymphoblastoid cells that expressed the HLA-B*4403 subtype were lysed very well, whereas little if any lysis was observed on the B*4402 lines. We conclude that recognition by CTL 22/31 is restricted to the B*4403 subtype.

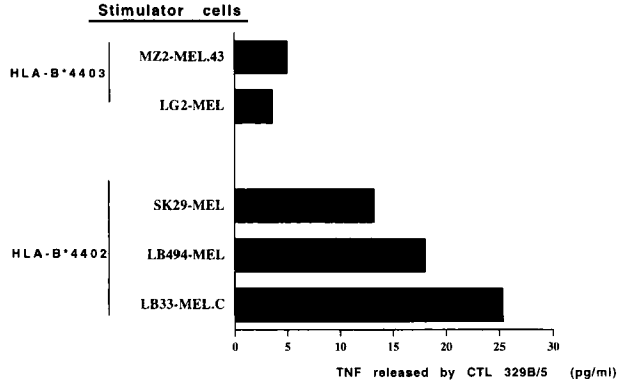

Figure 7. Recognition by CTL 329B/5 of melanoma cell lines expressing the tyrosinase and HLA-B*4402 or -03 subtypes. The cell lines were incubated with CTL 329B/5. One day later, the concentration of TNF in the supernatant was measured.

3.5 Peptide SEIWRDIDF can be recognized by CTL on the HLA-B*4402 molecule

We examined whether the tyrosinase peptide recognized on the B*4403 molecule can also be presented by the B*4402 molecule. The peptide was used as a competitor for a previously defined peptide which is recognized by autologous CTL on a HLA-B*4402 melanoma [37]. The tyrosinase peptide competed efficiently (data not shown), indicating that it binds to HLA-B*4402.

By stimulating $CD8^+$ T lymphocytes of a HLA-B*4402 healthy individual with autologous macrophages and dendritic cells pulsed with peptide SEIWRDIDF, we obtained a CTL population that lysed cells pulsed with the peptide. From this responder population we derived CTL clone 329B/5, that proved able to lyse EBV-transformed cells pulsed with the peptide provided they expressed the HLA-B*4402 subtype (Fig. 6). The ability to stimulate TNF release by CTL 329B/5 was also much higher for B*4402 melanomas than for B*4403 lines (Fig. 7).

4 Discussion

Two peptides encoded by tyrosinase and recognized on HLA-A2 melanoma cells by autologous CTL clones have been described previously [14]. We report here the identification of another tyrosinase peptide which is presented by HLA-B*4403 and forms antigen MZ2-C, which is also recognized by autologous CTL. The peptide corresponds to amino acids 192–200. Other epitopes encoded by tyrosinase are also recognized by autologous tumor-infiltrating lymphocytes (TIL) on HLA-A24 and by $CD4^+$ T cells on HLA-DR4 [38, 39].

A consensus motif for anchor residues binding to HLA-B44 has been recently inferred from the sequence of eluted peptides [40]. The reported motif shows a predominance for Glu at position 2, Tyr or Phe at positions 9 or 10 and hydrophobic residues at position 3. The tyrosinase nonapeptide 192–200 fits this consensus very well with Glu at position 2, Phe at position 9 and Ile at position 3. Other peptides recognized by CTL on HLA-B44 molecules, such as the EBNA3C protein of EBV [41], the p24gag of HIV [42], the nucleocapsid protein of hepatitis C virus [43] and a tumor antigen generated by a point mutation [37] also fit this consensus motif.

Among cells expressing tyrosinase, those of the HLA-B*4403 subtype are lysed by the anti-MZ2-C CTL clone, whereas those of the B*4402 subtype undergo little if any lysis. Such differences between HLA-B*4402 and B*4403 are in agreement with the observation of a strong allograft rejection between a donor and a recipient differing with respect to these two major subtypes of HLA-B44, which differ by a single Asp (B*4402) to Leu (B*4403) substitution in position 156 of the α2 domain [44, 45]. The restriction of anti-MZ2-C CTL by a particular B44 subtype could *a priori* be explained either by the binding of the peptides to only one subtype or by structural differences in the bound peptide induced by differences in the groove of the two subtypes. We observed that the tyrosinase nonapeptide binds to both subtypes. This is consistent with the finding that the consensus motif are similar for both sub-

types B*4402 and B*4403, since consensus motifs appear to be based on anchoring residues [40]. It appears, therefore, that different conformations of the peptide in the B*4402 and -03 grooves account for the difference in the CTL recognition. The substituted residue in position 156, which has been located at the edge of pocket D of HLA-A2, ought to interact with residues 5 or 6 of the bound peptide [46]. The tyrosinase nonapeptide contains in position 5 a positively charged residue (Arg). One can speculate that this residue cannot bind to the hydropobic Leu residue of HLA-B*4403 binding groove, so that it is free to interact with the T cell receptor. In the B*4402 groove, the Arg residue of the peptide could bind to the negative Asp residue, so that it would be buried in the groove. Thus, the T cell receptor would have to fit a different epitope.

The tyrosinase gene is expressed in all melanoma samples [13] and HLA-B44 individuals are very frequent in Caucasian populations (24 %) [47]. The polymorphism which affects codon 192 of the tyrosinase gene does not alter CTL recognition of the corresponding peptide. Among the subtypes which have been described for HLA-B44 [45, 48–50], the two major subtypes are HLA-B*4402 (15 % of Caucasians) and B*4403 (8 %) [49]. Taken together, our data suggest that melanomas expressing tyrosinase and HLA-B44 can be recognized by autologous CTL, in the context of both HLA-B*4402 and B*4403. Therefore, almost all patients typed positive for HLA-B44 could be eligible for pilot studies involving immunization against this tyrosinase epitope. The considerable number of different epitopes encoded by tyrosinase and the frequent occurrence of their presenting HLA molecules make it a good candidate for immunotherapy of melanoma, but the possible negative consequences of over-immunization against tyrosinase, such as destruction of normal melanocytes, remain to be evaluated. Previous observations that antibodies against differentiation protein such as gp75 are found in the sera of some melanoma patients are in line which the notion that differentiation antigens can constitute targets for autologous immune responses [51]. The observation that vitiligo occurring in some melanoma patients is associated with good prognosis [52, 53] suggests that immune responses directed against melanocyte differentiation antigens like tyrosinase may contribute to the elimination of tumor cells in melanoma patients.

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