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Tumour-specific MHC-class-II-restricted responses after in vitro sensitization to synthetic peptides corresponding to gp100 and Annexin II eluted from melanoma cells

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Abstract In a search for potentially tumour-specific MHC-class-II-restricted antigens, the immunogenicity of endogenous peptides that had been eluted from HLA-DR molecules of the human melanoma cell line FM3 (HLA-DRB1*02x, DRB1*0401) was tested in vitro. Two 16-mers representing gp100 positions 44–59, and annexin II positions 208–223 bound well to isolated DRB1*0401 molecules and are discussed here. HLA-DR-matched normal donors' T cells were cultured with peptide-pulsed artificial antigen-presenting cells (CHO cells cotransfected with genes for HLA-DRB1*0401 and CD80 and coexpressing high levels of both human molecules). Specific sensitization was achieved against both peptides, as measured in assays of autocrine proliferation and interleukin-2 secretion. Moreover, responses to native autologous melanoma cells but not to autologous B cells were also observed. In view of the expression of fas by the activated T cells and of fas ligand by the melanoma cells, blockade of potential fas/fas-ligand interactions was undertaken using monoclonal antibodies (mAb). The antagonistic fas-specific mAb M3, but not the fas agonist M33, caused a markedly enhanced T cell response to FM3 cells. These results demonstrate that synthetic peptide antigens are able to sensitize T cells in vitro for effective MHC-class-II-restricted recognition of melanoma cells.

Key words HLA-DR-restricted tumour antigens · Synthetic peptide antigens · Tumour escape from immune responses · Apoptosis · Melanoma

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Introduction

Many types of tumours can be immunogenic and the resulting specific T cell immune responses can have antitumour activity. Tumour antigens can be presented to T cells by either MHC class I or class II molecules. $CD8^+$ class-I-restricted CTL recognize and destroy tumour cells in vitro and in vivo, but in animal models commonly require $CD4^+$ MHC-class-II-restricted T cell help for optimal responses [37], particularly for maintenance of immunological memory [19]. In addition to their helper function for CTL, CD4 cells secrete cytokines that may have antitumour effects, and some $CD4^+$ T cells can also exert cytolytic activity themselves [13]. Therefore, class-II-restricted T cells may interact directly with the tumour cells as well as with host cells presenting tumour antigen. Nonetheless, most of our knowledge of tumour-specific responses and of peptide epitopes suitable for use in adoptive or active immunotherapy is limited to the class-I-restricted pathway [28]. However, class-II-restricted responses are now beginning to be characterised in a number of solid tumours and leukemias. Thus, Heike et al. reported the derivation of T cell clones specific for a human sarcoma that were exclusively $CD4^+$. The (unidentified) antigens recognized were presented by HLA-DR, although presumed HLA-DQ and -DP-restricted clones (not blocked by anti-DR mAb) were also found [13]. In leukemias, oncogenic fusion proteins may represent tumour-specific products

that can be recognized in a class-II-restricted fashion [4, 24, 35], and can act as target antigens for the recognition of the native tumour cells [36]. More sensitive techniques for measuring CD4 cell activation may reveal more about immune-system/tumour-cell interactions. CD4 cells propagated from tumour-infiltrating lymphocytes (TIL) in breast and colon carcinoma, or B lymphomas, may secrete cytokines with potential antitumour activity such as granulocyte/macrophage-colony-stimulating factor, tumor necrosis factor α or interferon γ in a tumour-specific MHC-class-II-restricted fashion, without showing other signs of activation [14, 32, 33].

At least two groups have also reported isolation of class-II-restricted melanoma-specific T cells from TIL in several different patients [9, 20, 39]. Le Drean et al. [16] obtained HLA-DQ- in addition to -DR-restricted CD4 T cell clones recognizing tumour antigens shared between several different melanomas, but did not identify any of the peptides recognized. Topalian et al. reported class-II-restricted recognition of a common melanoma antigen encoded by the tyrosinase gene [38] and went on to show the existence of two discrete tyrosinase-derived peptides recognized by CD4 T cells [40]. We have previously eluted and sequenced endogenous peptides bound to HLA-DR molecules expressed by melanoma cells [11] and presented preliminary data showing that normal T cells can be sensitized to the corresponding synthetic peptides in vitro in a system where antigen presentation was exclusively mediated by HLA-DR. The present study extends these results to show that T cells recognizing the gp100 or annexin-II-derived peptides presented by artificial antigen-presenting cells (APC) were also able to recognize the native melanoma cells. However, the melanoma cells expressed functional fas ligand, which induced apoptosis in T cells. Accordingly, the peptide-specific T cell response to tumour cell stimulation after mAb blockade of fas/fas-ligand interactions was investigated.

Materials and methods

Cells

Normal human peripheral blood mononuclear cells (PBMC) were isolated from buffy coats (courtesy of Tübingen University Hospitals' Blood Bank) by isopycnic centrifugation over Ficoll/Hypaque (Lymphoprep, Nyegaard, Oslo). The donor was typed HLA-DRB1*0401$^+$ according to standard molecular methods. The melanoma cell line FM3 [HLA-DR2 (DRB1*02x), DR4 (DRB1*0401), DQ1 subsplit DQ6 (DQB1*0602), DQ3 subsplit DQ7 (DQB1*0602), HLA-A2 (A2.1), 3, HLA-B7, 44] was established from a metastatic tumour of a 70-year-old woman. FM3 was shown to express several common melanoma antigens, including Melan-A, MAGE-1, MAGE-3, tyrosinase and gp100 [15]. An Epstein-Barr-virus-transformed B lymphoblastoid cell line (B-LCL), FB3, was obtained from the same patient. Cells were cultured in RPMI-1640 medium (Gibco, Eggenstein, Germany) supplemented with 5% fetal calf serum (Gibco, Eggenstein, Germany), 20 mM HEPES, 2 mM glutamine, and antibiotics and propagated in roller bottles or culture flasks. Chinese hamster ovary (CHO) K1 cells were transfected with human cDNA encoding HLA-DRB1*0401 and HLA-DRA, and cotransfected with CD80 in addition to these genes, as previously described [30]. Each batch of CHO transfectants was cryopreserved and one sample of each was checked for retention of expression of human gene products before that batch was released for use as APC. The level of expression of HLA-DR and CD80 was always high, and the ability of these cells to bind peptide and function as APC was excellent [22].

Peptides

Peptides were synthesized in-house on a multiple peptide synthesizer (model SMPS 350; Zinsser Analytik, Frankfurt/M., Germany), or on a Milligen 9050 Pepsynthesizer (Millipore, Eschborn, Germany). The latter allows peptides to be synthesized under a nitrogen atmosphere, which is important for protecting susceptible amino acids, particularly methionine, from oxidation. The fmoc/tBu strategy was used for both synthesizers and crude peptides were purified by reversed-phase HPLC (Merck-Hitachi) to a purity of more than 90%. The sequences of the purified peptides were checked by mass spectrometry (model API III; Sciex, Toronto, Canada). Peptides employed were the 16-mers gp100(44–59), WNRQLYPEWTEAQRLD, and annexin II(208–223), DVPKWISIMTERSVPH. These two peptides had been identified among those eluted from the HLA-DR molecules from the FM3 cells [11, 21]. Briefly, HLA-DR molecules from FM3 cells were purified by means of affinity chromatography using L243-coupled CNBr-activated Sepharose (Pharmacia, Uppsala, Sweden). The cell lysate containing 2% (w/w) NP-40 was cycled overnight through a precolumn (CNBr-activated Sepharose saturated with TRIS/glycine), three anti-HLA-DR mAb (L243, ATCC) columns and one TÜ22 (anti-DQ) column. After extensive washing, self peptides were eluted from intact HLA-DR molecules with a 100 mM sodium phosphate buffer containing 0.1% Zwittergent-12, pH 11.0. The self-peptide pool was separated by reversed-phase HPLC and matrix-assisted laser desorption ionisation mass spectrometry (Finnigan MAT, La Jolla, Calif.) served to determine the masses and the homogeneity of the collected peaks. Homogeneous peaks were directly submitted to the Edman protein microsequencer (ABI 476A, Weiterstadt, Germany). The resulting peaks were spotted directly on a polyvinylidene difluoride sequencing membrane (Immobilon-P, Millipore, Eschborn, Germany), and sequenced [11]. The sequencing data were compared with the Swissprot protein sequence data base at the European Bioinformatics Institute, Hinxton Hall, Cambridge, UK, to identify the sources of the peptides.

In vitro sensitization to synthetic peptides

A protocol was developed using APC constructed by cotransfecting HLA-DR and CD80 genes into CHO cells. The CHO-APC were preincubated for 2 h at room temperature at pH 7.0 with annexin II(208–223) or gp100(44–59) synthetic peptides at 10 μg/ml, and then fixed for 2 min with 0.025% glutaraldehyde. Pulsed (or unpulsed control) CHO cells were then cocultured with HLA-DRB*0401$^+$ PBMC. Coculture was carried out in 16-mm-diameter Costar wells, using 1×10^6 PBMC and 5×10^5 APC/well in 2 ml culture medium (RPMI-1640 medium supplemented with 10% heat-inactivated non-transfused human male serum). After 7 days, 1.5 ml medium was removed and replaced by fresh medium containing 5×10^5 peptide-pulsed (or unpulsed) APC to restimulate the responding cells. After a total of 14 days culture, cells were removed and plated at 2×10^4/well in triplicate into round-bottom microtiter plates to which the same number of CHO cells (glutaraldehyde-fixed), FM3 cells (irradiated 30 Gy) or FB3 cells (irradiated 80 Gy) were added as stimulators. The CHO cells were prepulsed with peptides or medium alone. The anti-fas mAb M3 or M33 (the kind gift of Dr. D. Lynch, Immunex, Seattle), respectively antagonistic and agonistic for fas-mediated apoptosis [1], was used at 10 μg/ml. After culture, each microtitre well received 37 kBq tritiated thymidine (Amersham-Buchler, specific activity 185 GBq/mmol) after 24, 48 or 72 h. Radioactivity incorporated into the

nuclei of proliferating cells was isolated by harvesting onto glass-fibre sheets and quantified by liquid scintillation counting.

Results

Sensitization of normal T cells to gp100(44–59) presented by CHO cells

The gp100(44–59) peptide binds well to HLA-DRB1*0401 molecules [11, 21]. CHO cells cotransfected with HLA-DRB1*0401 and DRA genes, as well as the costimulatory ligand CD80, were therefore employed as APC to present the gp100 peptide to normal T cells from HLA-DRB1*0401$^+$ donors. The cotransfected CHO cells, and untransfected CHO as controls, were pulsed with peptide, washed and fixed in glutaraldehyde before coculture with the HLA-DRB1*0401$^+$ PBMC. After 10 days, cells were harvested from the cocultures and re-stimulated with CHO transfectants alone, or pulsed with specific gp100 peptide, or with the annexin II(208–223) peptide (which also binds well to DRB1*0401 [11]). Figure 1 shows a kinetic study of autocrine proliferative responses generated by these gp100-sensitized T cells, confirming previous results [11]. The response to gp100-pulsed CHO cells was high after 42, 66 and 90 h culture, with the peak response seen around 66 h (Fig. 1). This response was gp100-specific because annexin-II-pulsed CHO transfectants did not stimulate significantly above the level seen with T cells sensitized against unpulsed CHO transfectants and restimulated with gp100-pulsed or annexin-II-pulsed cells. The response was also CD80-dependent, because CD80-negative CHO cells were ineffective sensitisers or restimulators (data not shown). These results confirm that gp100(44–59) is immunogenic for autocrine proliferating T cells (helper cells) and further show that the immunogenic epitope is present in the unprocessed gp100(44–59) peptide itself. This is presented by MHC class II HLA-DRB1*0401 molecules in a CD80-costimulation-dependent manner.

Reactivity of gp100(44–59)-sensitized normal T cells to tumour cell products

T cells sensitized as described were restimulated by CHO transfectants preincubated in lysates from either FM3 melanoma cells or a B lymphoblastoid cell line, FB3, derived from the same patient. Figure 2 shows the autocrine proliferative responses triggered by these stimuli at 42 h and at 66 h. Proliferative responses of gp100-sensitized T cells against CHO cells pulsed with FM3 cell lysate were notably higher than those stimulated by FB3-lysate-pulsed CHO cells (Fig. 2). These results show that material derived from FM3 melanoma can stimulate gp100-sensitized T cells.

Reactivity of gp100(44–59)-sensitized normal T cells to tumour cells

T cells sensitized against peptide-pulsed CHO APC were restimulated with irradiated whole FM3 melanoma cells or with irradiated EBV-transformed B cells from the same patient (FB3). Figure 3 shows the results of one such experiment measured at the peak assay time. The positive control, CHO transfectant + gp100, stimulated high levels of thymidine incorporation compared to CHO transfectants pulsed with the annexin II peptide as seen previously. The FM3 tumour cells stimulated less autocrine proliferation; however, this was still more than that stimulated by the FB3 B cells. These results therefore suggest that normal T cells sensitized to synthetic gp100 peptide in vitro may be capable of recognizing native gp100$^+$ tumour cells.

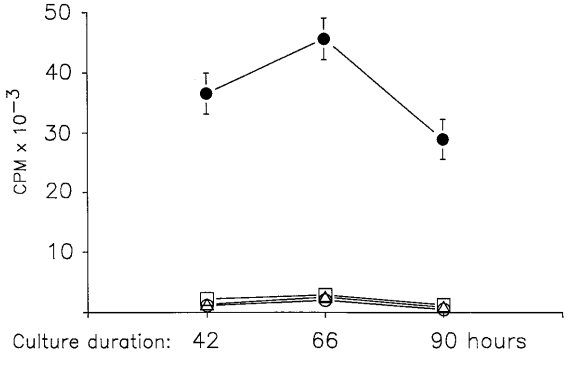

Fig. 1 Specific T cell sensitization to synthetic gp100(44–59) peptide in vitro. T cells primed to gp100-pulsed chinese hamster ovary (CHO) cells were restimulated by gp100-pulsed CHO (●) or annexin-pulsed CHO (□). Thymidine incorporation of replicate plates was measured at 42, 66 and 90 h. Responses of cells primed to CHO cells alone and then restimulated with gp100-pulsed CHO (△) or annexin-II-pulsed CHO (○) are also shown. The experiment was performed four times with different T cell donors, with similar results. Data points are background-corrected mean radioactivities (cpm) ± SEM of triplicate cultures

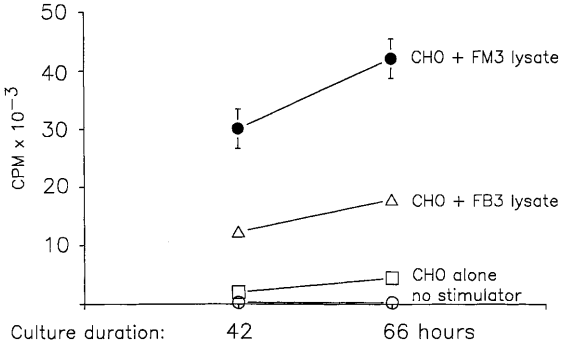

Fig. 2 Restimulation of gp100(44–59)-sensitized T cells by FM3 melanoma cell lysates. CHO antigen-presenting cells (APC) were preincubated with lysates from FM3 melanoma cells or FB3 B cells from the same patient (10^7 cell equivalents/10^6 CHO cells), washed, fixed and used as stimulators for the presensitized T cells. The experiment was performed twice with different donors, with similar results. Thymidine incorporation of replicate plates was measured at different times and data are shown as background-corrected means ± SEM of triplicate cultures

Reactivity of gp100(44–59)-sensitized normal T cells to tumour cells in the presence of antagonistic CD95 mAb

One possibility to explain the low stimulatory capacity of the FM3 cells was that, like the majority of melanomas, they were able to induce apoptosis of activated T cells because of their expression fas ligand. Cocultivation of FM3 cells with Jurkat cells resulted in the induction of apoptosis in the latter (data not shown), making it likely that FM3 cells would also be able to induce apoptosis in normal T cells. To test this possibility at the functional level, we took advantage of the existence of certain CD95 mAb that bind fas but do not induce apoptosis. Rather they block the fas apoptotic pathway, behaving as antagonists [1]. Figure 4 shows the results of an experiment in which gp100-specific T cells were restimulated by FM3 cells in the presence of a fas-antagonistic mAb, M3, and compared to T cells stimulated in the presence of an agonist, M33. In this experiment, the response to FM3 cells in the presence of M3 was found to be much enhanced compared to the response in the absence of mAb or in the presence of M33 (Fig. 4). These results suggest that gp100-sensitized cells can be stimulated by native melanoma cells but that this response is much enhanced when tumour-cell-induced apoptosis is prevented by fas/fas-ligand blockade.

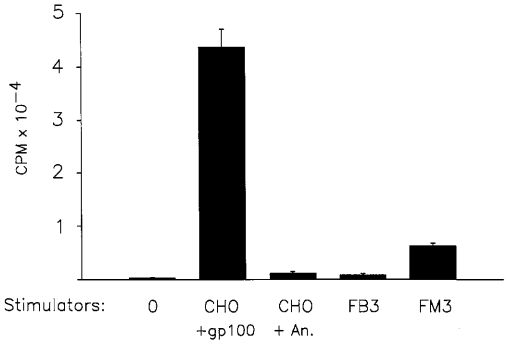

Fig. 3 Restimulation of gp100(44–59)-sensitized T cells by FM3 melanoma cells. T cells primed to gp100-pulsed CHO cells were restimulated by CHO cells pulsed with gp100 or annexin II peptide (*CHO + gp100*; *CHO + An.*), or by FM3 melanoma cells (*FM3*) or B cells from the same patient (*FB3*). This experiment was performed six times using various different donors, with comparable results, although the level of response to FM3 cells was variable. Thymidine incorporation was measured at 66 h and data are shown as background-corrected means ± SEM of triplicate cultures

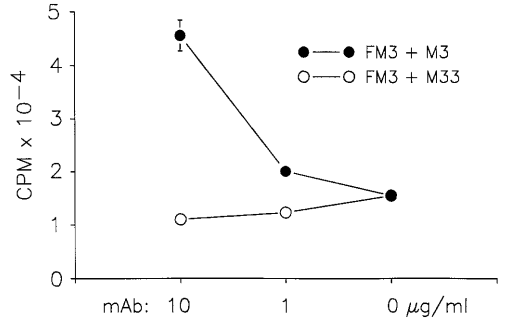

Fig. 4 Restimulation of gp100(44–59)-sensitized T cells by FM3 melanoma cells in the presence of CD95 mAb. The T cells were stimulated by FM3 melanoma cells (FM3) in the presence of titrated amounts of agonistic fas mAb M33 (*FM3 + M33*) or antagonistic fas mAb M3 (*FM3 + M3*). This experiment was repeated three times with similar results. Thymidine incorporation was measured at 66 h and data are shown as background-corrected means ± SEM of triplicate cultures

Reactivity of annexin-II(208–223)-sensitized normal T cells to tumour cells

Analogous experiments were also performed using the Annexin II(208–223) peptide instead of the gp100 peptide. It was demonstrated that annexin-II(208–223)-specific T cells could also be generated in vitro and that they were restimulated by annexin-II-pulsed but not gp100-pulsed CHO cells (Fig. 5). These annexin-II-peptide-sensitized T cells were then restimulated with FM3 melanoma cells, resulting in a response that could also be enhanced by the anti-fas mAb M3 but not M33 (Fig. 5). This again shows that fas blockade facilitates antitumour responses by peptide-sensitized T cells under these conditions, using a different tumour antigen.

Discussion

The technique of eluting endogenous peptides from MHC molecules was first developed to clarify the molecular nature of minor histocompatibility antigens [29, 42]. Since then, such approaches have become established techniques with which to analyse antigen presentation and recognition in the context both of class I and II [26]. Most usually, specifically sensitized T cells are

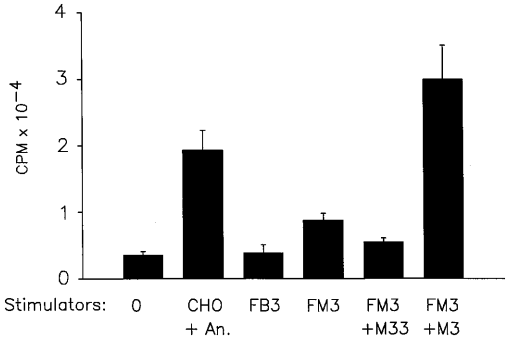

Fig. 5 Restimulation of annexin-II(208–223)-sensitized T cells by FM3 melanoma cells. T cells primed to annexin-II-peptide-pulsed CHO cells were restimulated by CHO cells pulsed with annexin-II-peptide (*CHO + An.*). The T cells were also stimulated by FM3 melanoma cells (FM3) in the presence of 10 μg/ml agonistic fas mAb M33 (*FM3 + M33*) or antagonistic fas mAb M3 (*FM3 + M3*). This experiment was repeated three times with similar results. Thymidine incorporation was measured at 66 h and data are shown as background-corrected means ± SEM of triplicate cultures

used as probes to identify fractions containing stimulating peptide and thence to identify the peptide itself. In tumour immunology, such T cells can be isolated from TIL and have been used for immunotherapy. This approach is not without difficulty because tumours may be able to inhibit T cells as a result of their evolution of manifold mechanisms for escaping the immune response. Thus, tumours can secrete immunosuppressive factors, induce anergy and apoptosis and cause dysregulated expression of T cell receptor components, to mention but a few of their functions [25]. Moreover, the T cells that can be isolated from patients may have been selected by cohabitation with the tumour and be specific only for a limited range of the potentially available tumour antigens. Because these T cells are obtained from patients in whom the immune response has already failed to prevent tumour growth, they may not represent the full repertoire of potential T cell responsiveness that could be exploited for immunotherapy.

An alternative approach to generating tumour-specific T cells may be to sensitize HLA-matched normal T cells to tumour cells in vitro, and this has occasionally been successfully carried out. However, problems arise because tumour cells also exert negative regulatory influences on T cells in vitro as well as in vivo. A possible solution to this problem is to sensitize T cells against tumour products presented by normal cells, and not against whole tumour cells themselves. This has been attempted with tumour cell lysates and class-I-binding peptides and, more recently, with certain synthetic peptides representing potential tumour antigens in the context of MHC class II [4, 5, 24, 35]. On the basis of the ability of the peptides to stimulate normal T cells in vitro, at least one group has begun clinical trials of active immunization [6, 7]. These studies all employed peptides representing potential tumour antigens selected on theoretical grounds but not known actually to be presented by the tumours. There is now some evidence beginning to appear that suggests that peptide-specific T cells may, on occasion, be able to recognize native leukemic and pancreatic tumour cells [8, 36].

In the present study, we have used directly isolated peptides bound to melanoma cells' HLA-DR molecules. It is likely that these are available in sufficient amounts for recognition by the immune system, because technical limitations are such that we can still only sequence those peptides bound to the MHC molecules in great abundance. Many of the peptides eluted from the DR molecules of melanoma FM3 have previously been found on DR molecules from B cell lines and are clearly not tumour-specific [11, 21]. However, several candidates were identified that may be expressed preferentially or exclusively by the tumour cells. Two of these, representing one sequence from gp100 and one from annexin II, were synthesized, characterized and used to sensitize normal T cells. The gp100 molecule is a lineage-restricted melanocyte marker, several peptides from which are well known as melanoma antigens in the context of recognition by HLA-A2-restricted T cells. A high proportion of TIL from HLA-$A2^+$ donors is specific for one of the five defined epitopes of gp100, but no class-II-restricted recognition has been reported thus far [28]. Annexin II, on the other hand, is a protein present in most cells, but may be overexpressed in tumours [27, 41]. In this way it may also function as a potential tumour antigen, as is the case with some other normal self molecules that are overexpressed on tumour cells [3].

The gp100 peptide bound very well to a range of HLA-DR alleles, but not to all of the alleles tested [11, 21]. Binding was strongest to HLA-DRB1*0401, with good binding also to HLA-DRB1*0101 and 0301, but very weak on HLA-DRB1*1101. These and other observations agree with the consensus binding motif for HLA-DRB1*0401 [12], which requires a large hydrophobic or aromatic residue at position i, a T or S at position $i+5$ and a small residue at position $i+8$. The gp100 peptide is, therefore, optimal at relative positions i and $i+5$, with the amino acid at position $i+8$ being tolerated [21]. Competition assays with the annexin II peptide also revealed good binding to HLA-DRB1*0401 [11]. We were therefore assured that these synthetic peptides would bind well to the HLA-DRB1*0401-transfected CHO cells that we intended to use as APC in these experiments.

The CHO:HLA-DR/CD80 cotransfectants are excellent APC in primary and secondary responses of TCR1 ($\gamma\delta$) and TCR2 ($\alpha\beta$) T cells stimulated by mitogens, superantigens or alloantigens [21, 30]. Although the DR molecules of the transfectants do not appear to be completely empty of peptide [2], they are capable of binding larger amounts of exogenous peptides than are DR molecules of the same allele from B cell lines [22]. It was therefore predicted that the transfectants would be able to present the peptides used here and, further, that they would be able to sensitize T cells to these antigens by virtue of their copresentation of the important costimulatory ligand CD80. The use of xenogeneic HLA-DR4-transfected APC was expected to focus the immune response onto the human peptides bound to the DR molecules in the absence of any other HLA antigens or competing human peptides. Accordingly, T cell lines were generated by culturing peptide-pulsed CHO cells together with PBMC from an HLA-matched DRB1*0401^+ donor. Restimulation of these resultant polyclonal lines with CHO cells revealed a peptide-specific component of the response. The autocrine proliferative capacity of the responding cells was very likely to have been supported by their secretion of interleukin-2 (IL-2), because assaying the supernatants of stimulated cells revealed the presence of IL-2 but not IL-4 (Zeuthen et al., unpublished results). Autoreactivity towards HLA-DR antigens by the peptide-sensitized T cells was excluded by the finding that there was no response to the $DR4^+$ B-LCL FB3 from the same patient who supplied the FM3 melanoma line. It is important to note that the sensitized T cells responded more strongly in most experiments to the FM3 tumour cells than to FB3 B cells. These results show that T cells primed to gp100 synthetic

peptide in vitro using CHO transfectants as APC can respond (usually weakly) to HLA-DRB1-matched $gp100^+$ melanoma cells in the absence of added peptide, although they are unable to respond to B cells from the same patient. Similar results were obtained with the annexin II peptide.

The ability of native tumour cells to stimulate the peptide-sensitized cells in the present experiments was commonly low compared to stimulation by antigen-pulsed CHO cells. Of the many possible reasons for this, one was investigated here. The inclusion in the cultures of an antagonistic anti-fas mAb, M3, which can block fas-dependent-activation-induced cell death [1], was found greatly to enhance the response to FM3 tumour cells. Enhancement of the antimelanoma response by fas blockade is consistent with a number of recent reports of the expression of fas ligand by melanoma [10] and other tumours [18, 34], and the suggestion that tumours can escape immunosurveillance by inducing apoptosis of antitumour T cells [17]. The FM3 cells used here were positive for fas ligand mRNA by Northern blotting, whereas the B cell line was not (Adibzadeh et al., manuscript in preparation). The FM3 but not FB3 cells were also able to induce DNA fragmentation in the $CD95^+$ indicator T cell line Jurkat, a functional assay for fas ligand positivity as measured in the terminal deoxynucleotidyltransferase-mediated dUPT nick-end labelling assay (Adibzadeh, manuscript in preparation). Early apoptosis induction was also indicated by the induction of annexin V binding to JURKAT cells after coculture with FM3 cells (Heinzel, unpublished results). T cells activated and cultured in the way described here are $CD95^+$ and are susceptible to fas-mediated lysis, suggesting that mAb M3 enhanced responses by preventing fas/fas-ligand-mediated melanoma-induced apoptosis of the sensitized T cells. The inclusion of the M3 mAb in cultures of T cells restimulated by B cells did not result in enhanced responses, whereas M3 was also able to enhance stimulation by immobilized CD3 mAb, which otherwise results in fas/fas-ligand-mediated-activation-induced cell death [23].

The sequences of the gp100 and annexin II peptides eluted from melanoma DR molecules both correspond to those of normal, unmutated, proteins. Despite this, both were readily recognized by normal donors' T cells. In the case of gp100, this may well be because the lineage restriction of its expression has prevented tolerance induction. In the case of annexin II, the amount of peptide expressed on healthy tissue is perhaps too low to trigger tolerance induction [31]. It remains to be seen whether melanoma patients' T cells also show gp100 or annexin II reactivity, or whether such T cells have been deleted from the repertoire. If this is not the case, the binding properties of both gp100 and annexin II to the majority of HLA-DR alleles suggest that these peptides may represent general-class-II-restricted tumour antigens on melanomas, and therefore may be useful for immunotherapy, possibly in combination with class-I-binding peptides.

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