ABSTRACT  The identification of antigenic peptides presented on the tumor cell surface by HLA class I molecules and recognized by tumor-specific cytotoxic T lymphocytes may lead to a peptide vaccine capable of inducing protective cellular immunity. We demonstrate that both HLA-A2-restricted breast and ovarian tumor-specific cytotoxic T lymphocytes recognize shared antigenic peptides. At least one of these peptides is derived from the oncogene product of HER2/neu, which is overexpressed in 30–40% of all breast and ovarian cancers. T cells sensitized against this nine-amino acid sequence demonstrate significant recognition of HLA-A2", HER2/neu" tumors. Since 50% of the tumor-cell population is HLA-A2" and many different tumors express HER2/neu, this peptide may be widely recognized and have many clinical applications.

Cytotoxic T lymphocytes (CTLs) are potent cellular effectors of the immune system. These T cells possess memory and, therefore, can respond to rechallenges by the same antigenic assaillant. This arm of the immune system, if induced by a tumor vaccine, could be effective in providing long-term protective immunity. CTLs recognize short peptide sequences presented by the HLA class I molecules on the tumor cell surface (1). Thus far, human tumor-associated antigens (TAA) that could potentially be useful for a vaccine have been demonstrated only indirectly, with the exception of the MAGE (melanoma antigen) system (2) and, most recently, Pmel 17 (3) and MART (melanoma antigen recognized by T cells) (4) in melanoma. The discovery of these genes and peptides substantiates the existence of such antigen systems, but unfortunately, melanoma accounts for only 3% of all malignancies and 1% of all cancer deaths (5).

HLA-A2 is expressed in approximately 50% of Caucasians (6) and has been demonstrated to play a critical role in antigen presentation of both viral antigens (7) and tumor antigens from a variety of cancers (8–10). HER2/neu is a 185-kDa transmembrane glycoprotein with tyrosine kinase activity and extensive homology to the epidermal growth factor receptor (11). HER2/neu is ubiquitously expressed in many tumors and known to be overexpressed in approximately 30–40% of all ovarian and breast cancers (12).

We have shown that tumor-specific CTLs can be demonstrated in the tumor-infiltrating lymphocytes (TILs) isolated from ovarian cancers (13). We have also found that HLA-A2 presents TAA in this disease (14). The level of expression of the HER2/neu oncogene in ovarian cancer positively correlates with recognition by HLA-A2" ovarian tumor-specific CTLs, and HLA-A2" melanoma cells transfected with the HER2/neu gene become sensitive to ovarian cancer-specific CTLs (15). Therefore, HLA-A2-presented, HER2/neu-derived antigenic peptides may be recognized by ovarian cancer-specific CTLs and, potentially, by breast cancer-specific CTLs as well.

In this study, we have searched the HER2/neu sequence (16) for HLA-A2-binding peptides (17), and we have found that both ovarian and breast cancer-specific CTLs recognize a nine-amino acid peptide from the transmembrane portion of the HER2/neu protein (GP2; amino acids 654–662). This peptide is widely expressed in HER2/neu" tumors and is capable of inducing HLA-A2-restricted, tumor-specific CTL populations in vitro.

METHODS

Generation of Tumor-Specific CTLs. Tumor-specific CTLs were generated from fresh tumor specimens obtained through the Departments of Surgery, Gynecologic Oncology, and Pathology at Brigham and Women's Hospital (BWH) and Beth Israel Hospital in Boston, under approval of the Institutional Review Boards. Solid tumor specimens were processed as previously described (14). Briefly, specimens were minced manually and enzymatically digested, and the lymphocytes and tumor cells were separated by centrifugation over discontinuous Ficoll (Organon Teknika–Cappel) gradients. TILs were suspended in RPMI-1640 medium (BioWhittaker) supplemented with 10% fetal calf serum (BioWhittaker) and antibiotics. Cultures were suspended at 5 × 10^6 cells per ml on solid-phase, anti-CD3 polystyrene plates (Orthoclone OKT3, Ortho Pharmaceuticals) for 48 h. T cells were then maintained in culture with 50 international units per ml of interleukin 2 (IL-2) (Amgen Biologicals). At 1, 3, and 5 wk, autologous, irradiated (10,000 rad from cesium source; 1 rad = 0.01 Gy) tumor cells were added to the T-cell cultures at a 10:1 lymphocyte-to-tumor cell ratio.

Phenotype Analysis. The HLA-A2 status of the tumor cells was determined by indirectly staining 10 μl of a 1:10 dilution of culture suspension with anti-HLA-A2 monoclonal antibodies (mAbs) BB 7.2 and MA 2.1 (American Type Culture Collection) at 4°C for 30 min, followed by a 30-min incubation with goat anti-mouse mAb conjugated with fluorescein isothiocyanate (FITC) (Becton Dickinson) and analysis with a Coulter Epics C Cytometer. HER2/neu expression was determined by indirect immunofluorescence staining with TA-1 anti-HER2/neu mAb (Oncogene Science) under the same conditions.

Tumor Cell Lines. The HLA-A2" ovarian and breast tumor cell lines were started from the fresh tissue specimens by suspending dissociated tumor cells in RPMI-1640 medium supplemented with 10% fetal calf serum and 5 μg of insulin per
ml. The HLA-A2- ovarian tumor lines were the kind gift of the Gynecology/Oncology Laboratory at BWH, and the HLA-A2- breast tumor lines (1897 and 1902) were purchased from American Type Culture Collection. All tumor cell lines had comparable HER2/neu expression as determined by flow cytometry.

Cytotoxicity Assays. Cytotoxicity was determined by standard 4-h chromium release assays as previously described (14). Briefly, targets were labeled with 50–100 μCi (1 Ci = 37 GBq) of sodium chromate (New England Nuclear) for 1 h at 37°C, washed, and plated at 2500 cells per well in a volume of 100 μl. Effectors were added at designated effector-to-target (E:T) ratios in a volume of 100 μl per well. After 4 h of incubation, culture supernatant was collected (Skatron, Sterling, VA) and radionuclide release measured with a γ counter (Gamma Tnc 1191, TM Analytic, Elk Grove, IL). All determinations were done in triplicate. Results are expressed as percent specific lysis defined as follows: ([experimental mean cpm – spontaneous mean cpm] / [maximum mean cpm – spontaneous mean cpm]) × 100.

Peptide-Pulsed Cytotoxicity Assays. For these experiments, we used the T2 cell line, which is a human T-cell/B-cell fusion product containing an antigen-processing defect in the TAP (transporter associated with antigen presentation) proteins, such that their HLA-A2 molecules can be effectively loaded with exogenous HLA-A2-binding peptides (18). The T2 cells were labeled with chromium as above, washed, and then incubated with peptide for 1 h at 37°C prior to continuing with the standard cytotoxicity assay.

Acridine Elution Studies. These studies were performed as outlined in ref. 19. Briefly, tumor cells were washed with a pH 3.3 citrate/phosphate buffer (0.131 M citric acid/0.066 M Na2HPO4) for 1 min at room temperature. The acrid was centrifuged to remove cell debris and then concentrated on C18 SepPak cartridges (Waters). Peptides were eluted from the column with 60% (vol/vol) acetonitrile and lyophilized. The peptides were reconstituted in the citrate/phosphate buffer and filtered through a Centricon-3 (Amicon) ultrafiltration device. The flowthrough was then injected onto a C18 analytical column (Rainin, Woburn, MA). Reversed-phase HPLC was performed, and the peptides were eluted with a linear 0–60% (vol/vol) acetonitrile (Pierce) gradient. The resulting fractions were lyophilized to remove organic solvents, resuspended in 150–200 μl of Hank’s balanced salt solution, and used in peptide-pulsed cytotoxicity assays.

Synthetic Peptides. Peptides were synthesized in the Biopolymer Laboratory at BWH on an Applied Biosystems 430 peptide synthesizer. Crude products were dissolved and injected onto C18 reversed-phase HPLC columns (4.6-mm i.d.; Rainin) and eluted with linear trifluoroacetic acid (TFA)/acetonitrile gradients (solvent A = 99.92% distilled H2O/0.08% TFA, and solvent B = 99.94% acetonitrile/0.06% TFA; 0–5 min, 100% A; 5–10 min, 0–10% B in A; 10–60 min, 10–60% in A; flow rate of 1.0 ml/min). Identity and purity of final materials were established by amino acid analysis, mass spectrometry, and analytical reversed-phase HPLC. Peptide GP1 (amino acids 650–658) was determined to be 97% pure, while peptide GP2 preparations (amino acids 654–662) could only be made ≈40% peptide by weight, as determined by amino acid analysis.

mAb-Blocking Assays. Prior to standard cytotoxicity assays, peptide-pulsed T2 cells were incubated with anti-HLA-A2 mAb BB 7.2 or anti-HLA-A,B,C mAb W6/32 (American Type Culture Collection) for 30 min at 4°C.

In Vitro Sensitization Experiments. Fresh TIL populations were split and cultured in parallel after initial anti-CD3 activation. The control cultures were maintained in a low concentration of interleukin 2 (50 international units per ml) and expanded as required. The peptide-educated cultures were stimulated with irradiated T2 cells pulsed with the GP2 peptide weekly for 3 wk. These cultures were then tested for their ability to recognize T2, GP1-pulsed T2, GP2-pulsed T2, and HLA-A2+ and HLA-A2− allogeneic tumor targets in standard cytotoxicity assays.

PCR Amplification and cDNA Sequencing. Approximately 2–5 × 106 tumor cells from ovarian cancers were lysed and total cellular RNA was isolated by ultracentrifugation in guanidinium isothiocyanate/cesium chloride. First strand cDNA was generated from 1 μg of total RNA by using oligo(dT) and reverse transcriptase in a reaction volume of 20 μl. PCR was performed using a mixture of reaction buffer (Perkin–Elmer/Cetus), deoxynucleotides, Taq DNA polymerase (Perkin–Elmer/Cetus), cDNA, and primers in a final reaction volume of 25 μl. Primers were constructed to amplify approximately 200 bp containing the transmembrane portion of the HER2/neu protein (forward primer was 5′-CATCAACTGACCCACCTCCT and reverse primer was 5′-GCAGGATGCTCCCGCATCCTG). One cycle of PCR consisted of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and elongation at 72°C for 2 min. A total of 30 cycles were performed, and the last cycle was followed by a 7-min extension at 72°C. For sequencing, 15 μl of the primary PCR reaction mixture was resolved on a 1.5% low-melting-point agarose gel, and appropriately amplified PCR products were isolated using the Quiax gel extraction kit (Quiagen, Chatsworth, CA). Sequencing was performed by using a modification of the dyeoxy chain termination method with Sequenase Version 2.0 (United States Biochemical). Samples were loaded onto a 6% polyacrylamide denaturing gel containing 7 M urea. Dried gels were autoradiographed using X-Omat AR film (Eastman Kodak).

RESULTS

HLA-A2-Restricted Crossreactivity of Breast and Ovarian Tumor-Specific CTLs. To determine if a common antigen system exists between ovarian and breast cancers, we isolated TILs from a series of HLA-A2+, HER2/neu+ ovarian (OvTIL) and breast (BrTIL) cancer specimens. The TILs were cultured with repeated stimulation by autologous tumor cells until the cultures revealed tumor-specific cytotoxicity as previously described (13). As shown in Fig. 1A, the HLA-A2+ OvTIL1, OvTIL2, and OvTIL3 lines recognized the HLA-A2+ ovarian tumor lines significantly better (P < 0.05) than the HLA-A2− lines, confirming our previous report (14). Likewise, the HLA-A2+ BrTIL1, BrTIL2, and BrTIL3 lines significantly (P < 0.05) recognized only HLA-A2+ breast cancer lines (Fig. 1B). More importantly, the OvTILs recognized the breast cancer lines in a HLA-A2-restricted manner, and the BrTILs recognized the ovarian cancers similarly (Fig. 1C and D, respectively, P < 0.05). Neither the OvTILs nor the BrTILs recognized HLA-A2+ melanomas, nor did either HLA-A2+ OvTILs or BrTILs lyse the HLA-A2+ cancer lines (data not shown). The HER2/neu expression in the HLA-A2+ and HLA-A2− tumors was comparable (data not shown). These data suggest that HLA-A2 serves as a restriction element in breast cancer also, but more importantly, at least one common tumor antigen system that is presented by the HLA-A2 allele exists between these closely related malignancies.

Acridine Elution of Endogenously Expressed, Commonly Recognized TAA. The existence of antigenic peptides presented on the tumor cell surface by HLA class I can be directly demonstrated. We have loaded acid-eluted (19) and HPLC-fractionated peptides on the T2 cell line and found that multiple peptides exist that induced recognition by ovarian tumor-specific CTLs (Fig. 2A). Several of the peptide fractions acid-eluted from ovarian tumor cells were also recognized by BrTILs (Fig. 2B), thus confirming by another method the existence of shared TAA between these two cancers.
HER2/neu-Derived Synthetic Peptides. As previously stated, the HER2/neu sequence was searched for HLA-A2-binding peptides, and in the transmembrane portion of the protein there is a peptide with the same HLA-A2-binding dominant residues (17) as found in the immunogenic influenza matrix peptide 58–66 (XIXXXXVXL) (20). Therefore, we synthesized the peptide (654–662) with the sequence IISAV-VGIL (GP2), to test if this peptide constitutes a T-cell epitope. We also constructed a control peptide from the sequence (650–658) PLTSIIASV (GP1), which has a known binding affinity for HLA-A2 (21). The T2 cell line (18) was loaded with the synthetic peptides, and four HLA-A2+ ovarian cancer-specific TIL lines recognized the GP2 peptide but not the GP1 peptide or the unloaded T2 \( (P < 0.05) \) (Fig. 3A). The level of killing ranged from 10 to 20% but was generally lower than the cytotoxicity against an allogeneic tumor control. Of extreme interest, three HLA-A2+ breast cancer-specific TIL lines also recognized the GP2 peptide but not the GP1 peptide or unloaded T2 \( (P < 0.05) \) (Fig. 3B). The level of recognition of the peptide-pulsed T2 was comparable to that of an HLA-A2+ allogeneic control. The level of recognition of GP2 was significant, consistent, and reproducible in multiple experiments with these CTL lines.

Both OvTILs and BrTILs recognized the GP2 peptide in a dose–response manner with maximal lysis requiring approximately 100 \( \mu \text{g} \) of peptide per ml (data not shown). No significant recognition of GP1 was found at any concentration. The relative insolvability of the very hydrophobic GP2 peptide makes it difficult to give an accurate concentration of peptide in solution, so concentrations are reported on the basis of the weight of peptide added to solution. The identity of this peptide was further confirmed by demonstrating that the recognition of GP2-pulsed T2 was HLA-A2-restricted by using mAb-blocking studies. The anti-HLA-A2 mAb BB 7.2 completely inhibited the recognition of GP2 by both OvTIL and

![Fig. 1. Shared TAA in HLA-A2+, HER2/neu+ ovarian and breast cancers.](image)

**Fig. 1.** Shared TAA in HLA-A2+, HER2/neu+ ovarian and breast cancers. (A) HLA-A2 restriction and shared antigens in ovarian cancer as recognized by ovarian tumor-specific CTLs. HLA-A2+ OvTILs were isolated from fresh surgical specimens and cultured with repeated autologous tumor stimulation. OvTILs were tested in standard 4-h chromium release assays at an E:T ratio of 20:1 against two HLA-A2+ and two HLA-A2− allogeneic ovarian cancer lines that were all comparable HER2/neu+. (B) HLA-A2 restriction and shared antigens in breast cancer as recognized by breast tumor-specific CTLs. HLA-A2+ BrTILs were generated as above and similarly tested against two HLA-A2+ and two HLA-A2− breast cancer lines with equivalent levels of HER2/neu expression. (C) OvTILs recognize HLA-A2+, HER2/neu+ breast cancer lines. Three HLA-A2+ ovarian tumor-specific CTL lines were tested against two HLA-A2+ and two HLA-A2−, HER2/neu+ breast cancer lines in cytotoxicity assays at an E:T ratio of 20:1. The OvTIL lines did not recognize HLA-A2+ melanoma lines. (D) BrTILs recognize HLA-A2+, HER2/neu+ ovarian cancer lines. Three HLA-A2+ breast tumor-specific CTL lines were tested against two HLA-A2+ and two HLA-A2−, HER2/neu+ ovarian cancer lines in cytotoxicity assays at an E:T ratio of 20:1. The BrTIL lines did not recognize HLA-A2+ melanoma lines. This series of experiments is representative of three separate experiments. ND, not done; ALLO, allogeneic; Ov, ovarian; Br, breast; A2+, HLA-A2+; A2−, HLA-A2−.

![Fig. 2.](image)

**Fig. 2.** OvTILs and BrTILs recognize many of the same acid-eluted, ovarian-derived peptides. (A) OvTILs recognize multiple antigenic peptides. Endogenously produced peptides were acid-eluted from the HLA molecules on an ovarian tumor cell line and fractionated by reversed-phase HPLC. The fractions were loaded onto the HLA-A2+, antigen-processing mutant, T2, and tested in chromium release assays at an E:T ratio of 10:1. (B) BrTILs recognize several ovarian cancer-derived peptides. BrTILs were used in standard cytotoxicity assays against T2 cells pulsed with the same acid-eluted, ovarian-derived fractions as above at an E:T ratio of 20:1.
more, this tumor recognition was specific and HLA-A2-restricted (Fig. 4) with significantly greater recognition of HLA-A2+ tumor cells as compared with HLA-A2- tumor targets \((P < 0.05)\).

**Endogenous Expression of GP2 in Ovarian Cancer.** According to one report, there is a valine instead of an isoleucine at position 655 (11); therefore, to confirm in ovarian cancer the expression of the peptide 654–662 as previously described in breast (22, 23) and gastric (16) cancers, we constructed PCR primers predicted to amplify the transmembrane domain of the HER2/neu mRNA. The amplified cDNA was directly sequenced, and in three consecutive ovarian tumors, isoleucine was found at position 655. These data confirm the expression of the GP2 peptide in ovarian cancer.

**DISCUSSION**

Finding a specific peptide derived from the HER2/neu sequence that is recognized by tumor-specific CTLs confirms that this oncogene serves as a tumor antigen system. HER2/neu is widely expressed in many different tumors, and the GP2 peptide has been sequenced from breast (22, 23) and gastric (16) cancers, and now from ovarian cancers as well. The isoleucine at position 655 is likely to be the most common amino acid residue; however, a neutral substitution of valine at position 655 has been described in the HER2/neu expressed in normal tissue by Coussens et al. (11). Since a valine at position 2 of the GP2 peptide would drastically decrease its affinity to HLA-A2 (17), the GP2 peptide would be theoretically a tumor-specific target. Unfortunately, due to the extremely low level of HER2/neu expression in normal tissue, we have not been able to confirm the sequence in normal tissue. However, even if this peptide is not tumor-specific, the difference in levels of HER2/neu expression between normal tissue and tumors would still allow this peptide to be utilized therapeutically.

The elution experiments demonstrate that there are other peptides that induce a CTL response. The HPLC fractions may contain antigenic peptides from mutated proteins, exclusively expressed tumor proteins, or other overexpressed normal proteins like HER2/neu that may be amplified as much as 20-fold in ovarian and breast cancers (12). The HER2/neu gene has been shown to transform NIH 3T3 cells (23); therefore, the immune system may recognize this potentially harmful protein when it is overexpressed. Recent reports suggest that normal HER2/neu-derived peptides may be recognized by CD4+ T cells (24) and ovarian tumor-specific CTLs (25). However, in the latter study, the 14-amino acid peptides recognized by a single CTL line would not be predicted to bind HLA-A2 as suggested (17, 21). Normal proteins have been documented, however, to be recognized by the immune system in melanoma (26). In fact, all of the melanoma TAA described thus far are also expressed in normal tissue (2–4). Therefore, the exploitation of CTL-recognized normal peptides must be based on the differential expression between tumors and normal tissue.

Of utmost interest for the design of tumor vaccines is the fact that there are shared TAA between ovarian and breast cancers which may be targeted. This finding, though previously unproven, may not be overly surprising, given the tumors' similarities. One group has recently described cross-recognition of HLA-A2+ pancreatic and colon carcinomas by HLA-A2+ ovarian cancer-specific CTLs (27). These tumors also express HER2/neu, as does non-small-cell lung cancer (12, 28). Data from our laboratory indicate that HLA-A2+, HER2/neu+ lung cancer-specific CTLs recognize HER2/A2+, HER2/neu+ ovarian cancers and the GP2 peptide (32). These findings suggest that the recognition of the GP2 peptide may be widespread and of extreme clinical importance.
We have shown that effective therapeutic TIL populations can be established with repeated tumor stimulation (13); however, this is potentially dangerous with the risk of inadvertent transfer of viable tumor cells back to the patient and quite often logistically impossible, due to the lack of viable tumor material from small surgical specimens. As we have shown, the GP2 peptide is efficient at stimulating the proliferation of CTL populations (D.C.L. et al., unpublished data), and these cultures demonstrate HLA-restricted, tumor-specific cytotoxicity (Fig. 4). The synthetic peptide can be mass produced and theoretically exploited for stimulating therapeutic tumor-specific CTLs for any HER2/neu+ malignancy expressing the peptide. This list currently includes breast, ovarian, and gastric cancers, and non-small-cell lung cancer will probably soon be added. Additional cancers to be tested include pancreatic and colon cancers. The level of expression of the GP2 peptide in these tumors will obviously impact the effectiveness of any therapeutic modalities targeting the peptide; however, highly sensitive and specific CTL cultures can be generated. The current limitations of the GP2 peptide, apparent in the level of cytotoxicity demonstrated in Fig. 3, are related to its relative insolubility and only moderate affinity for the HLA-A2 molecule. Both of these factors can be addressed by simple modifications of this peptide, which will potentially further increase its effectiveness for in vitro sensitization of therapeutic CTL populations.

Because GP2 is highly expressed in tumor tissue and is efficiently recognized by CTLs, the peptide may also be useful as a tumor vaccine. Soluble peptide vaccines have been shown to be effective with and without immunoadjuvants (30, 31) and could be potentially utilized to induce an endogenous antitumor immune response in high-risk patients, such as lymph node-positive breast cancer patients. In addition, just as genetically modified tumors are being injected and utilized for the in vivo sensitization of tumor-draining lymph node T cells for adoptive transfer (29), a peptide-loaded carrier cell could be utilized similarly. The peptide would be much safer if the carrier cell were innocuous, such as an autologous fibroblast or B-cell line. Furthermore, there would be no need to excise the vaccine injection site, as is required with the tumor cell option, simplifying the in vivo sensitization method.

The discovery of a widely expressed, oncogene-derived peptide that is highly expressed in tumor tissue and is recognized by tumor-specific CTLs offers many exciting future therapeutic options for the treatment of malignancies like breast and ovarian cancer for which there are currently no effective systemic therapies for advanced disease.

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