The melanosomal protein tyrosinase is considered as a target of specific immunotherapy against melanoma. Two tyrosinase-derived peptides are presented in association with HLA-A2.1 (Wölfl et al., Eur. J. Immunol., 24, 759–764 (1994)). Peptide 1-9 (MLLAVLYCL) is generated from the putative signal sequence. The internal peptide 369-377 is posttranslationally converted at residue 371, and its presentation is dependent on functional TAP transporters and proteasomes [Mosse et al., J. exp. Med.187, 37–48 (1998)]. Herein, we report on the processing and transport requirements for the signal sequence-derived peptide 1-9 that were studied in parallel to those for peptide 369-377. After infection of TAP-deficient (T2) and TAP-positive (T1) cells with a Modified Vaccinia Ankara construct carrying the human tyrosinase gene (MVA-hTyr), we found that recognition by CTL against peptide 1-9 did not require TAP function as opposed to recognition by CTL against peptide 369-377. When target cells with intact processing and transport functions were infected with MVA-hTyr, lysis by CTL against peptide 1-9 was not impaired by lactacystin, a specific inhibitor for the proteasome, whereas lysis by CTL against peptide 369-377 was completely abrogated. Taken together, peptide 1-9 derived from the signal sequence of tyrosinase is presented in a TAP-independent fashion and does not require proteasomes for processing. Cellular immune responses against this hydrophobic peptide can be monitored with lymphokine spot assays as documented in the case of a patient with metastatic melanoma, in whom we observed a preferential T-cell response against tyrosinase peptide 1-9 subsequent to chemotherapy. Independence of cytosolic processing and transport pathways and potentially enhanced expression levels make signal sequence-derived peptides and their carrier proteins important candidates for specific immunotherapy.

MATERIAL AND METHODS

Cells

Melanoma cell lines were maintained in DMEM (GIBCO, Grand Island, NY) containing 10 mM Hepes buffer, L-arginine (116 mg/ml), L-asparagine (36 mg/ml), L-glutamine (216 mg/ml), penicillin (10 IU/ml), streptomycin (100 µg/ml) and 10% FCS. EBV-B lines as well as T1, T2 (Salter et al., 1985) and T2 cells transfected with rat TAPIα and TAPIβ genes (T2/TAPIα+2) (Momburg et al., 1992) were cultured in RPMI-1640 (GIBCO), supplemented as described above for DMEM. Cell cultures were kept in a water-saturated atmosphere with 5% CO₂ at 37°C.

CTL clones IVS B and 210/9, directed against distinct HLA-A2.1-presented tyrosinase peptides, were derived from the peripheral blood of melanoma patients SK29(AV) and LB24, respectively (Wölfl et al., 1994). They were maintained in long-term culture by transferring every 4–7 days 2×10⁵ CTL to 2 ml cultures containing 5×10⁵ autologous melanoma cells (SK29-MEL-1 for IVS B and LB24-MEL for CTL210/9) as stimulators and 2×10⁵ allogeneic EBV-B lymphocytes as feeders in 24-well tissue culture plates (Greiner, Nürtingen, Germany). Both stimulator and feeder cells were irradiated prior to CTL culture with 100 Gy from a 137Cesium source. AK-EBV-B cells served as feeders for SK29-CTL and LG2-EBV-B cells as feeders for LB24-CTL. As culture medium for maintenance of CTL clones, we used RPMI-1640 medium supplemented as described above, but with 10% human serum and human natural IL-2 (25 U/ml) (a generous gift from Dr. Schwulera, Bioteest, Dreieich, Germany).

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Peripheral blood mononuclear cells (PBMC) were separated from heparinized blood samples of melanoma patient XC by centrifugation on a Ficoll-Paque gradient (Pharmacia, Uppsala, Sweden) and were cryopreserved at −70°C until use. Informed consent for T-cell frequency analysis on his PBMC was obtained from the patient.

**Viruses**

MVA-wt, the vaccinia virus MVA cloned isolate F6 and the recombinant virus MVA-hTyr expressing the human tyrosinase gene (Drexler et al., 1999) were purified by ultracentrifugation through a 36% sucrose cushion to give high-titer virus stocks.

**51Cr-release assay**

Lysis of target cells by CTL clones recognizing tyrosinase peptides 1-9 and 369-377 was tested in 4 hr standard 51Cr-release assays (Woelfel et al., 1997). HLA-A*0201-positive target cells (each 1×10⁶ cells) were infected for 3 hr with MVA-wt and MVA-hTyr at a multiplicity of 20, washed once, labeled for 1.5 hr at 37°C with 100μCi Na(51Cr)0 4 (Amersham Buchler, Braunschweig, Germany) and then washed 3 times. Labelled target cells were plated in round-bottomed 96-well plates at cell densities indicated in the figure legends and incubated for an additional 7.5 hr at 37°C. Twelve hours after infection, effector cells were added to the target cells at various E:T ratios to a total volume of 160 μl per well. After 4 hr, 80μl of supernatant per well was harvested and specific 51Cr release was determined. Data are given as means of duplicates.

In parallel to MVA infection, targets were exposed to lactacytins or Brefeldin A at concentrations applied earlier in comparable experiments (Bai and Forman, 1997). Incubation with lactacytin (purchased from Dr. J.I. Corey, Department of Chemistry, Harvard University, Boston, MA) was started at different concentrations (1 μM, 10 μM and 100 μM) 2 hr prior to MVA infection. Incubation with Brefeldin A (Sigma Chemical, St. Louis, MO) was initiated at the same time as infection with MVA (at 3.57°C). T2 cells are derived from the human cell hybrid T1 and have a large homozygous deletion within the MHC class II region, including all of the functional class II genes. They lack TAP1 and TAP2 genes and are therefore defective in peptide transport, which is not the case for parental T1 cells (Salter et al., 1985; Ribedery and Cresswell, 1992). The additional loss of LMP2 and LMP7 genes in T2 cells is compensated by other proteasome subunits permitting processing of most antigens reasonably efficiently (Beilich and Trowsdale, 1995). HLA-A2.1 molecules on T2 cells carry high level of a limited set of endogenous peptides that are processed from signal sequences and are presented in a TAP-independent fashion (Wei and Cresswell, 1992). T2 cells have been applied in various models to distinguish between TAP-dependent and -independent presentation of peptides (Anderson et al., 1991; Lee et al., 1996). Peptide 1-9 (MLLAVLYCL) is located within the putative signal sequence of tyrosinase, was previously found to be recognized by anti-melanoma CTL in association with HLA-A2.1 (Wölfel et al., 1994) and is therefore a candidate for TAP-independent presentation.

**IFN-γ ELISPOT assays**

Multiscreen HA plates (Millipore, Bedford, MA) were coated with 10 μg/ml of MAb anti-human IFN-γ (1-DIK; Mattech, Stockholm, Sweden) in PBS overnight at 4°C. Unbound Ab was removed by 3 washings with PBS. After blocking the plates with RPMI/10% human serum (1 hr, 37°C), CD8+ T cells, positively isolated from frozen PBMC by immunomagnetic CD8 MicroBeads (Miltenyi, Bergisch Gladbach, Germany), were seeded in triplicates at 10⁶ cells/well. Purity of isolated T cells was typically >95% according to FACS analyses using directly fluorescein-conjugated Abs (Miltenyi). T2 cells (7.5×10⁵ cells) preloaded overnight at 37°C with 100 μg/ml of peptides in serum-free RPMI-1640 medium supplemented with 10 μg/ml β₂-microglobulin (Sigma) were added. Control wells contained unstimulated T cells. T cells in the presence of unloaded T cells or peptides alone. Culture medium was RPMI-1640 supplemented with 10% heat-inactivated human serum at a final volume of 200 μl/well. After incubation at 37°C in 5% CO₂ for 20 hr, cells were removed by 6 washings with PBS/0.05% Tween 20 (PBS/T). Captured cytokine was detected by incubation for 2 hr at 37°C with biotinylated MAb anti-hIFN-γ (7-B6-1; Mabtech) at 2 μg/ml in PBS/0.5% BSA. Plates were washed 6 times with PBS/T, and Avidin Peroxidase-Complex (1/100; Vectastain Elite Kit, Vector, Burlingame, CA) was added for 1 hr at room temperature. Unbound complexes were removed by 3 successive washings with PBS/T and 3 with PBS alone. Peroxidase staining was performed with 3-amino-9-ethyl-carbazole (Sigma) for 4 min and stopped by rinsing the plates under running tap water. Spot numbers were automatically determined with the use of a computer-assisted video image analyser (Herr et al., 1997) equipped with software KS ELISPOT (Version 4.1.146) (Zeiss-Kontron, Jena, Germany). Indicated spot numbers per seeded CD8+ lymphocytes represent mean values of triplicates. To calculate the number of CD8+ T cells responding to a particular peptide, the mean numbers of spots induced by T2 cells alone were subtracted from mean spot numbers induced by peptide-loaded T2 cells. For statistical evaluation a t-test for unpaired samples was used. Values of p < 0.05 were considered as significant.

Peptides applied herein were tyrosinase 1-9 (MLLAVLYCL), tyrosinase 369-377 (YMDGTMSEQV) (Wölfel et al., 1994), Melan-A/MART-1 26-35 (EAAAGIGLV) (Romero et al., 1997), gp100 154-162 (KTWGGYWQV), gp100 457-466 (LLDG-TATRL) (Skipper et al., 1999) and HIV reverse transcriptase (RT) peptide 476-484 (ILKEPVHGY) (Tosimides et al., 1991). They were synthesized on solid-phase using Fmoc for transient protein and characterized by mass spectrometry.

**RESULTS**

**Presentation of tyrosinase peptide 1-9 by HLA-A2.1 is independent of TAP expression**

T2 cells are derived from the human cell hybrid T1 and have a large homozygous deletion within the MHC class II region, including all of the functional class II genes. They lack TAP1 and TAP2 genes and are therefore defective in peptide transport, which is not the case for parental T1 cells (Salter et al., 1985; Ribedery and Cresswell, 1992). The additional loss of LMP2 and LMP7 genes in T2 cells is compensated by other proteasome subunits permitting processing of most antigens reasonably efficiently (Beilich and Trowsdale, 1995). HLA-A2.1 molecules on T2 cells carry high levels of a limited set of endogenous peptides that are processed from signal sequences and are presented in a TAP-independent fashion (Wei and Cresswell, 1992). T2 cells have been applied in various models to distinguish between TAP-dependent and -independent presentation of peptides (Anderson et al., 1991; Lee et al., 1996). Peptide 1-9 (MLLAVLYCL) is located within the putative signal sequence of tyrosinase, was previously found to be recognized by anti-melanoma CTL in association with HLA-A2.1 (Wölfel et al., 1994) and is therefore a candidate for TAP-independent presentation.

TAP-positive T1 cells, TAP-deficient T2 cells and T2/TAP1+2 cells, transfected with rat TAP1b and TAP2 genes (Momburg et al., 1992) express HLA-A2.1. They were infected with an MVA construct encoding full-length tyrosinase cDNA (MVA-hTyr) or with wild-type MVA (MVA-wt). Two distinct anti-tyrosinase CTL clones restricted by HLA-A2.1 were available for testing.

CTL210/9 was derived from the peripheral blood of patient LB24 and was directed against peptide 1-9. CTL IVS B was isolated from the peripheral blood of patient SK29AV and recognized the internal peptide 369-377 (Wölfel et al., 1994). As shown in Figure 1, both CTL clones lysed T1 cells infected with MVA-hTyr. T2 cells infected with MVA-hTyr were only recognized by CTL210/9 but not by IVS B. T2/TAP1+2 cells infected with MVA-hTyr were sensitive to lysis by both CTL clones. As a control, MVA-wt did not confer recognition by tyrosinase-reactive CTL. This indicated that infection of T1 with MVA-hTyr led to presentation of both tyrosinase peptides on the cell surface. In TAP-deficient T2 cells, only peptide 1-9 was transported to the cell surface but not peptide 369-377. The latter observation is not due to the lack of LMP2 and LMP7 in T2 cells because peptide 369-377 was presented on the surface of T2 cells transfected with TAP1 and TAP2 genes. After infection with MVA-hTyr, T2/TAP1+2 cells were less efficiently lysed by CTL against peptide 369-377 than T1 cells. This is in accordance with data in the initial report on T2/TAP1+2 that indicated a suboptimal capacity of this transfec-
Presentation of tyrosinase peptide 1-9 by HLA-A2.1 is independent of proteasome activity

The melanoma cell line NA8-Mel expresses HLA-A2.1, but does not express tyrosinase (Skipper et al., 1996). NA8-Mel cells were infected with MVA-hTyr and were simultaneously exposed to lactacystin, a highly specific proteasome inhibitor, and Brefeldin A, which disrupts the Golgi complex. Lysis by IVS B (recognizing peptide 369-377) was blocked by lactacystin, whereas lysis by CTL210/9 (recognizing peptide 1-9) was resistant to lactacystin. Lactacystin effects were already seen at 10 μg/ml (not shown), but were complete at 100 μg/ml. Brefeldin A blocked lysis by both CTL clones (Fig. 2). Identical results were obtained with pancreatic carcinoma cell line MZ-PC-2 (Wo¨lfel et al., 1993) (data not shown).

Induction of a T-cell response to peptide1-9 occurring in vivo

Patient XC with stage IV melanoma experienced a complete and durable response of intraabdominal and thoracical lymph node metastases to chemoimmunotherapy with cisplatinum, dacarbazine, IFN-α and IL-2 [schedule B in Keilholz et al. (1993)]. In IFN-γ ELISPOT assays, we tested CD8+ T lymphocytes isolated from the patient’s peripheral blood over a period of 15 months for reactivity against different HLA-A2.1-restricted melanoma peptide antigens derived from tyrosinase, gp100 and Melan-A/MART-1.

Reactivities were measured without prior expansion in ex vivo lymphocytes. Background spot formation results from spontaneous IFN-γ release and from allo-reactivity against T2 cells (Herr et al., 1996, 1997). In the samples collected over time, background spot formation was rather constant (15–47/105 CD8+ T cells), indicating comparable spot forming capability (Fig. 3).

Predominant reactivity was observed against tyrosinase peptide 1-9 that appeared at the end of therapy and increased during the following months. The maximum frequency was seen 5 months after the last treatment cycle (125 peptide-responsive T cells per 10^5 CD8+ lymphocytes above background). Low but statistically significant reactivities were also seen against gp100 peptide 154-162 (KTWGQYWQV) and Melan-A/MART-1 peptide 26-35 (EAAGIGILTV). No reactivity was found against tyrosinase peptide 369-377, gp100 peptide 457-466 and HIV reverse transcriptase (RT) peptide 476-484 in any of the test samples (Fig. 3).

DISCUSSION

Tyrosinase is a type I integral membrane glycoprotein finally located in melanosomes. In general, synthesis of secretory and membrane-bound proteins begins in the cytosol on ribosomes not bound to membranes. N-terminal signal sequences emerging from the ribosomes bind to signal sequence receptors located in endoplasmatic reticulum (ER) membranes. The signal sequence is cleaved in the ER lumen by signal peptidase, while the protein is elongated and is finally transferred into the ER (Walter et al., 1984). Characteristically, signal sequences have one or more positively charged amino acids near their N-termini followed by a continuous stretch of hydrophobic amino acids, as it is the case for the predicted signal sequence of tyrosinase, but otherwise have little homology to each other. Sorting signals in the cytoplasmic domain of tyrosinase finally divert the protein to the melanosomal compartment (Calvo et al., 1999).

Processing of secretory and membrane-bound proteins is complex. The generation of peptide 369-377 (YMDGTMSQV) re-
quires translocation of tyrosinase from the ER, where residue 371 is posttranslationally converted from N-D, back to the cytosol and is TAP- and proteasome-dependent (Mosse et al., 1998). Signal sequence-derived peptides are processed in different ways. Typically, they find access to nascent HLA I molecules through the translocon pathway and are TAP- and proteasome-independent (Henderson et al., 1992; Bai and Forman, 1997). Final trimming occurs in the ER (Elliott et al., 1995; Snyder et al., 1994; Yewdell et al., 1998). Some leader peptides enter the cytosolic pathway and depend on TAP function. These are peptides derived from the

**Figure 2** – Processing of HLA-A2.1-restricted tyrosinase peptides. HLA-A2.1-positive NA8-MEL cells were infected with MVA-hTyr and simultaneously exposed to lactacystin or to Brefeldin A (concentrations indicated in parentheses). HLA-A2.1-restricted CTL clones against distinct tyrosinase peptides (peptide specificity given in parentheses) were added at a 20:1 effector-to-target ratio in a 4 hr ^51^Cr assay. Target cells were seeded at 10^5/well. Data are means of duplicates.

**Figure 3** – Reactivity of peripheral blood T lymphocytes of melanoma patient XC to HLA-A2.1-restricted peptides from melanocyte differentiation antigens. PBMC were separated on 5 occasions over a period of 15 months from peripheral blood of HLA-A2-positive melanoma patient XC and were frozen. During the first 70 days, the patient was treated with 3 cycles of chemoimmunotherapy. PBMC were thawed, CD8^+^ T cells were isolated with magnetic beads and tested in 20 hr IFN-γ ELISPOT assays to detect and quantitate reactivity against the indicated peptides loaded on T2 cells (7.5 × 10^5/well). Empty bars: spot numbers per 10^5^ CD8^+^ T cells in response to T2 cells alone; filled bars: spot production per 10^5^ CD8^+^ T cells in response to T2 cells loaded with the respective peptide. Data represent means of triplicates ± SD. Asterisks indicate significant peptide reactivity above background (p < 0.05).
leaders of MHC class I molecules and are presented by QA-1 (Aldrich et al., 1994) or its human homologue HLA-E (Braud et al., 1997). Their presentation depends on functional TAP and tapasin but is proteasome-independent (Lee et al., 1998; Bai and Forman, 1997; Bai et al., 1998). Currently the way of processing and transport for individual signal sequence-derived peptides cannot be predicted. Moreover, how and why some leader peptides enter the cytosolic pathway is not understood (Bai et al., 1998). Herein we demonstrate (Fig. 1) that tyrosinase peptide 1-9 (ML-LAVLYCL) is presented in a TAP-independent fashion.

At least one example of a leader-derived peptide has been described that is proteasome-dependent (Gallimore et al., 1998). Lactacystin covalently modifies the highly conserved amino-terminal threonine of proteasome beta-subunit MBL1 and is a highly specific, irreversible inhibitor of proteasomes (Fentenay et al., 1995). It did not affect the generation of tyrosinase peptide 1-9, whereas it efficiently inhibited the generation of peptide 369-377. Brefeldin A, a fungal metabolite known to shut off anterograde traffic out of the Golgi complex and to simultaneously enhance retrograde traffic to the ER (Klausner, 1992), inhibited recognition of both tyrosinase peptides. We conclude from these data (Fig. 2) that peptide 1-9, in contrast to peptide 569-377, does not require proteasome activity for being presented on the cell surface. As expected, both peptides are transported via the Golgi complex to the cell surface.

The analysis of T-cell responses to leader sequence-derived peptides is affected by their hydrophobicity. Enzyme-linked immunospot assays, intracellular lymphokine staining and flow cytometry with tetrameric MHC-peptide complexes are currently regarded as the most sensitive techniques giving results with good statistical correlation (Murai-Krishna et al., 1998; Tan et al., 1999). The complexing of HLA-A2.1 tetramers containing ML-LAVLYCL is hampered by the peptide’s hydrophobicity. Therefore, assays based on lymphokine production in response to exogenous peptide seem to be better suitable. As shown in Figure 3, we verified by an IFN-γ ELISPOT assay the development of a specific and preferential T-cell response against peptide 1-9 in a patient with a durable complete clinical remission of metastatic melanoma after chemoimmunotherapy. The magnitude of this response was clearly above what we observed so far in random screening of healthy individuals and melanoma patients. There we detected significant reactivity against tyrosinase peptides 1-9 and 369-377, Melan-A/MART-1 peptide 26-35, gp100 peptide 154-162 in about 30% of the donors at frequencies of up to 40 spot-forming lymphocytes per 10⁵ CD8⁺ T-cells above background (not shown).

Aside from determination by the individual T-cell repertoire, preferential T-cell reactivity against peptides targeted to the ER without proteasome and TAP-function might develop, if stimulating cells are deficient in peptide processing and transport. Both deficiency of proteasome components and of TAP molecules have been observed in various human tumors and are regarded as mechanisms of immune escape (Restifo et al., 1993; Maeurer et al., 1996; Seliger et al., 1997). Usually they do not affect the presentation of ER-targeted peptides and might even lead to predominant presentation of such peptides (Henderson et al., 1993). In the case of patient XC, we had only access to his primary tumor, surgically removed in 1981, which was immunohistochemically TAP-1 positive (not shown). But we did not obtain sufficient tissue from the inguinal lymph node metastasis, surgically removed 15 years after initial diagnosis, for further analysis. Especially for melanomas with TAP and proteasome deficiencies, ER-targeted tumor-associated peptides are of particular immunotherapeutic interest. However, for the time being we cannot predict whether preferential T-cell responses against this category of peptides correlate with anti-tumor activity.

In addition to signal sequences, membrane proteins also carry sorting signals, which in the case of tyrosinase confer transport to melanosomes (Calvo et al., 1999). As demonstrated for the mouse brown locus product gp75/tyrosinase-related protein-1, such sorting signals can target epitopes to the endocytic pathway, thus leading to presentation by class II MHC molecules to helper T-cells (Wang et al., 1999). Growing knowledge about the role of signal and sorting sequences for the generation of T-cell-recognized peptides from membrane proteins will help to understand fully the antigenic potential of individual proteins and the relative importance of different epitopes. In addition, it might help to design more efficient vaccine constructs. It has been observed that expression of ER-targeted peptides by viral constructs containing ER leader sequences strongly increases the efficiency of their presentation by up to 2000-fold, which enhances their potential to induce T-cell responses even when expressed on nonprofessional antigen-presenting cells (Yewdell et al., 1998).

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REFERENCES


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