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Identification of New HER2/*neu*-Derived Peptide Epitopes That Can Elicit Specific CTL Against Autologous and Allogeneic Carcinomas and Melanomas[1]

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Twenty-two new HLA-A2.1-binding peptides derived from the protooncogene HER2/*neu* were identified and analyzed for their capacity to elicit peptide and tumor-specific CTL responses. We used peptide-pulsed autologous DC from the ascites of patients with ovarian carcinomas to induce CTL. Of the 22 tested new HER2/*neu*-derived epitopes that could bind HLA-A2 with high (IC_{50} < 50 nM) or intermediate (50 nM < IC_{50} < 500 nM) affinity, we report the recognition by CTL of at least four novel epitopes, including HER2(9_{435}), HER2(9_{665}), HER2(9_{689}), and HER2(10_{952}), and confirm that of the known HER2 (9_{369}) epitope. These epitopes were able to elicit CTL that specifically killed peptide-sensitized target cells and, most importantly, a HER2/*neu*-transfected cell line and the autologous tumor cells. We also confirm that HER2/*neu* is overexpressed in several melanoma lines, and as a new finding, report that some of these lines are sensitive to CTL induced by the HER2 (9_{369}), HER2(9_{435}), and HER2(9_{689}) epitopes. Finally, CTL clones specific for HER2 (9_{369}), HER2(9_{435}), and HER2(9_{689}) epitopes were isolated from tumor-specific CTL lines, further demonstrating the immunodominance of these epitopes. These findings broaden the potential application of HER2/*neu*-based immunotherapy. ***The Journal of Immunology,* 1999, 163: 1037–1044.**

It is now well established that small peptide epitopes bound to the MHC, derived from processed proteins synthesized by the tumor cells, can be recognized as Ags by CTL. Tumor-specific CTL, adoptively transferred or activated in vivo by tumor-associated CTL epitopes, have therapeutic activity and can induce regression of established tumors or micrometastases (1, 2). The development of immunotherapeutic methods to treat cancer is critically dependent on the identification of tumor-associated Ags. Several immunodominant peptide epitopes, recognized by CTL lines and clones, have been defined from human carcinomas (3–8), mainly using a genetic method based on genomic or cDNA expression libraries (5, 6, 8), but also using a biochemical approach based on HPLC-derived (5) peptide fractions (4). Tumor-specific CTL, raised from tumor-infiltrating lymphocytes of cancer patients by culturing in IL-2 (6) or from PBMC of cancer patients after repetitive stimulation with the autologous tumor (5, 8), have been critical reagents for these studies.

As an alternative to the genetic and biochemical approach for identifying tumor- associated CTL epitopes, a reverse immunology method has been developed, which is not constrained by the availability of tumor-specific CTL, and thereby not limited to the analysis of only immunodominant epitopes already recognized by cancer patients during the course of their disease. In this method, MHC class I-binding epitopes are identified and corresponding synthetic peptides are tested for their capacity to induce peptide- and tumor-specific CTL derived from healthy individuals or cancer patients. This approach has recently been used for the definition of several new CTL epitopes in different melanoma Ags (7, 9–11).

The identification of tumor-associated Ags in melanomas has been paralleled by the demonstration that CTL derived from solid tumors or ascites of patients with ovarian cancer are capable of recognizing autologous and HLA class I-matched allogeneic tumors (12–16). The HER2/*neu* gene, which encodes a 185-kDa transmembrane glycoprotein with tyrosine-specific kinase activity and has a similarity in structure and sequence to the epidermal growth-factor receptor (17), is frequently recognized by tumor-specific CTL lines and clones derived from patients with carcinomas. HLA-A2-restricted CTL epitopes in the HER2/*neu* protein recognized by ovarian (12, 13) and breast cancer-specific (18, 19) CTL have previously been defined. Our previous work (20) has recently resulted in the identification of two new HLA-A2.1-restricted CTL epitopes from HER2/*neu*.

In the present study, we have tested a set of new HLA-A2-binding HER2/*neu*-derived peptides, using the reverse immunology approach, for their ability to elicit peptide- and tumor-specific CTL responses in patients with ovarian carcinomas. A method was developed in which peptide-loaded DC derived from the ascitic

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fluid of patients were used to induce peptide-specific CTL. We found that HER2/*neu*-specific CTL, elicited by several HER2/*neu*-derived peptides or isolated from the ascitic fluid of patients with ovarian carcinoma, were able to kill autologous and allogeneic HLA-A2^{+} ovarian and colorectal carcinoma cells in a tumor-specific manner. Furthermore, as a new finding, we report that melanomas, previously reported to overexpress HER2/*neu* (21), are also efficiently lysed by HER2/*neu*-specific CTL. The findings that several new HER2/*neu*-derived epitopes can efficiently elicit tumor-specific CTL against carcinomas and melanomas by using T cells and dendritic cells (DC)[5] from cancer patients broaden the potential application for epitope-based immunotherapy based on this protooncogene.

Materials and Methods

Ovarian tumor cells

Tumor cells were isolated as described (22) from the ascitic fluid of HLA-A2.1^{+} patients with advanced epithelial ovarian cancer. All patients had undergone various regiments of chemotherapy due to recurrent disease. The ascitic fluid was collected in sterile heparinized containers. The samples were centrifuged at 800 $\times$ *g* for 10 min, and the cell pellet was resuspended in PBS containing 5% FBS, 100 U/ml penicillin, and streptomycin. Ascitic cells were then isolated by centrifugation on Ficoll-Hypaque and cultured at 10^6 cell/ml. Lymphocyte growth was inhibited by adding 1 μg/ml cyclosporin A to the medium. Nonadherent cells and debris were removed and the tumor cells were cultured in RPMI 1640 medium (Life Technologies, Auckland, NZ), supplemented with 10% FBS (Life Technologies), 2 mM glutamine (Sigma-Aldrich, Tyresö Sweden), 20% autologous ascitic fluid, 2.5 μg/ml insulin, and 1% penicillin-streptomycin (Sigma-Aldrich). Every 5 to 6 days, the medium was replaced with fresh supplemented medium.

Preparation of DC

DC were produced from monocytes according to a previously described protocol (23–26). Briefly, after Ficoll-Hypaque separation, cells from ascitic fluid were resuspended in RPMI 1640 medium, supplemented with 10% FBS, 2 mM L-glutamine, and 1% penicillin-streptomycin. The cells (10^6 cell/ml) were incubated overnight at 37°C, and the nonadherent cells were removed by gentle pipetting. The adherent cells were cultured in RPMI 1640 and supplemented with 10% FBS medium containing 1000 U/ml GM-CSF (Schering-Plough, Stockholm, Sweden) and 1000 U/ml IL-4 (Schering-Plough, Brinny, Cork, Ireland). After 9–12 days, floating DC-like cells were harvested in PBS and used as stimulators after peptide loading. Cell morphology was determined by standard microscopic techniques.

Peptide-specific CTL lines and antitumor CTL clones

DC were incubated with 25 μg/ml peptide for 12 h at room temperature, then irradiated and washed twice to remove excess of peptide, before being used as stimulator cells. Lymphocytes derived from tumor-associated lymphocytes (TAL) were stimulated with irradiated autologous peptide-loaded DC cells in AIM-V medium (Life Technologies) containing 12.5 U/ml IL-2 (kindly supplied by Dr. P. Simon, DuPont Merck Pharmaceutical, Glenolden, PA) and 10 ng/ml IL-7 (Becton Dickinson, Stockholm, Sweden). Stimulator to T cell ratio was 1:20. After at least four rounds of stimulation at weekly intervals, the cytotoxicity of CTL was measured by a standard ^{51}Cr release assay. Antitumor CTL clones were then obtained from reactive CTL by limiting dilution after three weekly stimulations with irradiated autologous tumor cells.

Peptide synthesis and HLA-A2.1-binding assay

HER2/*neu*-derived peptides were identified on the basis of the HLA-A2.1-binding motif using a computer program (27). Nonamer and decamer peptides were synthesized by a solid phase method, using a multiple peptide synthesizer, and purified by HPLC, as previously described (10, 11). Peptide binding to HLA-A2 was measured as described (27, 28). Briefly, various doses of the test peptides (ranging from 100 μM-1 nM) were coincubated with 0.5 nM radiolabeled HBVc18–27 (FLPSDYFPSV) peptide and HLA-A2.1 heavy chain and β_2-microglobulin for 2 days at room temperature in the presence of a mixture of protease inhibitors. Percentage of MHC-bound radioactivity was determined by gel filtration, and the IC_{50}, the concentration required to inhibit 50% of the radiolabeled peptide binding, was calculated for each peptide.

Cell lines

CAOV-4, SW-626 ovarian tumor cell lines and colon carcinoma cell line SW-620 were furnished by the American Type Culture Collection (ATCC, Manassas, VA). OVA-9301, OVA-0826, OVA-6906, OVA-320929, OVA-360622, OVA-1226 ovarian tumor lines, and BL, BE, DFB melanoma lines were established at Microbiology and Tumor Biology Center in Karoliska Institute. FM-55 and FM-3D melanoma lines were kindly provided by Dr. J. Zeuthen (Danish Cancer Society Research Center, Copenhagen, Denmark). FMS melanoma line was from Dr. Gaudernack (Norwegian Radium Hospital, Oslo, Norway). C1R/A2, a MHC class I-defective LCL cell line transfected with HLA-A2 (29) was provided by Dr. M. Mosac, Karolinska Institute. The TAP-defect HLA-2.1 T2 cell line derived from the human T cell leukemia/B cell LCL hybrid 174 (30) was a generous gift of Dr. P. Cresswell (Yale University School of Medicine, New Haven, CT).

Transfections

Transfections were performed using Lipofectin (Life Technologies, Grand Island, NY), according to the manufacturer's protocols. The C1R/A2 cell line was transfected in our laboratory with the gene encoding for the HER2/*neu* protooncogene (LTR-2/*erbB-2* encoding a full-length HER2/*neu* cDNA) kindly provided by Dr. J. Pierce (Laboratory of Cellular and Molecular Biology, National Cancer Institute). The HER2/*neu*-transfected C1R/A2 line (C1R/A2. HER2) was selected in medium containing 1 μg/ml mycofenolic acid, 250 μg/ml xantine, 15 μg/ml hypoxantine, 10 μg/ml thymidine, and 2 μg/ml aminopterine (Sigma-Aldrich). The ovarian tumor cell line SW-626 was transfected with the HLA-A2 expression vector containing the full-length HLA-A2.1 cDNA. The transfected cells were selected with 200 μg/ml hygromycin B (Boehringer Mannheim, Mannheim, Germany).

mAbs and FACS analysis

The anti-HER2/*neu* (mAb TA-1) recognizing the extracellular domain of HER2/*neu* was purchased from Oncogene Science (New York, NY). The mAb BB7.2 (HB82, ATCC) was used to detect HLA-A2.1. FITC-conjugated goat anti-mouse IgG was purchased from Becton Dickinson (Mountain View, CA). For cytometric analysis of tumor cells, 1 $\times$ 10^5 cells per staining were washed in PBS, incubated with a primary murine anti-human mAb for 20 min on ice. Cells were washed twice before incubation with a secondary FITC-labeled rabbit anti-mouse IgG Ab for 20 min. Cells were then washed twice before being resuspended in PBS containing 1% paraformaldehyde and 1% FBS.

FITC-labeled anti-CD3 (mAb UCHT1), CD4 (mAb MT310), and CD8 (mAb DK25), plus PE-labeled anti-CD4 (mAb MT310), CD8 (mAb DK25), and CD56 (mAb MOC-1) were purchased from Dako (Dakopatts, Älvsjö, Sweden). After staining, the cells were fixed with 1% paraformaldehyde and kept at 4°C until analysis by FACS (Becton Dickinson). For every staining, isotype-matched control mAb was used as a negative control.

Cytotoxic assay

To analyze tumor recognition, tumor cells were labeled for 1 h at 37°C with 125 μCi/10^6 cells ^{51}Cr (Amersham, Amersham Sweden, Solna). For peptide recognition, T2 cells or C1R-A2 cells were incubated overnight at 26°C together with 25 μg/ml peptide, washed, and then labeled. Cytotoxic assays were performed by incubating ^{51}Cr-labeled target cells with effector cells at various E:T ratios at 37°C for 4 h. In some experiments, blocking with an anti-HLA-A2 Ab was performed by incubating the target cells for 20 min with 1/50 dilution of the supernatant of the BB-7.2 hybridoma. Cold target inhibition was also done using nonradiolabeled T2 loaded with HER2/*neu* peptide or with the HLA-A2-binding irrelevant peptide MP58–66 (GILGFVFTL) used as negative control. The cold target to hot target (^{51}Cr labeled) ratio was 10:1, unless otherwise indicated. Supernatants were harvested and radioactivity was determined using a gamma counter. The percentage of ^{51}Cr release was calculated according to the following formula: percent lysis = 100 $\times$ (experimental release $-$ spontaneous release)/(maximum release $-$ spontaneous release). All determinations were made in triplicates.

[5] Abbreviations used in this paper: DC, dendritic cell; LCL, lymphoblastoid cell line; TAL, tumor-associated lymphocyte.

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Table I. *Relative binding affinity of HER-2/*neu *derived peptides to HLA-A2.1*[a]

Peptide	Sequence	A*0201 Binding[b] IC_{50} (nM)
HER2(9_{665})	VVLGVVFGI	14.3
HER2(9_{106})	QLFEDNYAL	17.2
HER2(9_{689})	RLLQETELV	20.8
HER2(9_{153})	VLIQRNPQL	22.7
HER2(9_{369})	KIFGSLAFL	33.3
HER2(9_{653})	SIISAVVGI	69.4
HER2(9_{435})	ILHNGAYSL	74.6
HER2(10_{952})	YMIMVKCWMI	83.3
HER2(9_{5})	ALCRWGLLL	100.0
HER2(10_{785})	LLGICLTSTV	102.0
HER2(10_{5})	ALCRWGLLLA	138.9
HER2(9_{789})	CLTSTVQLV	147.1
HER2(9_{48})	HLYQGCQVV	147.1
HER2(9_{952})	YMIMVKCWM	217.4
HER2(9_{466})	ALIHHNTHL	238.1
HER2(9_{767})	ILDEAYVMA	238.1
HER2(10_{434})	RILHNGAYSL	277.8
HER2(10_{773})	VMAGVGSPYV	277.8
HER2(10_{144})	SLTEILKGGV	333.3
HER2(10_{106})	QLFEDNYALA	357.1
HER2(9_{508})	GLACHQLCA	416.7
HER2(10_{654})	IISAVVGILL	416.7

[a] Results based on a previous report (20).

[b] Concentration of peptides inhibiting 50% of the binding of 0.5 nM standard peptide HBVc18-27 to the HLA-A2.1 molecules (see *Materials and Methods*). According to affinity data, peptides are arbitrarily ranked as high (IC_{50} < 50 nM), intermediate (50 nM < IC_{50} < 500 nM), and weak (IC_{50} > 500 nM, not shown) binders.

Results

*Induction of CTL responses to HER2/*neu*-derived peptides*

We have previously screened the amino acid sequence of the HER2/*neu* protooncogene for the most probable HLA-A2 nona- and deca-mer peptide epitopes by the use of a computer program that takes into account the presence of main HLA-A2.1-specific anchor residues, and specific secondary anchor residues (20, 27). Of 165 peptides (9 mers and 10 mers) containing HLA-A2.1-binding motifs, 23 were found to bind with an IC_{50} < 500 nM to purified HLA-A2.1 molecules, and 22 of these were included in the present analysis (Table I). The HER2 (9_{369}) peptide previously reported as a CTL epitope in cancer patients (14, 31) and also the HER2 (9_{689}) epitope previously found by us to be immunodominant in tumor-specific tumor-infiltrating lymphocytes from gastric cancer (32) were among the highest affinity HLA-A2.1-binding peptides.

The 22 peptides were tested for their capacity to elicit HLA-A2-restricted CTL from TAL derived from ascitic fluid of patients with advanced ovarian carcinoma. TAL were stimulated at least four times over a period of 4–6 wk with synthetic peptides pulsed onto autologous DC also derived from the ascitic fluid. These cells had typical DC morphology when observed by standard light microscopy, and expressed high levels of MHC class II and of the costimulatory molecules CD54, CD80, CD86, and CD40 and did not express or expressed only very low levels of CD14, as measured by immunofluorescence and flow cytometry (data not shown). The resulting CTL lines were composed of a majority of $CD8^+$ cells (>75%), the remaining cells being $CD4^+$ (data not shown).

Three of the five high affinity binding (IC_{50} < 50) peptides, HER2(9_{665}), HER2(9_{689}), and the previously defined HER2(9_{369}) (14, 31), were able to induce peptide-specific lysis of T2 by CTL derived from at least four of eight different patients with ovarian carcinomas, as shown with CTL from patients OVA-320929 and OVA-360622 (Fig. 1, data not shown for HER2(9_{665})). Six among the seventeen intermediate binders (IC_{50} = 51–500 nM), including HER2(9_{48}), HER2(9_{435}), HER2(9_{767}), HER2(10_{785}), HER2(9_{789}), and HER2(10_{952}), were able to elicit peptide-specific CTL in at least three of eight different patients analyzed (Fig. 1, data only shown for HER2(9_{435})).

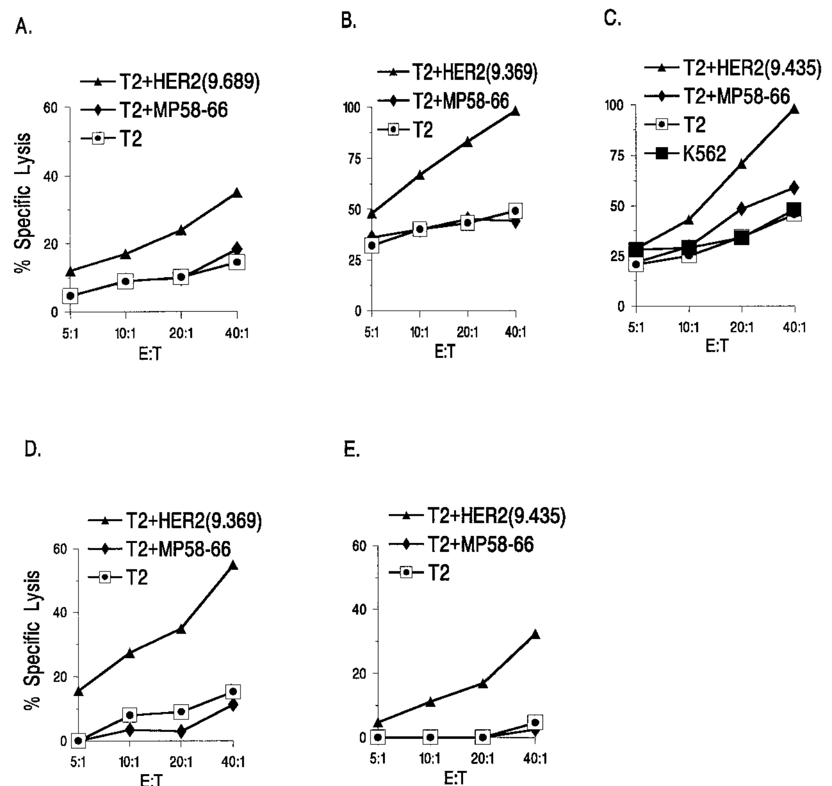

FIGURE 1. Induction of CTL against HER2/*neu*-derived peptides. *A*, *B*, and *C*, Cytotoxicity of CTL induced by peptide-pulsed DC cells (DC to T cell ratio, 1:20) in the presence of IL-2 and IL-7. CTL were produced as described in *Materials and Methods* and tested against T2 target cells pulsed with the same HER2/*neu* peptide HER2(9_{689}) (*A*), HER2(9_{369}) (*B*), and HER2(9_{435}) (*C*) epitopes as used for the stimulation, with an irrelevant peptide (MP58–66) or against unpulsed T2 and/or K562 as control. *D* and *E,* Induction of CTL against HER2(9_{369}) (*D*) and HER2(9_{435}) (*E*) using a high ratio of DC to T cells (1:2.5–5) and with IL-7 alone without IL-2, tested as described above. CTL line A, B, and D from OVA-320929 patient; C and E from OVA-360622 patient.

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The majority of these epitopes induced specific CTL as they did not kill, or killed to a much lesser extent, either the corresponding nonpulsed target cell or target cells pulsed with an irrelevant HLA-A2-binding peptide (Fig. 1). The peptide specificity of these CTL was observed even in the presence of a 20-fold excess of K562 cells (data not shown). In our initial protocol, stimulations were conducted in the presence of IL-2 (12.5 U/ml) and with a ratio of DC to T cells of 1:20 (Fig. 1, *A–C*). By adapting this to a protocol in which stimulations were conducted without IL-2 and with a higher ratio of DC to T cells (1:2.5–5) in the presence of 10 ng/ml IL-7, less nonspecific killing of nonpulsed T2 was observed (Fig. 1, *D* and *E*).

Thus, our CTL induction protocol made it possible to produce HER2/*neu*-derived peptide-specific CTL from TAL of four of eight tested HLA-A2$^+$ ovarian carcinoma patients, using nine different HLA-A2-restricted epitopes from HER2/*neu*. Notably, all four of the eight patients, OVA-9301, OVA-0826, OVA-320929, and OVA-360622, from which HER2/*neu*-specific CTL were generated, had tumors that expressed HER2/*neu* (data not shown).

*HER2/*neu*-specific CTL can specifically recognize cells expressing HLA-A2 and HER2/*neu

To establish whether peptide-induced CTL could recognize HLA-A2-associated naturally processed epitopes, we next transfected the HLA-A2$^+$ gene in the HLA-A2$^-$, HER2/*neu*-expressing ovarian carcinoma line SW-626. Six peptide-specific CTL lines from patient OVA-9301, selected on the basis of availability of cells, were tested against this HLA-A2$^+$ transfectant and its control. The CTL raised against peptide HER2(9_{369}), HER2(9_{435}), HER2(9_{665}), HER2(9_{689}), and HER2(10_{952}) all showed cytotoxic activity against the HLA-A2$^+$ transfectant of SW-626, although the cytotoxic activity of the CTL raised against HER2(9_{435}) was low in this experiment. The HLA-A2$^-$ control line was relatively resistant to cytotoxicity (Fig. 2*A*). As control, the influenza virus peptide (MP58–66)-specific CTL were not able to lyse these targets.

To further establish that natural processing generated these epitopes, the C1R/A2 cell line was transfected by the full-length HER2/*neu* cDNA. This transfectant expressed levels of HER2/*neu* as high as those seen in HER2/*neu* high-expressing tumor lines (data not shown). When the same CTL lines from patient 9301 induced by the five peptides, as listed above, were tested, all showed cytotoxic activity against the HER2/*neu* transfectant of C1R/A2 with little or no activity against C1R/A2 control cells (Fig. 2*B*). Again, the cytotoxic activity of the CTL raised against HER2(9_{435}) was low in this experiment. Similarly, MP58–66 peptide-specific CTL did not lyse the transfectant.

Our data therefore show that the CTL lines induced by at least five of the high or intermediate HER2/*neu* HLA-A2 binders can induce specific CTL that also recognize naturally processed MHC class I-associated peptides.

*HER2/*neu*-specific CTL can specifically recognize autologous and allogeneic carcinomas overexpressing HER2/*neu

We succeeded in establishing tumor lines from patients 9301 and 0826. Both of these lines were found to overexpress HER2/*neu* (data not shown). HER2/*neu*-specific CTL were tested for their capacity to kill autologous tumor cells, while the HLA-A2$^-$, HER2/neu$^+$ ovarian tumor line SW-626 was used as control. CTL from patient 9301 induced by peptides HER2(9_{369}), HER2(9_{435}), HER2(9_{665}), HER2(9_{689}), and HER2(10_{952}) were able to kill the autologous tumor, while the HLA-A2$^-$ SW-626 tumor or the NK-sensitive K562 line was relatively resistant to lysis by the same CTL (Fig. 3). HER2(10_{785}) peptide-specific CTL did not specifically lyse autologous tumor cells (data not shown). Similarly, the CTL lines from patient OVA-0826 induced by the same four peptides showed cytotoxic activity against the autologous tumor, although this tumor was in general more resistant to cytotoxicity (data not shown).

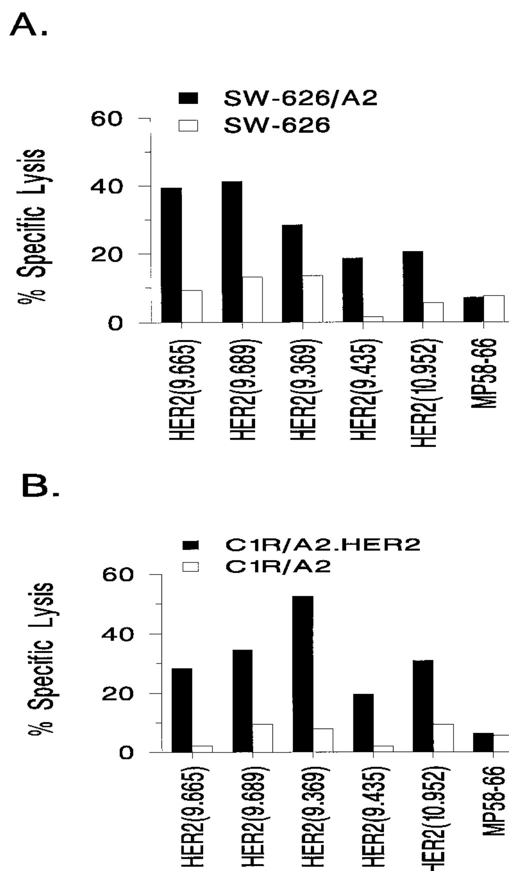

FIGURE 2. CTL induced by HER2/*neu*-derived peptides can recognize naturally processed peptides on the surface of HLA-A2$^+$ target cells. Five different HER2/*neu* peptide-specific CTL lines from patient OVA-0826 were tested against the HLA-A2-transfected, HER2/*neu*$^+$ ovarian tumor line SW-626 (SW-626/A2) (*A*) or HER2/*neu*-transfected C1R/A2 (C1R/A2. HER2) (*B*) at an E:T ratio of 20:1. SW-626 (*A*) and C1R/A2 (*B*) were used as controls, respectively. An influenza virus peptide (MP58–66)-specific CTL derived from a healthy HLA-A2$^+$ donor was included as control (not tested in the same experiment as the HER2/*neu* peptide-specific CTL). This line lysed 36% of T2 cell-pulsed MP58–66 (data not shown).

The CTL from donor 0826 induced by peptide HER2(9_{369})-loaded DC cells were further tested on a panel of semiallogeneic (sharing HLA-A2) and allogeneic carcinomas, which had been typed for their expression of HER2/*neu* and HLA-A2 (Table II). Among the ovarian carcinomas, CAOV-4 and OVA-6906, both of which are HLA-A2$^+$ and HER2/*neu*$^+$, were lysed, while the SW-626 (HLA-A2$^-$, HER2/*neu*$^+$) was very poorly recognized and OVA-1226 (HLA-A2$^-$*neu*$^-$) were not lysed at all. The colorectal cancer line SW-620 (HLA-A2$^+$, HER2/*neu*$^+$) was also highly sensitive to these HER2/*neu*-specific CTL. These data confirm the widespread expression of the HER2/*neu* Ag in various types of carcinomas, and show that it is processed and presented to MHC class I-restricted CTL in all of the recognized carcinomas. The carcinoma-specific cytotoxicity of this HER2(9_{369})-specific CTL line was further established by blocking with HLA-A2-specific mAb (data not shown).

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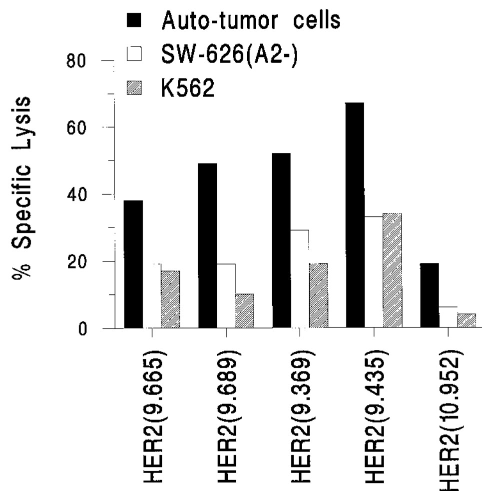

FIGURE 3. CTL induced by HER2/*neu*-derived peptides can recognize naturally processed peptides on the surface of the autologous tumor cell. CTL lines derived from patient OVA-9301 were tested in a standard ^{51}Cr release assay against the autologous tumor line or as control the HLA-A2$^-$ ovarian line SW-626, or the NK-sensitive target K562 at an E:T ratio of 20:1.

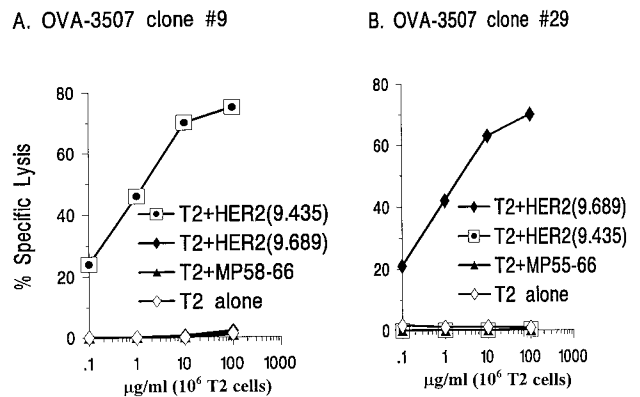

FIGURE 4. Peptide specificity of anti-HER2/*neu* CTL clone OVA-3507 9 and 29. The T2 cells were preincubated for 2 h with the indicated peptide together with ^{51}Cr in a final volume of 0.5 ml, and then washed twice and used as target cells at an E:T ratio of 10:1.

*HER2/*neu*-specific CTL can specifically recognize HLA-A2 allogeneic melanomas overexpressing HER2/*neu

In the next series of experiments, five of eight melanoma lines, BL, BE, FMS, FM-3D, and FM-55, were found to express HER2/*neu*, further confirming (21) that this molecule can also be expressed in tumors of neuroectodermal origin (Table II). Two of these melanoma lines (FMS and BL) that were HLA-A2$^+$ and HER2/*neu*$^+$ were also highly sensitive to the HER2(9_{369})-specific CTL line from donor OVA-0826 (Table II), from OVA-320929, and to the HER2(9_{689})- and HER2(9_{435})-specific CTL lines from donor OVA-320929, and from donor OVA-360622 (data not shown). However, two of the HLA-A2$^+$ and HER2/*neu*$^+$ melanoma lines (BE and FM-3D) showed weak sensitivity to the HER2(9_{369})-specific CTL (Table II). The HLA-A2$^+$, HER2/*neu*$^-$ melanoma line DFB was resistant (Table II) or only weakly sensitive (data not shown) to HER2/*neu*-specific CTL.

Table II. *Cytotoxicity of OVA-0826 CTL induced by HER2(9_{369})-loaded autologous DC on allogeneic tumor lines*

Target Name	HLA-A2[a]	HER-2/*neu*[a]	% Lysis 15:1[b]	30:1[b]
Ovarian carcinomas				
CAOV-4	+	+	24	42
OVA-6906	+	+	39	54
SW-626/A2	+	+	54	61
SW-626	−	+	7	15
OVA-1226	−	−	0	0
Colon carcinoma				
SW-620	+	+	49	43
Melanomas				
FMS	+	+	30	43
BE	+	+	12	18
FM-3D	+	+	13	18
BL	+	+	50	50
DFB	+	−	0	0
Other				
K562	−	−	4	7

[a] Expression of HLA-A2.1 and HER2/*neu* as detected by flow cytometry, and categorized as negative (−) or positive (+) as compared to the control.
[b] E:T ratio.

The specificity of the CTL lines against HER2/*neu*$^+$ melanomas was further tested by adding an excess of nonlabeled cold T2 cells pulsed with the specific peptide to the mixture of CTL and ^{51}Cr-labeled melanoma targets. Recognition of the melanoma targets by the CTL lines specific for HER2(9_{689}), HER2(9_{369}), or HER2(9_{435}) was significantly inhibited (20–40% inhibition) by unlabeled T2 cells only when pulsed with the cognate peptide, but not by unlabeled T2 cells pulsed with an irrelevant HLA-A2.1-binding peptide or by unlabeled control T2 cells. These results further confirm that a substantial part of the cytotoxicity of the HER2/*neu*-specific CTL is directed against naturally processed HER2-derived epitopes presented at the surface of the HLA-A2$^+$ HER2/*neu*$^+$ melanomas.

*CTL clones specific for new HER2/*neu*-derived epitopes expressed on ovarian carcinomas can be isolated from ovarian-specific CTL lines*

We next asked whether CTL clones specific for HER2/*neu* epitopes on ovarian carcinomas and melanomas can be isolated from ovarian-specific CTL lines that were derived from expanding ascitic derived T cells in IL-2 without stimulation with synthetic peptides. A tumor-specific CTL line (OVA-3507) produced by repeated stimulation with the autologous tumor and previously shown to be cytotoxic against the autologous tumor and against other HLA-A2.1-expressing allogeneic tumors (31) was cloned by limiting dilution. Seven of fifty-eight clones derived from the OVA-3507 line were shown to be cytotoxic for HLA-A2-expressing ovarian carcinoma lines (data not shown). Three of them were further analyzed for their cytotoxicity against the panel of HER-2/*neu*-derived peptides loaded onto T2 cells. Clone 9 recognized HER2(9_{435}), and clone 29 recognized the HER2(9_{689}) epitopes (Fig. 4) when loaded at concentrations of 100–0.1 μg/ml to T2 cells, thus demonstrating that these two epitopes can also be immunodominant, as we already have shown for the HER2(9_{689}) epitope (32). None of these two clones recognized other HLA-A2-binding peptides (Fig. 4). One of the three clones (43) was found to recognize the HER2(9_{369}) peptide (data not shown), thus confirming previous results (31).

The CTL clone 9 from the 3507 line recognizing the HER2(9_{435}) epitope and the clone 29 recognizing the HER2(9_{689}) epitope were tested for cytotoxic activity against a panel of carcinoma and melanoma lines (Fig. 5*A*). A similar pattern of recognition was observed for both of these CTL clones, in which the carcinomas CAOV-4 and SW-620 (HLA-A2.1$^+$, HER2/*neu*$^+$) and the melanoma lines FMS and BL (HLA-A2.1$^+$, HER2/*neu*$^+$) were lysed,

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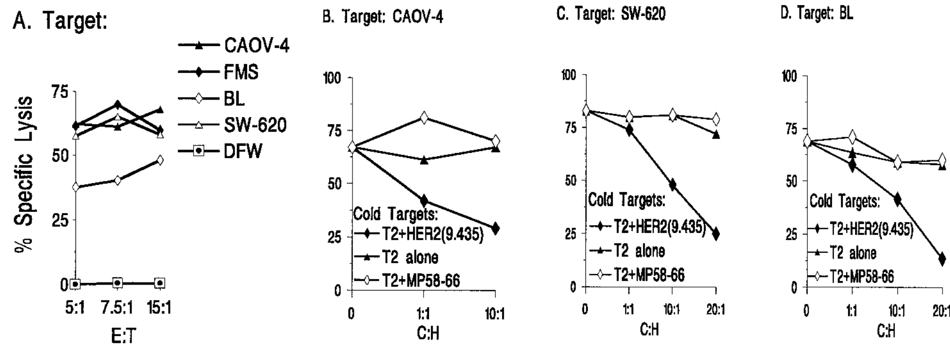

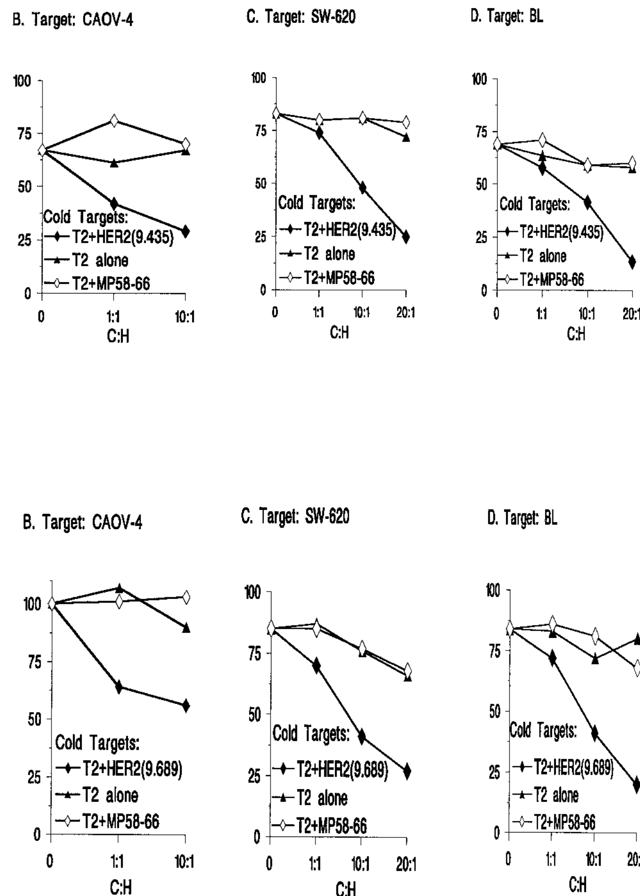

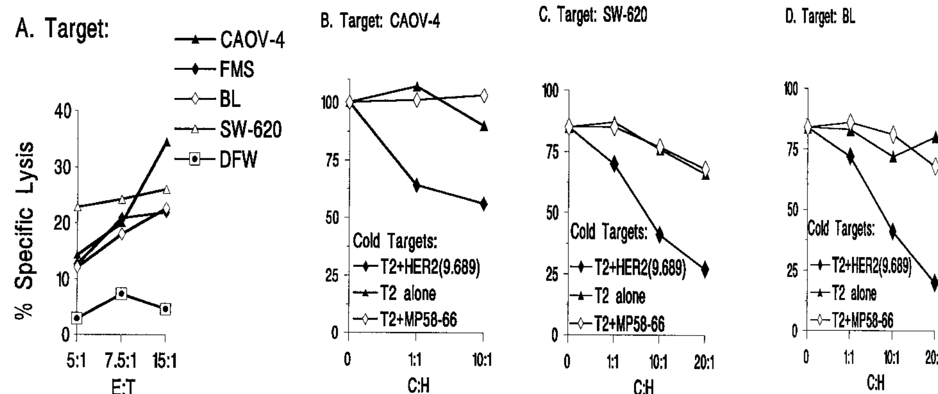

FIGURE 5. Demonstration of specificity of the anti-HER2/*neu* CTL clone OVA-3507 9 and 29. Cytotoxicity of the CTL clones 9 and 29 against the HLA-A2$^+$ and HER2/*neu*$^+$ ovarian tumor cell line CAOV-4, colon carcinoma SW-620, and melanoma cell line FMS, BL, and against the control HLA-A2$^+$ and HER2/*neu*$^-$ melanoma DFW (*A*). The HLA-A2$^+$ and HER2/*neu*$^+$ tumor cell lines CAOV-4 (*B*), SW-620 (*C*), and BL (*D*) were ^{51}Cr labeled and mixed with unlabeled T2 pulsed with the specific peptide HER2(9_{435}) or HER2(9_{689}) or with the irrelevant peptide (MP58–66) or with unlabeled T2 cells without peptide. C:H = cold target:hot target ratio.

while the HLA-A2.1$^+$, HER2/*neu*$^-$ melanoma line DFW was not. To confirm that the antitumor cytotoxic activity of the OVA-3507-derived clones 9 and 29 (Fig. 5) was specific for the cognate peptide, an excess of nonlabeled cold T2 cells pulsed with the corresponding specific HER2 peptide was added to the mixture of CTL and ^{51}Cr-labeled ovarian carcinoma CAOV-4 (Fig. 5*B*), colon carcinoma SW-620 (Fig. 5*C*), and melanoma BL (Fig. 5*D*) targets. Recognition of the ovarian carcinoma and melanoma targets was significantly inhibited by unlabeled T2 cells pulsed with the cognate HER2-derived peptide, but not or much less by unlabeled T2 cells or T2 cells pulsed with an irrelevant HLA-A2.1-binding peptide (MP58–66).

Discussion

We report in this study several findings of relevance to HER2/*neu*-based cancer immunotherapy. First, we identified new HLA-A2.1-restricted CTL epitopes of HER2/*neu*, and showed that T cells from patients with advanced tumors overexpressing this molecule are still able to develop CTL against these epitopes. Second, we have shown that HER2/*neu*-specific CTL raised against synthetic peptide epitopes or isolated from TAL in the ascitic fluid of patients can recognize naturally processed peptides on ovarian carcinomas and, as a new observation, also on melanomas. Third, we have shown that ascitic fluid can be utilized as a source of both T cells and DC for CTL induction experiments. Last, we have confirmed the immunodominance of HER2/*neu* epitopes, and demonstrate that T cells specific for these epitopes are present in tumor-specific CTL lines.

Epitope identification was performed by the reverse immunology approach, previously used to define other CTL epitopes from human tumor-associated Ags. Herein, besides the known HER2(9_{369}) epitope (12, 31), we report the identification of at least four more HER2/*neu*-derived epitopes, including HER2(9_{435}), HER2(9_{665}), HER2(9_{689}), and HER2(10_{952}), that were able to elicit CTL in patients with ovarian cancer. These epitopes elicited CTL that specifically killed peptide-sensitized target cells and, most importantly, autologous tumor cells. The HER2(9_{689}) epitope was recently found to be immunodominant in gastric cancer-specific CTL (32). The HER2(9_5), HER2(9_{369}), and HER2(9_{435}) peptides were shown to be able to elicit peptide- and tumor-specific CTL from PBL of HLA-A2.1$^+$ healthy donors (20), although a different T cell sensitization protocol was used. Two of these peptides, HER2(9_{369}) and HER2(9_{435}), were also found to elicit CTL in cancer patients. Our CTL induction protocol, with repeated stimulations over an extended period of time, does however not allow us to draw conclusions regarding the immunodominance of these epitopes in the tumor-specific CTL repertoire of the patients. The response in healthy donors may represent an example of an in vitro induction of a primary response, or alternatively, a weak secondary response following sensitization in vivo by peptides expressed on HER2/*neu*$^+$ normal tissues. A comparison of epitope-specific CTL precursor frequencies between cancer patients and healthy donors would seem necessary to establish whether the ascitic fluid does indeed contain enhanced levels of HER2/*neu*-specific CTL precursors.

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Three additional epitopes that were able to elicit peptide-specific CTL were defined, including HER2(9_{48}), HER2(9_{767}), and HER2(9_{785}) (data not shown).

T cells induced in vitro with APC pulsed with peptides selected for their ability to bind to MHC class I alleles frequently have been shown to recognize peptide-pulsed target cells, but not target cells presenting naturally processed epitopes (33, 34). Presumably, this is due to the low levels of epitope presented by the latter. It is therefore of particular interest that CTL generated against several of the new HER2/*neu*-derived epitopes characterized in this study were able to recognize the autologous HER2/*neu*$^+$ HLA-A2$^+$ ovarian carcinomas, but not HLA-A2$^-$ ones (Fig. 3). The specificity of these peptide-induced lines was further established by their lysis of C1R cells transfected with HER2/*neu* and HLA-A2 genes (Fig. 2*B*) and by analyzing a HLA-A2 transfectant of a HLA-A2$^-$, HER2/*neu*-expressing ovarian carcinoma (Fig. 2*A*). CTL specific for the HER2(9_{369}) (31, 35), HER2(9_{971}) (12), and HER2(10_{654}) (35, 36) epitopes were previously shown to recognize HLA-A2$^+$, HER2/*neu*$^+$ tumor targets, demonstrating that several epitopes from this molecule appear to be naturally processed and presented on the surface of carcinomas. Our method for CTL generation may favor the outgrowth of high affinity CTL precursors bearing the capacity to recognize cell surface epitopes expressed at low density.

Immunodominance was addressed by testing CTL clones developed from patient-derived CTL lines stimulated solely with autologous tumor cells. In agreement with previous results (12, 31), one CTL clone was found to be specific for the HER2(9_{369}) epitope, while two additional CTL clones from the same donor also recognized the HER2(9_{435}) and HER2(9_{689}) epitopes, further confirming that HER2/*neu*-derived epitopes constitute a dominant part of the tumor-specific CTL response against ovarian carcinomas. Cold target competition assays confirmed that the cytotoxicity of these clones against HLA-A2$^+$ carcinomas and melanomas was specific for the cognate naturally processed peptide. The immunodominance of HER2/*neu* could be explained by the fact that this molecule is a surface receptor (37) that may recycle into the cytoplasm and therefore be easily accessible to the MHC class I Ag presentation pathway. We have recently defined immunodominant CTL epitopes from another cell surface receptor (38), the MC1R receptor expressed on the majority of human melanomas (39). Thus, it is possible that cell surface receptors in general may frequently generate CTL epitopes.

Herein we also present the novel finding that HER2/*neu*-specific CTL lines and clones, recognizing epitopes HER2(9_{369}), HER2(9_{435}), or HER2(9_{689}), also can kill some HLA-$A_2$$^+$, HER2/neu$^+$ melanoma lines. Immunohistochemical studies have previously shown that human melanomas could express significant levels of HER2/*neu* (21). We find that five of the eight melanoma lines express significant levels of HER2/*neu*, as analyzed by flow cytometry, although their levels of expression were somewhat lower than that seen in the ovarian carcinoma lines. Two of the freshly isolated melanoma samples from which these tumor lines were established (BL and BE) were also analyzed for HER2/*neu* expression by immunohistochemistry and were found to express HER2/*neu* (data not shown), demonstrating that their HER2/*neu* expression was not induced by in vitro culture. Four of these melanoma lines were killed by our HER2/*neu*-specific CTL, although two (BE and FM-3D) only to a relatively low degree. The specificity of the killing of the melanoma lines by HER2/*neu*-specific CTL was further confirmed by cold target competition assays. Our finding of HER2/*neu*-specific CTL killing of melanomas may broaden the potential use of immunotherapy based on HER2/*neu*.

It should be noted that as we have not tested any low affinity binding HER2/*neu* epitopes, we cannot exclude that some of these may also be able to induce CTL with our CTL sensitization protocol. Our results demonstrate that the reverse immunology approach can be utilized to greatly broaden the repertoire of epitopes available to specific immunotherapy, allowing to tap into a rich collection of subdominant epitopes. Several of these epitopes would remain unavailable for exploitation if one were limited to only the most dominant epitopes.

Others have used the ascites as a source of T cells to generate tumor-specific CTL (22, 40). As a new approach, we have in this study used the ascites as a source for both T cells and DC for the generation of peptide-specific CTL. The ascitic fluid from patients with ovarian and other types of tumors (colon cancer, liver cancer, and pancreatic cancer) may therefore be a useful starting source of T cells and DC cells for the generation of large numbers of CTL to be used in protocols of adoptive immunotherapy.

Our data clearly establish that HER2/*neu* is an immunodominant molecule. DC cultures can be isolated from ascitic material, loaded with peptides corresponding to the dominant and subdominant epitopes, and used to develop CTL lines from T cell cultures originating from autologous ascitic material. We hope that this approach will be useful in developing protocols for epitope-based immunotherapy against various carcinomas.

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