Recognition of Breast Cancer Cells by CD8⁺ Cytotoxic T-Cell Clones Specific for NY-BR-1

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Abstract

Immunotherapy for breast cancer using cytotoxic T cells (CTL) is hindered by the lack of well-characterized breast cancer antigens that are expressed in most breast tumor cells and recognized by CD8⁺ CTL. A recently described breast tissue differentiation antigen, NY-BR-1, is expressed in >80% of breast tumors and elicits a humoral response in a subset of breast cancer patients. To identify potential NY-BR-1 epitopes that are recognized by CTL, CD8⁺ T cells were stimulated in vitro with autologous dendritic cells pulsed with NY-BR-1 peptides that were predicted to bind to HLA-A2. In multiple normal female donors and breast cancer patients, specific CD8⁺ CTL responses were detected by enzyme-linked immunospot assay against several NY-BR-1 peptides after two cycles of stimulation. CD8⁺ CTL clones against three NY-BR-1 epitopes were isolated and recognized peptide-pulsed target cells with high avidity. T-cell clones specific for one of the NY-BR-1 epitopes (p904) also recognized breast tumor cells expressing NY-BR-1, NY-BR-1⁺ cells transfected with a cDNA encoding the NY-BR-1 protein, and autologous dendritic cells pulsed with oposinized NY-BR-1⁺ breast tumor cells. Taken together, these results show that the p904 epitope derived from NY-BR-1 is efficiently processed and presented endogenously and identify NY-BR-1 as a promising target for T-cell-based immunotherapy for breast cancer. (Cancer Res 2006; 66(13): 6826-33)

Introduction

Immunotherapy for cancer using T cells specific for MHC-bound peptides derived from unique or overexpressed tumor-associated antigens (TAA) has the potential to selectively target tumor cells for elimination without the toxicity associated with chemotherapy. The adoptive transfer of CD8⁺ T cells specific for defined tumor antigens can eliminate established tumors in animal models (1, 2). Encouraging results have also been achieved in clinical trials, in which autologous T cells that were reactive with melanocyte differentiation antigens and expanded from the blood or tumor biopsies were infused into patients with metastatic melanoma (3, 4). Extending this approach to other malignancies, such as breast cancer, will require the identification of target antigens and the isolation and expansion of tumor-reactive T cells.

Several nonmutated self-proteins that contribute to cell proliferation or survival, such as HER-2/neu, Mucin-1, telomerase, cyclin B1, survivin, and mdm2, have been suggested as potential targets for T-cell therapy of breast cancer (5–11). The most extensively studied of these is HER-2/neu, which is overexpressed in 25% to 30% of breast tumors and has been successfully used as a target for antibody therapy (12). Patients with HER-2/neu⁺ tumors may exhibit HER-2-specific antibody and CD4⁺ T-cell responses, but these endogenous responses are insufficient to prevent tumor growth (13). Efforts to boost HER-2-specific T cells in breast cancer patients by vaccination with peptides selected using computer algorithms that predict MHC binding have predominantly elicited peptide-specific T cells that failed to recognize tumor cells (14, 15). This may reflect the difficulty overcoming mechanisms of T-cell tolerance to widely expressed self-proteins that limit the repertoire of high-avidity T cells to prevent autoimmunity (16–18). Thus, the discovery of additional antigens that are expressed by breast tumors but have limited expression in normal tissues may provide more effective targets for T-cell immunotherapy.

Several candidate TAAs have been identified by screening serum samples from breast cancer patients for antibody responses to proteins expressed from a tumor cDNA library (19). A gene, termed NY-BR-1, which is composed of 37 exons and has an open reading frame of 4.0 to 4.2 kb, was identified by this approach. The polypeptide encoded by NY-BR-1 has a predicted molecular weight of 150,000 to 160,000 and contains a bipartite nuclear localization signal motif and a bZIP site (DNA-binding site followed by leucine zipper motif), suggesting that it may serve as a transcription factor. NY-BR-1 mRNA is expressed in >80% of breast tumor specimens but very few in nonmammary tumors (19). By quantitative real-time PCR analysis, only one of three normal breast samples had low-level NY-BR-1 mRNA expression compared with high levels in most breast tumors (20). Apart from the low-level expression in normal breast, NY-BR-1 is not expressed in other normal tissues, except testis. However, a related gene (NY-BR-1.I), which has 54% amino acid homology to NY-BR-1, is expressed in brain (19).

The identification of NY-BR-1 as a tissue-specific protein that is recognized by a humoral immune response in breast cancer patients led us to examine whether this protein could also serve as a target for CD8⁺ cytotoxic T cells (CTL). We report here that CD8⁺ CTL clones specific for three NY-BR-1 epitopes presented by HLA-A*0201 can be isolated from normal female donors and breast cancer patients. Of the three NY-BR-1 peptides that are recognized by CD8⁺ CTL, one is efficiently processed and presented endogenously by breast tumor cells and could also be cross-presented by tumor-loaded dendritic cells. These findings show that the T-cell repertoire contains high-avidity T cells reactive with NY-BR-1 and identify NY-BR-1 as a new TAA to target in T-cell immunotherapy trials for breast cancer.

Materials and Methods

Peripheral blood samples. Peripheral blood was obtained from HLA-A2⁺ healthy female volunteers and from patients with stage II to IV...
breast cancer by either leukapheresis or venipuncture according to protocols approved by the Institutional Review Board. All subjects gave written informed consent. Donors were initially screened for HLA-A2 expression by flow cytometry of peripheral blood mononuclear cells (PBMC) using an A2-specific monoclonal antibody (mAb; BB7.2). The expression of HLA-A2 was subsequently confirmed by molecular typing in the Clinical Immunogenetics Laboratory at the Seattle Cancer Care Alliance (Seattle, WA) and maintained as adherent monolayers with RPMI 1640 supplemented with 10% fetal bovine serum. Expression of MHC class I molecules and HLA-A2 was determined by flow cytometry using the mAbs W6/32 and BB7.2, respectively. Expression of NY-BR-1 was examined using reverse transcription-PCR (RT-PCR) with primers 5'-TCAAGAGCAGCATAGGAA-3' and 5'-CAGAACCTTTAGCTGGCACCAC-5' that amplify a 350-bp region of the NY-BR-1 cDNA. The PCR products were done in an iCycler (Bio-Rad, Hercules, CA) for 35 cycles of denaturation at 94°C for 15 seconds followed by annealing at 53.6°C for 30 seconds and extension at 68°C for 30 seconds. The magnesium concentration for the reaction was 1.5 mmol/L.

Primers 5'-GGAGGTTCGATGCGTACA-3' and 5'-CTGACTCTCCTGTGTCGA-5' that amplify a 470-bp region of β-actin were used as controls for amplification. The hybridoma cell line MB40.5 was obtained from ATCC, and the corresponding mAb (IgG1), which is specific for HLA-A, HLA-B, and HLA-C, was purified at the Biologics Facility of Fred Hutchinson Cancer Research Center (Seattle, WA) according to published protocols. EBV-transformed B-cell lines (B-LCL) were generated as described previously.

Dendritic cell and T-cell preparations. PBMCs were isolated from blood samples by Ficol-Hypaque density gradient centrifugation. Dendritic cells were derived from PBMC by culture of adherent monocytes for 5 days in AIM-V medium (Invitrogen, Carlsbad, CA) supplemented with 800 units/mL granulocyte macrophage colony-stimulating factor and 500 units/mL interleukin (IL)-4 and then matured for 24 to 48 hours with a cocktail of cytokines consisting of 10 ng/mL tumor necrosis factor-α, 10 ng/mL IL-6, 1,000 ng/mL prostaglandin E2, and 2 ng/mL IL-1β. Mature dendritic cells were pulsed for 4 hours in serum-free medium with synthetic peptides, each at the concentration of 20 μg/mL, washed, irradiated at 32 Gy, and used as stimulator cells. CD8+ T lymphocytes were harvested from PBMC using Miltenyi microbeads according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA) and cultured in a ratio of 10:1 with peptide-pulsed autologous dendritic cells in the presence of 10 ng/mL IL-2 and 5 ng/mL IL-12 for 8 to 10 days in RPMI 1640 supplemented with 25 mmol/L HEPS, 4 mmol/L L-glutamine, 25 mmol/L L-mercaptoethanol, and 10% heat-inactivated human serum. Cultures were restimulated every 8 to 10 days with peptide-pulsed dendritic cells under similar conditions, except IL-6 and IL-12 that were replaced by IL-7 (10 ng/mL) and IL-2 (10 units/mL) in the second stimulation cycle. Alternatively, CD8+ T cells were stimulated with peptide-pulsed autologous dendritic cells in 96-well plates in the presence of 10 ng/mL IL-12. IL-15 was added on day 7 at a final concentration of 10 ng/mL, and each well was assayed on day 12 for recognition of peptide-pulsed T2 cell in a standard 4-hour 51Cr release assay.

Tumor cell uptake and dendritic cell maturation. The breast tumor cell line MDA-453 (NY-BR-1) was first treated with 150 units/mL IFN-γ for 3 days to increase the surface expression of MHC class I. Pretreated MDA-453 cells (2 × 10^6) were then incubated with anti-class I mAb MB40.5 (10 μg/mL) for 30 minutes, washed twice with PBS, irradiated at 30 Gy to arrest cell growth, and cocultured with immature dendritic cells at 1:1 ratio for 18 to 24 hours. Dendritic cells were subsequently matured for 48 hours with the cytokine cocktail above and used as stimulators for autologous CD8+ T cells.

Enzyme-linked immunospot assay. The enzyme-linked immunospot (ELISPOT) assay was used to quantify antigen-specific IFN-producing effector cells as described previously (21). Briefly, nitrocellulose-bottomed 96-well plates (Multiscreen MAIP N45, Millipore, Bedford, MA) were coated with an anti-IFN mAb (clone 1-DIK, Mabtech, Stockholm, Sweden), and nonspecific binding was blocked using 0.5% bovine serum albumin in RPMI 1640. Effector cells were added with peptide-pulsed autologous PBMC at ET ratio of 5 and incubated overnight at 37°C. After two washes, biotinylated IFN mAb (clone 7-B6-1, Mabtech), the conjugate (avidin-peroxidase complex; Vectastain avidin-biotin complex method Elite kit; Vector Laboratories, Burlingame, CA), and substrate (Vectastain 3-amin-9-ethylcarbazole substrate) were used according to the manufacturer's instructions. Spot-forming cells were counted by using the ImmunoSpot (Cellular Technology Ltd., Cleveland, OH) optical reader and are expressed per 10^5 input cells. T cells stimulated with phytohemagglutinin (final concentration, 2 μg/mL; Sigma-Aldrich, St. Louis, MO) served as the positive control.

IFN-γ capture and T-cell cloning by limiting dilution. Detection and subsequent capture of IFN-γ-secreting cells by flow cytometric sorting were done using an IFN-γ secretion detection kit according to the manufacturer's instructions (Miltenyi Biotec). Effector cells were incubated with autologous PBMC pulsed with 10 μg/mL peptide at ET ratio of 5. After overnight incubation, a bispecific antibody was used to capture cells that secrete IFN-γ in response to peptide stimulation. These cells were subsequently labeled with a second IFN-γ-specific antibody conjugated to R-phycocerythrin for detection and sorting by fluorescence-activated cell sorting (FACS). The FACS-sorted cells were then plated in limiting dilution cultures in 96-well round-bottomed plates with anti-CD3 mAb (30 ng/mL), γ-irradiated PBMC (7.5 × 10^5 per well) and LCL (1 × 10^6 per well) as feeder cells, and IL-2 (50 units/mL). Wells with positive growth were tested for cytolytic activity in a standard 4-hour 51Cr release assay using peptide-pulsed target cells. Clones with specific lytic activity were further expanded and characterized.

Cytotoxicity assay. Target cells were labeled with 51Cr overnight or for 2 hours, washed, and then pulsed with peptides at the indicated concentration for 2 hours or added directly to 96-well plates in triplicate wells at 2,000 per well at various E/T ratios. After 4-hour incubation at 37°C, supernatants from each well were counted for 51Cr release and the percentage of specific lysis was determined according to the following formula: ([51Cr release sample − spontaneous 51Cr release]/ (maximum 51Cr release − spontaneous 51Cr release) × 100. In the cold target inhibition assay, unlabeled T2 cells were first pulsed for 2 hours with 10 μg/mL of indicated peptides, washed thrice, and then mixed with 51Cr-labeled HTB-21 at equal cell numbers per well.

cDNA transfection. A 1.4-kb COOH-terminal portion of NY-BR-1 was amplified from the human testis total RNA (Clontech, Palo Alto, CA) by RT-PCR using the primers 5'-GGCGCCACCATGTTTCTACAGAAG-GATGTTGTG-3' and 5'-GAGATGATGTTGCTGACGCT-5' corresponding to the published cDNA sequence of NY-BR1 from 2,626 to 3,927 and cloned into the TOPO vector (Invitrogen). The insert was sequenced to confirm that no mutations were introduced and subcloned into the pEAK10 expression vector (Edge Biosystems, Gaithersburg, MD) under the control of EF-1α promoter. The pEAK10-NY-BR-1 construct was transfected into HLA-A2- B-LCL by Nucleofection (Amaxa Biosystems, Cologne, Germany). Transfected cells were selected by culturing in 600 ng/mL puromycin beginning 24 hours after transfection and used as targets in cytotoxicity assays 3 days after selection.

Results

Isolation of CD8+ cytotoxic T-cell clones against multiple NY-BR-1 epitopes. We used two epitope prediction algorithms2 to scan the NY-BR-1 sequence for nonamer peptides predicted to bind efficiently to HLA-A*0201 (22, 23). Twelve peptides from NY-BR-1 with the highest predicted binding scores by both algorithms were synthesized and assayed for binding to HLA-A*0201. The peptides were pulsed with β2-microglobulin onto TAP-deficient T2 cells, and stabilization of HLA-A2 expression on the surface of T2 cells detected by staining with a FITC-labeled HLA-A2-specific mAb (BB7.2) was used as an index of binding (8). Incubation of T2 cells with each of the 12 peptides increased HLA-A2 expression,
Table 1. Binding of NY-BR-1-derived peptides to HLA-A2

<table>
<thead>
<tr>
<th>NY-BR-1 peptides</th>
<th>Bioinformatics and Molecular Analysis Section (BIMAS) score</th>
<th>SYFPEITHI score</th>
<th>Mean fluorescence (% increase)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p1274 NMVLLQQLVL</td>
<td>417</td>
<td>19</td>
<td>155</td>
</tr>
<tr>
<td>p35 FLVDRKQQL</td>
<td>403</td>
<td>22</td>
<td>60</td>
</tr>
<tr>
<td>p1043 YLLHENCML</td>
<td>364</td>
<td>24</td>
<td>265</td>
</tr>
<tr>
<td>P1293 KITIDHFL</td>
<td>130</td>
<td>23</td>
<td>54</td>
</tr>
<tr>
<td>p1269 QLQSKNMWL</td>
<td>117</td>
<td>21</td>
<td>48</td>
</tr>
<tr>
<td>p1262 SLQKLFQQL</td>
<td>114</td>
<td>27</td>
<td>424</td>
</tr>
<tr>
<td>p904 SLKILDTV</td>
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<td>370</td>
</tr>
<tr>
<td>p68 ILIDSGADI</td>
<td>73</td>
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<td>60</td>
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<tr>
<td>p94 EILSVVAKL</td>
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</tr>
<tr>
<td>p200 QIMETIRKL</td>
<td>20</td>
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</tr>
<tr>
<td>p1057 MKLEIATL</td>
<td>20</td>
<td>27</td>
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</tr>
<tr>
<td>p445 SIYQKMEI</td>
<td>11</td>
<td>26</td>
<td>113</td>
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</tbody>
</table>

NOTE: The entire protein sequence of NY-BR-1 was scanned using the epitope prediction algorithms (BIMAS, http://bimas.dkrnig.gov/molbio/hla_bind; SYFPEITHI, http://syfpeithi.bmi-heidelberg.com) to identify nonamer peptides predicted to bind HLA-A2. To assess peptide binding, 12 peptides with high scores by both algorithms were synthesized and pulsed with β2-microglobulin onto TAP-deficient T2 cells. HLA-A2 expression on control and peptide-pulsed T2 cells was determined by flow cytometry using mAb BB7.2. Results are expressed as percentage increase of mean fluorescence in the presence of peptide over empty T2 cells. A HLA-B8 peptide from UTY (LPHNHTDL) was used as a negative control (37).

although the degree of stabilization was variable and did not correlate with the predicted binding scores (Table 1).

To determine if CD8+ T cells specific for any of these putative NY-BR-1 epitopes could be elicited from the T-cell repertoire of normal female donors, we stimulated purified CD8+ T cells obtained from six HLA-A2+ donors with autologous dendritic cells pulsed with three separate pools of NY-BR-1 peptides. After two stimulations, the presence of CTL specific for individual NY-BR-1 peptides in the pool used for stimulation was examined by measuring IFN-γ secretion in an ELISPOT assay. Responses to 3 of the 12 NY-BR-1 peptides (designated p904, p1057, and p1293) were detected with frequencies of spot-forming cells ranging from 0.35% to 0.51% of the plated T cells in the ELISPOT assay (Fig. 1A). T-cell responses to these three NY-BR-1 peptides were detected in all six of the normal female donors tested.

CD8+ T cells specific for each of the three NY-BR-1 peptides were subsequently enriched from the polyclonal cultures using a bispecific antibody to capture cells that produce IFN-γ after peptide stimulation (24) and then cloned by limiting dilution. Cloning wells with evident T-cell growth were screened for lysis of T2 cells pulsed with the respective NY-BR-1 peptides, and a panel of 10 to 17 CD8+ CTL clones specific for each of the three NY-BR-1 peptides that exhibited the highest level of lysis in the screening assay was selected for further analysis. The avidity of the CTL clones was examined by measuring cytolytic activity against B-LCL or T2 cells pulsed with varying concentrations of peptide. The peptide concentration required for half-maximal lysis of B-LCL for most clones was between 1 and 10 ng/mL for all three epitopes (Fig. 1B), which is comparable with that reported for T-cell clones specific for viral antigens and at least one log lower than that observed with T-cell clones specific for other candidate breast tumor antigens, such as HER-2/neu, survivin, and mdm-2 (15). These data show that highly avid T cells specific for NY-BR-1 peptides are not deleted from the repertoire and can be detected and isolated from normal female donors after in vitro stimulation.

Recognition of B-LCL transfected with NY-BR-1 and breast tumor cells expressing NY-BR-1 by the CTL clones. One potential mechanism for the absence of tolerance to these three NY-BR-1 epitopes is a failure of the peptides to be generated by natural processing of the NY-BR-1 protein. To determine whether the epitopes were processed and presented from endogenously expressed NY-BR-1, we cloned a portion of the NY-BR-1 gene that encodes the COOH terminal, one third of the NY-BR-1 protein, including all three of the T-cell epitopes into the pEAK10 vector under the control of EF-1α promoter. The pEAK10-NY-BR-1 construct was then transfected into a HLA-A2+ B-LCL line, and the
transfectants were selected by incubation with puromycin for 72 hours. The transfected B-LCL expressed NY-BR-1 transcripts by RT-PCR and was recognized by CD8⁺ CTL clones specific for p904 but not for p1057 or p1293 (Fig. 2A). These results suggested that intracellular processing of endogenous NY-BR-1 protein efficiently generated only the p904 epitope.

We next examined the presentation of NY-BR-1 to CD8⁺ CTL by a panel of breast cancer cell lines obtained from ATCC. The HTB-21 cell line expressed NY-BR-1 by RT-PCR and was also positive for HLA-A2. Similar to the findings with the NY-BR-1-transfected B-LCL, T-cell clones specific for p904 but not for p1057 or p1293 lysed HTB-21 cells (Fig. 2B). CTL recognition was augmented by pre-treatment of HTB-21 with 150 units/mL IFN-γ for 48 to 72 hours, which up-regulated class I MHC and adhesion molecules (data not shown). MCF-7 tumor cells, which are HLA-A2⁺ but NY-BR-1⁻, were used as controls in these experiments and were not lysed by the p904-specific CTL clones. HTB-21 could be readily lysed by T-cell clones specific for p1057 or p1293 if the tumor cells were pulsed exogenously with the respective peptide, suggesting that the inability of these CTL to kill untreated HTB-21 was due to absence of the naturally processed epitope on the cell surface (data not shown). The recognition of HTB-21 by p904-specific CTL was blocked in a cold target inhibition assay by unlabeled T2 cells pulsed with the p904 peptide but not the p1293 peptide and by a mAb against HLA-A2 (BB7.2; Fig. 2C). A second tumor cell line, MDA-453, which is NY-BR-1⁻ but HLA-A2⁺, was not lysed by T-cell clones specific for any of the three NY-BR-1 peptides. However, after MDA-453 was transfected with a cDNA construct encoding HLA-A*0201, it was lysed by p904-specific CTL but not p1057 or p1293-specific CTL (Fig. 2D). These data show that HLA-A*0201⁺, NY-BR-1⁺ breast tumor cells endogenously process and present the p904 epitope and can be efficiently lysed by NY-BR-1-specific T cells.

**Cross-presentation of NY-BR-1 by dendritic cells.** Dendritic cells are highly specialized professional antigen-presenting cells and are pivotal for initiating cytotoxic T-cell responses. To determine whether p1057 and p1293 epitopes that were not processed by breast tumor cells could be processed and presented by dendritic cells, we used tumor-loaded dendritic cells to stimulate CD8⁺ T cells. We and others have recently shown that dendritic cells take up tumor cells opsonized with antibodies that bind to tumor-associated cell surface molecules via Fcγ receptor-mediated endocytosis and efficiently cross-present tumor epitopes for CTL activation (25, 26). MB40.5, a pan-class I mAb of IgG1 isotype, was used to coat the breast tumor cell line MDA-453 (NY-BR-1⁻ and HLA-A2⁻). MDA-453 cells were pretreated with IFN-γ to enhance surface expression of MHC class I molecules, incubated with the MB40.5 antibody, cocultured with dendritic cells, and used to stimulate autologous CD8⁺ T cells. After two stimulation cycles, the presence of NY-BR-1-specific CD8⁺ T cells in the cultures was determined nonamer peptides (Fig. 3). CD8⁺ T cells specific for the p904 peptide were readily detectable, but T cells to other NY-BR-1 epitopes, including p1057 and p1293, were not detected. The p904-reactive T cells derived by this method of stimulation were subsequently cloned after selection with a HLA-A2/p904 tetramer and recognized NY-BR-1-expressing target cells in a standard cytotoxicity assay (data not shown). These results show that dendritic cells can cross-present NY-BR-1 from breast tumor cells and provide further evidence that p904, but not p1057 or p1293, is naturally processed.

**Lack of recognition of the NY-BR-1.1 homologue by p904-specific CTL.** NY-BR-1.1 is expressed in brain tissues, testes, and normal breast and has 54% homology to the putative NY-BR-1 protein sequence (19). The predicted amino acid sequence of NY-BR-1.1 contains peptides TSLKILVAL and MLKLEVATL that are homologous to p904 (SLSKILDTV) and p1057 (MLKLEIATL) of NY-BR-1. Thus, we sought to determine whether the corresponding peptides from NY-BR-1.1 were recognized by the CTL clones.
generated in response to the NY-BR-1 peptides. The p904-1.1 peptide TLKILDAL, which has substitutions of T for S at position 1, A for T at position 8, and L for V at position 9, was not recognized by two different CTL clones specific for p904 when pulsed on B-LCL at concentrations up to 10^6 ng/mL (Fig. 4A). T2 cells pulsed with the p904-1.1 peptide were recognized poorly by p904-specific CTL, and the peptide concentration required for recognition was >10,000-fold higher than that of the p904 peptide (Fig. 4B). By contrast, p1057-1.1, which has only one substitution of V for I at position 6, was recognized by CTL clones specific for p1057 of NY-BR-1 with comparable avidity (Fig. 4A). There is no corresponding peptide in NY-BR-1.1 for p1293 of NY-BR-1. These data show that CTL specific for p904, the naturally processed NY-BR-1 epitope presented by HLA-A2, do not cross-react with the homologous NY-BR-1.1 sequence.

**CTL specific for NY-BR-1 can be isolated from breast cancer patients.** Patients with advanced breast cancer are often heavily treated with immunosuppressive chemotherapy. To determine whether NY-BR-1-specific T cells can be recovered from these patients, we obtained lymphocytes from HLA-A2+ patients with stage II to IV breast cancer. CD8+ T cells were enriched from PBMC of two patients who had received 6 months of dose-dense chemotherapy and reached a clinical complete remission. At the time the blood was obtained for analysis, the patients had been maintained on hormonal therapy alone for over a year. Autologous dendritic cells were pulsed with the p904 peptide and used to stimulate CD8+ T cells in vitro. After two stimulation cycles, T cells specific for p904 were easily detected by ELISPOT assay in both patients (Fig. 5A). There is no corresponding peptide in NY-BR-1.1 for p1293 of NY-BR-1. These data show that CTL specific for p904, the naturally processed NY-BR-1 epitope presented by HLA-A2, do not cross-react with the homologous NY-BR-1.1 sequence.

**Discussion**

A goal of T-cell-based immunotherapy for solid tumors is to engineer by vaccination or adoptive T-cell transfer, a high-avidity T-cell response that is specific for target antigens expressed by tumor cells (27). Adoptive T-cell therapy targeting melanocyte differentiation antigens, which are expressed in the majority of melanoma cells but only in a limited number of normal tissues, has been successful in a subset of patients (3, 4). However, this approach has not been extended to tumors, such as breast cancer, in part because few TAAs that are recognized by CD8+ T cells have been identified. Here, we provide the first demonstration that CD8+ T cells specific for NY-BR-1 can be isolated from breast cancer patients.
cytotoxic T cells, specific for the breast differentiation antigen NY-BR-1, can be isolated from both normal women and breast cancer patients and lyse breast tumor cells in vitro.

Expression cloning and peptide elution have been used extensively to identify tumor antigens recognized by tumor-specific T cells isolated from the blood or from T cells infiltrating the tumor site (28). In the absence of MHC-restricted tumor-reactive T-cell clones that can be used to screen expression libraries or peptide eluates, "reverse immunology," in which peptides are selected from candidate tumor-associated proteins based on MHC binding or other properties, provides an alternative approach to antigen discovery. NY-BR-1 is an attractive candidate antigen because it is expressed in the majority of breast tumors but expressed at low or undetectable levels in normal breast and nonbreast tissues (19, 20).

To identify potential antigenic epitopes in NY-BR-1, we used computer algorithms to select NY-BR-1 peptides that were predicted to bind to HLA-A*0201 and screened these peptides for the ability to activate CD8+ T cells in vitro. This approach has limitations because it only takes into account HLA binding and does not consider other requirements, including proteosomal digestion and peptide transport into the endoplasmic reticulum. Thus, T cells, elicited as a result of priming with exogenous peptide, may not recognize tumor cells expressing the cognate protein due to inefficient production of HLA/peptide complexes by the processing of the endogenous protein or due to low avidity of the T-cell receptor (reviewed in ref. 29). In our study, CD8+ T-cell clones specific for 3 of the 12 peptides were isolated reproducibly but only one peptide (p904) was efficiently processed and presented by breast tumor cells, NY-BR-1-transfected cell lines, and tumor-loaded dendritic cells. Although individual p904-specific CTL clones differed in avidity for peptide-pulsed autologous LCL by as much as two logs (Fig. 5D), all the p904-specific CTL clones were able to recognize NY-BR-1+ breast tumor cell lines. The failure of the T-cell clones specific for p1057 and p1293 to recognize endogenously processed NY-BR-1 did not seem to reflect differences in the avidity of the T-cell clones because they recognized target cells pulsed with equivalently low concentrations of peptide as p904-specific CTL (Fig. 1B). The computer program PAPoReC identifies multiple potential proteosome cleavage sites within both p1057 and p1293, suggesting that these epitopes may be destroyed during processing. However, our data do not exclude the possibility that these peptides might be presented at low copy number on tumor cells and could be recognized by T-cell clones of higher avidity than those generated in this study.

We initially analyzed T-cell responses to NY-BR-1 in healthy female donors because the repertoire in these individuals would only be influenced by normal mechanisms of tolerance. The isolation of T cells specific for TAAs from tumor-bearing patients is often more difficult than from normal donors due to a global reduction in the T-cell repertoire as a consequence of cytotoxic chemotherapy (30), the selective loss of high-avidity T cells as a consequence of tumor growth (31), and/or the expansion of regulatory cells in cancer patients that may diminish the frequency or reactivity of tumor-specific T cells (17). Our studies show that CD8+ T cells specific for the NY-BR-1 p904 epitope can be detected and isolated from three of

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**Figure 5.** NY-BR-1-specific CTL response in breast cancer patients. **A,** CD8+ T cells from two HLA-A2+ breast cancer patients (BC-1 and BC-4) were stimulated with autologous dendritic cells pulsed with the p904 peptide. IFN-γ-secreting cells were quantitated after two stimulations in an ELISPOT assay using autologous PBMC pulsed with either p904 peptide (filled columns) or an irrelevant peptide (open columns). Columns, mean of triplicate wells; bars, SD. **B,** CD8+ T cells were cloned under limiting dilution conditions after one stimulation in 96-well plates with p904-pulsed autologous dendritic cells. Clones positive for recognition of peptide-pulsed T2 target were selected and expanded. **C,** recognition of T2 cells pulsed with p904 (10 μg/mL) and breast tumor cell HTB-21 (NY-BR-1+) but not MCF-7 (NY-BR-1-) by clone 3A2 isolated from a breast cancer patient. **D,** lysis of autologous LCL pulsed with indicated amount of the p904 peptide by CTL clones from breast cancer patient (filled symbols) and healthy female donors (open symbols) at E:T ratio of 10.
four breast cancer patients, although the responses were weaker than those from normal female donors. Due to the lack of a mAb, it is unknown whether NY-BR-1 is overexpressed in the tumors of these patients. It remains possible that tumor expression of NY-BR-1 may limit the T-cell repertoire to NY-BR-1 due to tolerance (32) or conversely result in expansion of NY-BR-1-specific T cells as a consequence of in vivo priming (33). Efforts to generate an NY-BR-1-specific antibody are in progress to facilitate identification of patients with NY-BR-1+ tumors and define the effect of antigen expression on the ability to elicit NY-BR-1-specific T cells.

The expression of a gene (NY-BR-1.1) in the brain that is highly homologous to NY-BR-1 could pose a potential problem for targeting NY-BR-1 by vaccination or adoptive T-cell therapy. Our studies show the NY-BR-1.1 peptide corresponding to the naturally processed p904 NY-BR-1 epitope is recognized poorly by p904-specific CTL and only at a peptide concentration that is 10,000-fold higher than the concentration of p904 needed for sensitizing target cells. The more than 10,000-fold difference in T-cell avidity for the two peptides is most likely due to differences in T-cell receptor contact because the MHC anchor residues at positions 2 and 9 are conserved. Similar observations have been made for the minor histocompatibility antigen SMCY, where peptide binding to the MHC molecule is comparable with the homologous SMCX peptide, but the avidity of SMCY-specific CD8 T cells for SMCY and SMCX differs by 10,000-fold (34). The inability of p904-specific CTL to recognize the NY-BR-1.1 homologue peptide at physiologic concentration and the restricted access of lymphocytes to the central nervous system (CNS) in the absence of a disrupted blood-brain barrier (35) suggest that immunotherapy targeting NY-BR-1 is unlikely to cause CNS toxicity.

NY-BR-1 is a large protein and is likely to encode additional T-cell epitopes that are sufficiently distinct from those of NY-BR-1 and could also provide selective targeting of breast tumors. However, screening all potential epitopes using the reverse immunology approach used in this study is inefficient. Our work also shows that dendritic cells loaded with oponized tumor cells elicit tumor-reactive T cells in vitro, including p904-specific T cells (Fig. 3; ref. 26). We are currently using tumor-loaded dendritic cells to generate T cells that can then be screened against a panel of overlapping peptides derived from NY-BR-1. This approach may be more efficient for defining immunologically relevant epitopes, including those presented by class I HLA alleles other than HLA-A2. The results of these studies will provide the foundation to target NY-BR-1 in breast cancer patients by vaccination or T-cell therapy.

Note Added in Proof:

After initial submission of our manuscript, a report was submitted by Jager et al. and published in Cancer Immunology describing the p904 epitope as a naturally processed and presented peptide and recognized by a CTL clone isolated from a breast cancer patient (36).

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Recognition of Breast Cancer Cells by CD8+ Cytotoxic T-Cell Clones Specific for NY-BR-1

Wei Wang, Jennifer Epler, Lupe G. Salazar, et al.


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