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Article

Humoral and cellular immune responses against the breast cancer antigen NY-BR-1: Definition of two HLA-A2 restricted peptide epitopes

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Cancer immunotherapy depends on the identification of tumor-specific target antigens that are predominantly expressed in cancer cells and not in normal tissues. Here, we report the cloning and the expression analysis of the differentiation antigen NY-BR-1 that we have identified in a previous SEREX (serological analysis of recombinant cDNA expression libraries) screening. The cloning of the full length NY-BR-1 sequence led to the prediction of an open reading frame of 4.2 kb, encoding a protein of 158.9 kDa. NY-BR-1 mRNA expression analysis revealed tissue-specific expression in normal testis and breast tissues, as well as in 70% of breast tumors. We now show that NY-BR-1 is also sporadically expressed in normal prostate and in 32% of prostate tumors. Furthermore, we were able to identify two HLA-A2 restricted NY-BR-1 epitopes (p158-167 and p960-968) that are recognized by CD8+ T cell clones (NW1100-CTL-7 and NW1100-CTL-43, respectively), as determined by ELISPOT analysis and tetramer staining. Cotransfection assays of COS-7 cells also demonstrated that these two peptides are naturally processed and presented on HLA-A2 molecules. The identification of these two naturally processed NY-BR-1-specific CD8+ T cell epitopes opens the perspective for active immunotherapy of HLA-A2 positive patients with NY-BR-1 expressing tumors.

Introduction

Antigen-specific cancer immunotherapy approaches rely on the identification of antigens expressed by cancer cells that can be used as target structures for the interaction with the immune system. Most immunotherapy approaches focus on the induction of a cellular immune response (CD8+, CD4+ T cells) against tumor antigens. Ideally, activated antigen-specific CD8+ T cells mediate the regression of antigen-positive tumors in vivo. To prevent autoimmune reactions that potentially harm normal tissues, the expression of target antigens for immunotherapy should ideally be tumor-restricted. Different antigen cloning techniques were successful in identifying tumor antigens that can be used for active and passive immunotherapy in cancer.

The T cell epitope cloning technique introduced by Boon and colleagues (1) made it possible to identify and clone tumor antigens based on their recognition by cytotoxic T cells. The first antigen cloned from the melanoma system MZ-2 by this technique was the MAGE-1 antigen. Subsequently, other tumor antigens like the *BAGE* and *GAGE* gene family have been identified following the same cloning strategy (2, 3). The expression pattern of these antigens is restricted to normal germ cells and various tumor tissues. Therefore, antigens from this category were designated as "cancer-testis" (CT) antigens (4, 5). A second group of antigens representing potential target molecules for immunotherapy includes differentiation antigens which are expressed in differentiated normal tissue, i.e. melanocytes, and the malignant counterpart, i.e. melanoma. By using the same T cell epitope cloning technique, Melan-A (6), tyrosinase (7), gp100 (8), and gp75 (9) were identified. Tumor-restricted mutations of normal genes (*CDK4, p53*) and viral gene products expressed in different cancers were also identified as targets for CD8+ T cells (10, 11, 12, 13).

The T cell epitope cloning technique is dependent on cultured tumor and T cell systems. Since most epithelial cancers are difficult to grow in culture, a new antigen cloning technology, SEREX, was developed on the basis of humoral immune responses against tumor antigens expressed by recombinant cDNA libraries (14). SEREX analyses of different tumor types has led to the identification of a number of CT antigens, i.e. *HOM-MEL-40*, a gene identical to the synovial sarcoma/X breakpoint 2 gene (*SSX2*) involved in the t(X;18) translocation in synovial sarcoma (15), as well as other CT antigens, i.e. NY-ESO-1 (16), CT7/MAGE-C1 (17), SCP-1 (18), cTAGE-1 (19), OY-TES-1 (20), HOM-TES-85 (21), CAGE (22), and most recently NY-SAR-35 (23).

Autologous SEREX screening of a breast cancer library recently identified the breast cancer antigen NY-BR-1 (24). NY-BR-1 is a breast differentiation antigen expressed in normal breast and normal testis, and in about 70% of breast cancers, but not in other tumor types. Humoral immune responses against NY-BR-1 have been documented in several breast cancer patients but not in healthy individuals. In this study, we extend the analysis of NY-BR-1 expression and demonstrate that NY-BR-1 is also expressed in approximately 32% of prostate cancers. To analyze

cellular immune responses against NY-BR-1, we screened HLA-A2+ patients with NY-BR-1 positive breast and prostate cancers for spontaneous, NY-BR-1-specific CD8+ T cell responses. We identified two HLA-A2 restricted peptides that are recognized by CD8+ T cells. We further demonstrated that these two peptides represent naturally processed NY-BR-1 epitopes. Based on these results, these peptides are now being considered for active immunotherapy of HLA-A2 positive patients with NY-BR-1 expressing cancers.

Results

Analysis of NY-BR-1 expression

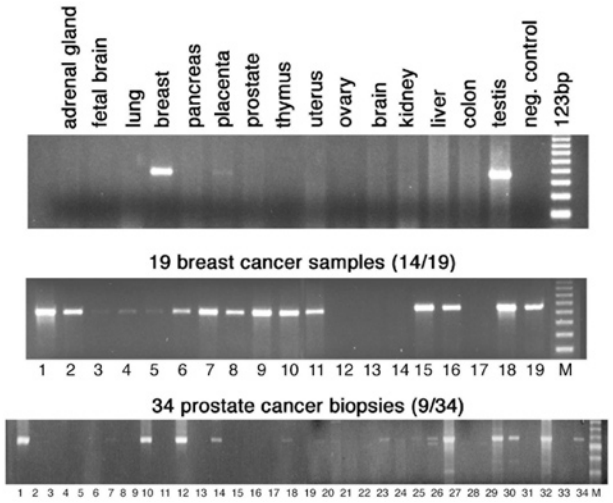
In addition to data reported previously (24), we extended the analysis for NY-BR-1 mRNA expression to a larger panel of normal and tumor tissues of different histological types (breast cancer, prostate cancer, urothelial cancer, colon cancer, melanoma, non-small cell lung cancer, squamous cell carcinoma, renal cancer, ovarian cancer, and sarcoma). NY-BR-1 mRNA was expressed in normal breast, normal testis, and inconsistently in normal prostate. All other normal tissues were NY-BR-1 mRNA negative by RT-PCR (Figure 1). In tumor tissues, NY-BR-1 mRNA expression was detected in 70% of breast cancers and 32% of prostate cancers, while all other tumors types were NY-BR-1 mRNA negative.

To evaluate differences in NY-BR-1 expression levels between normal and malignant tissues, we performed quantitative RT-PCR analysis using NY-BR-1-specific primer pairs and probe. Figure 2 shows the quantitative NY-BR-1 mRNA expression relative to 18S rRNA. In normal tissues, the highest NY-BR-1 mRNA levels were detected in normal testis, whereas normal breast and normal prostate tissues showed similar but significantly weaker expression compared to testis. In tumor tissues, NY-BR-1 mRNA expression was variable, with the highest expression being detected in a breast cancer tissue.

Cloning of the full length NY-BR-1 cDNA sequence

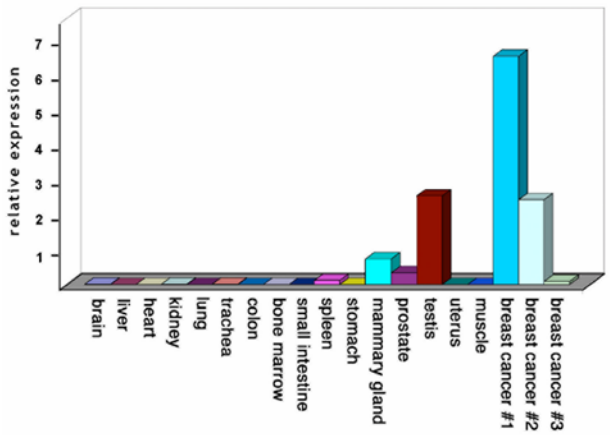
Using a combined approach of serological expression cloning, RACE-PCR, database analysis and nucleotide screening of a testicular library, an NY-BR-1 cDNA sequence of 4458 bp with a 3' untranslated region of 333 bp and a putative 5' untranslated region of 99 bp was defined (GenBank Accession No. AF269087) (24). No stop codon was found in the first 99 bp of the NY-BR-1 cDNA sequence, suggesting the possibility of additional 5' coding sequence. Comparison of the available NY-BR-1 cDNA sequence to a genomic sequence deposited in the database (GenBank Accession No. AC067744) extended the NY-BR-1 cDNA sequence in its 5' region, creating a new translation

Figure 1



NY-BR-1 mRNA expression. A panel of normal tissues (top), breast cancer samples (middle), and prostate cancer specimens (bottom) were assayed for NY-BR-1 expression by RT-PCR.

Figure 2



NY-BR-1 mRNA is overexpressed in some breast cancers relative to normal breast and testis. NY-BR-1 mRNA expression was determined by quantitative RT-PCR. Normal prostate is weakly NY-BR-1 mRNA positive.

Figure 3

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ATGGAGGAGATCTCTGCCGCCGCTGTCAAGGTCGTGCCGGGCCCGGAGCGCCCGAGCCCTTTCAGCCAGCTAGTCTATAC   80
CAGCAACGACTCCTACATCGTCCACTCTGGGGATCTTAGAAAGATCCATAAAGCTGCCTCCCGGGGACAAGTCCGGAAGC  160
TGGAGAAGATGACAAAGAGGAAGAAGACCATCAACCTTAATATACAAGACGCCCAGAAGAGGACTGCTCTACACTGGGCC  240
TGTGTCAATGGCCATGAGGAAGTAGTAACATTTCTGGTAGACAGAAAGTGCCAGCTTGACGTCCTTGATGGCGAACACAG  320
GACACCTCTGATGAAGGCTCTACAATGCCATCAGGAGGCTTGTGCAAATATTCTGATAGATTCTGGTGCCGATATAAATC  400
TCGTAGATGTGTATGGCAACATGGCTCTCCATTATGCTGTTTATAGTGAGATTTTGTCAGTGGTGGCAAAACTGCTGTCC  480
CATGGTGCAGTCATCGAAGTGCACAACAAGGCTAGCCTCACACCACTTTTACTATCCATAACGAAAAGAAGTGAGCAAAT  560
TGTGGAATTTTTGCTGATAAAAAATGCAAATGCGAATGCAGTTAATAAGTATAAATGCACAGCCCTCATGCTTGCTGTAT  640
GTCATGGATCATCAGAGATAGTTGGCATGCTTCTTCAGCAAAATGTTGACGTCTTTGCTGCAGATATATGTGGAGTAACT  720
GCAGAACATTATGCTGTTACTTGTGGATTTCATCACATTCATGAACAAATTATGGAATATATACGAAAATTATCTAAAAA  800
TCATCAAAATACCAATCCAGAAGGAACATCTGCAGGAACACCTGATGAGGCTGCACCCTTGGCGGAAAGAACACCTGACA  880
CAGCTGAAAGCTTGGTGGAAAAAACACCTGATGAGGCTGCACCCTTGGTGGAAAGAACACCTGACACGGCTGAAAGCTTG  960
GTGGAAAAAACACCTGATGAGGCTGCATCCTTGGTGGAGGGAACATCTGACAAAATTCAATGTTTGGAGAAAGCGACATC 1040
TGGAAAGTTCGAACAGTCAGCAGAAGAAACACCTAGGGAAATTACGAGTCCTGCAAAAGAAACATCTGAGAAATTTACGT 1120
GGCCAGCAAAAGGAAGACCTAGGAAGATCGCATGGGAGAAAAAAGAAGACACACCTAGGGAAATTATGAGTCCCGCAAAA 1200
GAAACATCTGAGAAATTTACGTGGGCAGCAAAAGGAAGACCTAGGAAGATCGCATGGGAGAAAAAAGAAACACCTGTAAA 1280
GACTGGATGCGTGGCAAGAGTAACATCTAATAAAACTAAAGTTTTGGAAAAAGGAAGATCTAAGATGATTGCATGTCCTA 1360
CAAAAGAATCATCTACAAAGCAAGTGCCAATGATCAGAGGTTCCCATCAGAATCCAAACAGAGAGAAGATGAAGAATAT 1440
TCTTGTGATCTCCGGAGTCTCTTGAGAGTTCTGCAAAGATTCAAGTGTGTATACCTGAGTCTATATATCAAAAAGTAAT 1520
GGAGATAAATAGAGAAGTAGAAGAGCCTCCTAAGAAGCCATCTGCCTTCAAGCCTGCCATTGAAATGCAAAACTCTGTTC 1600
CAAATAAAGCCTTTGAATTGAAGAATGAACAAACATTGAGAGCAGATCCGATGTTCCCACCAGAATCCAAACAAAAGGAC 1680
TATGAAGAAAATTCTTGGGATTCTGAGAGTCTCTGTGAGACTGTTTCACAGAAGGATGTGTGTTTACCCAAGGCTACACA 1760
TCAAAAAGAAATAGATAAAATAAATGGAAAATTAGAAGAGTCTCCTAATAAAGATGGTCTTCTGAAGGCTACCTGCGGAA 1840
TGAAAGTTTCTATTCCAACTAAAGCCTTAGAATTGAAGGACATGCAAACTTTCAAAGCGGAGCCTCCGGGGAAGCCATCT 1920
GCCTTCGAGCCTGCCACTGAAATGCAAAAGTCTGTCCCAAATAAAGCCTTGGAATTGAAAAATGAACAAACATGGAGAGC 2000
AGATGAGATACTCCCATCAGAATCCAAACAAAAGGACTATGAAGAAAATTCTTGGGATACTGAGAGTCTCTGTGAGACTG 2080
TTTCACAGAAGGATGTGTGTTTACCCAAGGCTGCGCATCAAAAAGAAATAGATAAAATAAATGGAAAATTAGAAGGGTCT 2160
CCTGTTAAAGATGGTCTTCTGAAGGCTAACTGCGGAATGAAAGTTTCTATTCCAACTAAAGCCTTAGAATTGATGGACAT 2240
GCAAACTTTCAAAGCAGAGCCTCCCGAGAAGCCATCTGCCTTCGAGCCTGCCATTGAAATGCAAAAGTCTGTTCCAAATA 2320
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AGAAATAGATAAAATAAATGGAAAATTAGAAGAGTCTCCTGATAATGATGGTTTTCTGAAGGCTCCCTGCAGAATGAAAG 2560
TTTCTATTCCAACTAAAGCCTTAGAATTGATGGACATGCAAACTTTCAAAGCAGAGCCTCCCGAGAAGCCATCTGCCTTC 2640
GAGCCTGCCATTGAAATGCAAAAGTCTGTTCCAAATAAAGCCTTGGAATTGAAGAATGAACAAACATTGAGAGCAGATCA 2720
GATGTTCCCTTCAGAATCAAAACAAAAGAAGGTTGAAGAAAATTCTTGGGATTCTGAGAGTCTCCGTGAGACTGTTTCAC 2800
AGAAGGATGTGTGTGTACCCAAGGCTACACATCAAAAAGAAATGGATAAAATAAGTGGAAAATTAGAAGATTCAACTAGC 2880
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AAAAATGGAACAAATGAAAAAGAAGTTTTGTGTACTGAAAAAGAAACTGTCAGAAGCAAAAGAAATAAAATCACAGTTAG 3040
AGAACCAAAAAGTTAAATGGGAACAAGAGCTCTGCAGTGTGAGATTGACTTTAAACCAAGAAGAAGAGAAGAAGAAAAT 3120
GCCGATATATTAAATGAAAAATTAGGGAAGAATTAGCAGAAGACAGCATAGAAAAGAGTTAGAAGTGAACTA 3200
ACAACTTGAACAGGCTCTCAGAATACAAGATATAGAATTGAAGATGTGTAGAAAGTAATTTGAATCAGGTTTCTCACACTC 3280
ATGAAAATGAAAATTATCTCTTACATGAAAATTGCATGTTGAAAAAGGAAATTGCCATGCTAAAACTGGAAATAGCCACA 3360
CTGAAACACCAATACCAGGAAAAGGAAAATAAATACTTTGAGGACATTAAGATTTTAAAAGAAAAGAATGCTGAACTTCA 3440
GATGACCCTAAAACTGAAAGAGGAATCATTAACTAAAAGGGCATCTCAATATAGTGGGCAGCTTAAAGTTCTGATAGCTG 3520
AGAACACAATGCTCACTTCTAAATTGAAGGAAAAACAAGACAAAGAAATACTAGAGGCAGAAATTGAATCACACCATCCT 3600
AGACTGGCTTCTGCTGTACAAGACCATGATCAAATTGTGACATCAAGAAAAAGTCAAGAACCTGCTTTCCACATTGCAGG 3680
AGATGCTTGTTTGCAAAGAAAAATGAATGTTGATGTGAGTAGTACGATATATAACAATGAGGTGCTCCATCAACCACTTT 3760
CTGAAGCTCAAAGGAAATCCAAAAGCCTAAAAATTAATCTCAATTATGCAGGAGATGCTCTAAGAGAAAATACATTGGTT 3840
TCAGAACATGCACAAAGAGACCAACGTGAAACACAGTGTCAAATGAAGGAAGCTGAACACATGTATCAAAACGAACAAGA 3920
TAATGTGAACAAACACACTGAACAGCAGGAGTCTCTAGATCAGAAATTATTTCAACTACAAAGCAAAAATATGTGGCTTC 4000
AACAGCAATTAGTTCATGCACATAAGAAAGCTGACAACAAAAGCAAGATAACAATTGATATTCATTTTCTTGAGAGGAAA 4080
ATGCAACATCATCTCCTAAAAGAGAAAAATGAGGAGATATTTAATTACAATAACCATTTAAAAAACCGTATATATCAATA 4160
TGAAAAAGAGAAAGCAGAAACAGAAAACTCATGA                                                4194
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Complete cDNA coding sequence of NY-BR-1.

initiation site 168 bp upstream to the previously predicted ATG initiation codon. To confirm that the additional NY-BR-1 cDNA is being transcribed, we designed intron spanning RT-PCR primer pairs with the forward primer starting at the predicted translation initiation site, which resulted in an RT-PCR product of the calculated size. Screening of a testis library using NY-BR-1 cDNA as a probe identified an NY-BR-1 clone that was incomplete in its 5' sequence, but extended the available 3' sequence in the 3' untranslated region. The complete NY-BR-1 cDNA sequence (Figure 3) would encode for a protein of 158.9 kDa.

Recognition of NY-BR-1 peptides by CD8+ T cells

Thirty-two potential HLA-A2 binding peptides derived from the NY-BR-1 protein sequence (Table 1) were synthesized and tested on T2 target cells for specific recognition by CD8+ T cells from patient NW1100 following presensitization of effector cells with the respective peptide. Of the 32 peptides tested, peptides p158-167 and p960-968 were recognized by CD8+ T cells from patient NW1100 after 6 days of presensitization. T cell clones NW1100-CTL-7 and NW1100-CTL-43 were obtained after repetitive stimulation with the p158-167 and p960-968 peptides respectively and displayed the same specificity (Figure 4).

COS cell transfection assays

To evaluate if NY-BR-1 p158-167 and p960-968 represent naturally processed and presented epitopes, COS-7 cells were cotransfected with the respective NY-BR-1 and HLA-A2 pcDNA3.1(-) constructs as described above. NW1100-CTL-7 and NW1100-CTL-43 were generated by repetitive stimulation with NY-BR-1 p158-167 and p960-968 respectively. COS-7 cells cotransfected with NY-BR-1 cDNA encoding p158-167 and HLA-A2 were able to stimulate the production of TNF-alpha by NW1100-CTL-7 cells. COS-7 cells cotransfected with NY-BR-1 cDNA encoding p960-968 and HLA-A2 stimulated the production of TNF-alpha by NW1100-CTL-43. COS-7 cells alone, COS-7 cells transfected with NY-BR-1 alone or HLA-A2 alone did not stimulate TNF-alpha production (Figure 5).

Tetramer analysis

The specificity of NW1100-CTL-7 and NW1100-CTL-43 for NY-BR-1 p158-167 and p960-968 respectively was confirmed by tetramer analysis. Homogenous staining of both NY-BR-1-specific CD8+ T cell clones supported the data obtained by ELISPOT analysis (data not shown).

Table 1

Potential HLA-A2 binding peptides derived from the NY-BR-1 sequence

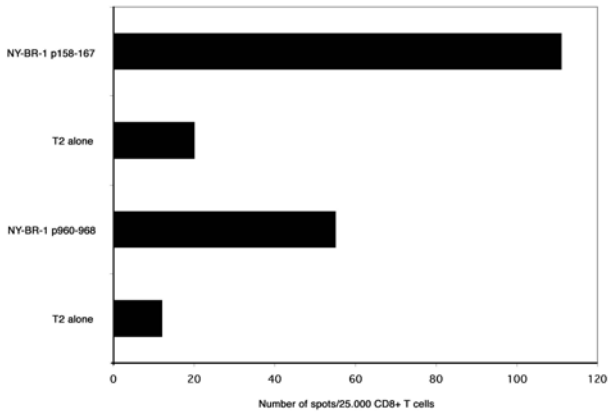
Sequence	Designation
FLVDRKCQLDVL	p91-102
ILIDSGADI	p124-132
AVYSEILSV	p146-154
ILSVVAKLL	p151-159
KLLSHGAVI	p157-165
LLSHGAVIEV	p158-167
FLLIKNANA	p190-198
MLLQQNVDV	p223-231
LLQQNVDVFA	p224-233
IAWEKKETPV	p417-426
SLFESSAKI	p486-494
CIPESIYQKV	p497-506
KVMEINREV	p505-513
ELMDMQTFKA	p743-752
SLSKILDTV	p960-968
KILDTVHSC	p963-971
ILNEKIREEL	p1043-1052
RIQDIELKSV	p1074-1083
YLLHENCML	p1099-1107
CMLKKEIAML	p1105-1114
AMLKLEIATL	p1112-1121
KILKEKNAEL	p1137-1146
VLIAENTML	p1170-1178
CLQRKMNVDV	p1230-1239
KMNVDVSST	p1234-1242
SLDQKLFQL	p1318-1326
KLFQLQSKNM	p1322-1331
QLQSKNMWL	p1325-1333
NMWLQQQLV	p1330-1338
WLQQQLVHA	p1332-1340
KITIDIHFL	p1349-1357
YQYEKEKAET	p1385-1394

Discussion

The analysis of humoral and cellular immune responses against breast cancer antigens has a long history, from evidence for immune responses against the murine mammary tumor virus (25), delayed type hypersensitivity and humoral immunity against T/Tn antigens (26) to more recent findings of humoral and cellular responses to p53 (11), HER2/neu (27) and NY-ESO-1 (16, 28, 29). The development of the SEREX technique opened new perspectives for the dissection of the spectrum of humoral immune responses against breast cancer antigens. It has been shown before that tumor antigens can elicit integrated immune responses involving simultaneous humoral and cellular immune responses (28, 30). Thus, the SEREX technique represents an excellent method for the identification of tumor antigens that can be recognized by CD4+ and CD8+ T cells and are therefore candidate target antigens for immunotherapy approaches in breast cancer.

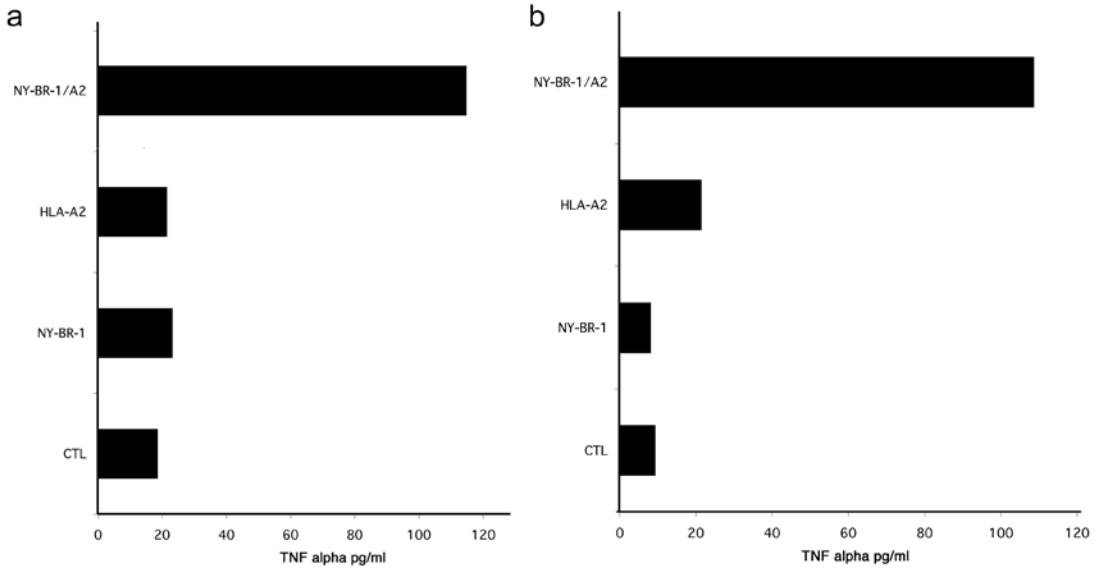
In a recent autologous breast cancer SEREX screening, a new differentiation antigen, NY-BR-1, was identified and characterized (24). NY-BR-1 mRNA was shown to be frequently expressed in breast cancers, but not in normal tissues except for breast and testis. In this study we extended the expression analysis to various other normal and tumor tissues and found inconsistent expression of NY-BR-1 in normal prostate, but consistent NY-BR-1 mRNA expression in 32% of prostate cancers, but not in other tumor types. The normal tissue RNA used for the RT-PCR expression analysis was purchased commercially. To clarify if the inconsistent RT-PCR signal in normal prostate reflects

Figure 4



Recognition of NY-BR-1 peptides by CD8+ T cells. Summary of the ELISPOT results demonstrating peptide-specific recognition (p158-167and p960-968) by CD8+ T cell clones NW1100-CTL-7 and NW1100-CTL-43.

Figure 5



Specific recognition of NY-BR-1 transfected COS-7 cells by T cell clones NW1100-CTL-7 and NW1100-CTL-43. Only HLA-A2 and NY-BR-1 transfected COS-7 cells were recognized by the two T cell clones, but not HLA-A2 negative or NY-BR-1 negative COS-7 cells. Results were confirmed by duplicate assays.

variable NY-BR-1 expression or was caused by contaminating occult prostate cancer cells, immunohistochemical staining with NY-BR-1-specific monoclonal antibody has to be performed.

To evaluate if the antibody defined antigen NY-BR-1 can also be recognized by CD8+ T cells, we screened HLA-A2 positive breast cancer patients with NY-BR-1 expressing tumors for spontaneous CD8+ T cell responses against potential HLA-A2 binding epitopes encoded by the NY-BR-1 cDNA sequence. We identified two peptides that were recognized by CD8+ T cells derived from breast cancer patient NW1100. Several breast cancer cell lines under consideration as potential targets for CD8+ T cells were tested for NY-BR-1 expression by RT-PCR. Consistent with NY-BR-1 being a differentiation antigen, there was no or very little expression in all cell lines analyzed (data not shown), which represented the most undifferentiated type of breast cancer cells. Lacking a suitable breast cancer cell line model we decided to use a NY-BR-1/HLA-A2 cotransfected COS-7 cell line model. We could demonstrate in COS-7 cell cotransfection assays that NY-BR-1 p158-167 and p960-968 are naturally processed and presented in the context of HLA-A2 molecules. These findings add to a previous observation that antigens identified by serological expression cloning may also represent target molecules for CD4+ and CD8+ T cell responses (16, 28, 30).

Active approaches to immunotherapy, such as peptide-based cancer vaccines, immunization with tumor DNA or viral constructs encoding peptide epitopes or the full length antigen, have shown that peptide/antigen-specific CD8+ T cell responses can be induced *in vivo* (31, 32, 33, 34). In some patients, strong immune responses against the vaccines were associated with tumor regressions. NY-BR-1 represents an attractive target antigen for immunotherapy in breast and prostate cancer. Active immunization in melanoma patients against melanocyte differentiation antigens has shown that potent CD8+ T cell responses can interact with melanocytes in the skin, resulting in vitiligo. By quantitative RT-PCR, NY-BR-1 mRNA expression can be detected in normal breast, prostate and testis tissues. Therefore, vaccine-induced CD8+ T cell responses may induce autoimmune reactions with MHC class I expressing normal breast and prostate tissues. A phase I clinical trial is projected to study the toxicity and immunological effects in HLA-A2 positive patients with NY-BR-1 expressing cancers.

Abbreviations

SEREX, serological analysis of recombinant cDNA expression libraries

Acknowledgements

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Materials and methods

Patients, blood and tissue samples

Breast cancer patient NW1100 was diagnosed with metastatic disease in 1999. The primary tumor was resected in 1997 (ductal carcinoma, pT2, pN2, M0, G2). Metastatic sites were lymph node, liver, and peritoneum. A complete remission of all metastases that lasted up to 16 months was achieved by chemo- and hormonal therapy.

Prostate cancer patient NW85 was diagnosed with metastatic disease in 1995. Biopsies of metastatic lesions and T cell lines were obtained at Krankenhaus Nordwest, Germany.

Patient specimens were obtained from the Klinik und Poliklinik für Onkologie, Universitätsspital Zürich, Switzerland, and Krankenhaus Nordwest, Frankfurt, Germany. Tissue samples, blood and serum samples were collected following patients' informed consent. The study was approved by the local ethic committees. All tissue samples were evaluated by the local pathology department of the individual institutions.

Cell lines

Breast cancer cell lines were derived from the repository of the LICR at Memorial Sloan Kettering Cancer Center, as well as from Krankenhaus Nordwest, Frankfurt. The following cell lines were tested for NY-BR-1 expression: MCF-7, 734B, ALAB, BM, BT00474, BT549, CAMA, DU 4474, GAKL, HTB 131, KS, MDA-MB 468, MDA-MB-231, MDA-MB-415, MDA-MB-435S, MDA-MB-436, MDA-MB-469, SK-BR-3, SK-BR-5, SK-BR-7, T47D:A18, T47D:C42W, ZR-75-1.

Analysis of NY-BR-1 expression by RT-PCR

To evaluate the mRNA expression pattern of NY-BR-1 cDNA in normal and malignant tissues, total RNA was extracted from tumor tissues and cell lines using the RNeasy kit (Qiagen, Hilden, Germany), and normal tissue RNA was obtained commercially (Clontech, Heidelberg, Germany). Gene-specific oligonucleotide primers were designed to amplify a cDNA segment of 573 bp in length (Roth, Karlsruhe, Germany): Fw, 5'-CAAAGCAGAGCCTCCCGAGAAG-3' and Rv, 5'-CCTATGCTGCTCTTCGATTCTTCC-3'. RT-PCR was performed using 30 amplification cycles in a thermal cycler (Eppendorf, Wesseling-Berzdorf) at an annealing temperature of 60°C, and the products were analyzed by 1.3% gel electrophoresis and ethidium bromide visualization.

Real-time quantitative RT-PCR analysis

Total RNA derived from 16 normal tissues (Clontech, Heidelberg, Germany) and 3 breast cancer specimens was used for quantitative RT-PCR analysis. 20 µg/l of RNA was reverse transcribed into cDNA using the TaqMan EZ RT-PCR kit (Applied Biosystems, Darmstadt, Germany). Gene-specific primers/probes were designed using the Primer Express 1.5a software (Applied Biosystems, Darmstadt, Germany). To exclude amplification of genomic DNA, the gene-specific primers were designed to span an intron-exon junction (Applied biosystems assay number Hs00369567_m1, assay location 1382, Exon 8 | Exon 9). As an endogenous control, 18S rRNA-specific primers and probe were purchased from Applied Biosystems. The PCR consisted of 40 cycles of 95°C denaturation (15 s), and 60°C annealing/extension (60 s). PCR reactions were prepared using 25 µl of cDNA diluted in TaqMan Universal PCR Mix supplemented with (Fam)-labeled gene-specific TaqMan probe and an optimal concentration of the gene-specific forward and reverse primers (300-900 nM). Thermal cycling and fluorescent monitoring were performed using an ABI7000 thermal cycler. All PCR reactions were run as triplicates.

Prediction of HLA-A2 binding peptides and peptide synthesis

The entire NY-BR-1 protein sequence was analyzed for HLA-A2 binding motifs using an MHC-binding predictor (35). All predicted HLA-A2 binding peptides were synthesized using a multiple peptide synthesizer (Abimed 422; Abimed, Langenfeld, Germany). Peptides were isolated and purified by repeated ether precipitations. The purity was determined by analytical reversed phase HPLC to be at least 90%. The integrity of the peptides was determined by laser desorption time-of-flight mass spectrometry on a Lasermat mass spectrometer (Finnigan MAT, UK).

Peptide presensitization and T cell cloning

CD8+ T lymphocytes were separated from peripheral blood lymphocytes (PBL) by antibody-coated magnetic beads (Minimacs; Miltenyi Biotec, Auburn, CA) and seeded into 48-well plates (Corning) at a concentration of 2.5 x 10^5 cells per well in RPMI 1640 medium supplemented with 10% human serum, L-asparagine (50 mg/l), L-arginine (242 mg/l), and L-glutamine (300 mg/l). PBLs depleted of CD8+ T cells were used as antigen presenting cells. After irradiation, these cells were incubated with 2.5 µg/ml beta2-microglobulin and 10 µg/ml peptide for 1 h at room temperature and added to plates at a concentration of 1 x 10^6 cells per well. After 24 h, IL-2 and IL-7 (2.5 ng/ml and 10 ng/ml, respectively; Biotest Pharma, Dreieich, Germany) were added to the culture wells. For ELISPOT analysis, CD8+ T cells were used on day 6 of presensitization. For tetramer analysis, CD8+ T cells were restimulated on day 6 with peptide and used on day 10 for the analysis. The NY-BR-1-specific CD8+ T cell clones were obtained by repetitive stimulation with NY-BR-1 peptides and limiting dilution as described (36).

ELISPOT assays

Flat-bottomed, 96-well nitrocellulose plates (Millititer; Millipore, Schwalbach, Germany) were coated with IFN-gamma mAb (2 µg/ml, 1-DIK; MABTECH, Stockholm, Sweden) and incubated overnight at 4°C. After washing with PBS, the plates were blocked with 10% human AB serum for 1 h at 37°C. Presensitized CD8+ T cells (1 x 10^3 to 5 x 10^4) and peptide-pulsed T2 cells (5 x 10^4) were added to each well and incubated for 20 h in RPMI 1640 medium lacking IL-2 and human serum. Plates were then washed thoroughly with PBS to remove cells, and IFN-gamma mAb (0.2 µg/ml, 7-B6-1-biotin; MABTECH) was added to each well. After incubation for 2 h at 37°C, the plates were washed and developed with streptavidin-alkaline phosphase (1 µg/ml; MABTECH) for 1 h at room temperature. After washing, substrate (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium; Sigma, Munich, Germany) was added and the plates incubated for 5 min. After final washes, plate membranes displayed dark-violet spots that were counted under the microscope.

Tetramer synthesis

HLA-A2 tetrameric complexes were synthesized as described previously. Briefly, purified HLA heavy chain and beta2-microglobulin were synthesized in a prokaryotic expression system (pHN1). The HLA heavy chain was modified by deletion of the transmembrane cytosolic tail and addition of a sequence containing the BirA enzymatic biotinylation site to the COOH-terminal. The HLA heavy chain, beta2-microglobulin, and peptide were refolded by dilution and biotinylated by BirA (Avidity, Denver, USA) in the presence of biotin, adenosine 5'-triphosphate. The 45 kDa refolded product was isolated by size-exclusion chromatography. Extravidin-phycoerythrin conjugate (Sigma, Munich, Germany) was added at a 1:4 molar ratio. Tetramers were assembled with NY-BR-1 derived peptides p158-167 and p960-968.

Tetramer assays

CD8+ T cells were presensitized with the respective NY-BR-1 peptide as described above. The cultures were refed with IL-2 (2.5 µg/ml) and IL-7 (10 ng/ml) on days 4 and 7, and the presensitization period was extended to 10 days. Sensitized (1 x 10^4 cells per sample) and nonsensitized (1 x 10^6 cells per sample) CD8+ T cells were stained with phycoerythrin-labeled tetramer for 15 min at 37°C before addition of Tricolor-CD8 mAb (Caltag, South San Francisco, CA, USA) for 15 min on ice. After washing, stained cells were analyzed by flow cytometry.

Eukaryotic expression cloning of NY-BR-1 fragments

Two fragments of the NY-BR-1 cDNA sequence (nucleotides 334-516 and 1825-4191 of the full length NY-BR-1 sequence) coding for polypeptides that contain the peptide sequences recognized by CD8+ T cells were amplified by PCR and cloned into the eukaryotic expression vector pcDNA3.1(-) (Invitrogen, Carlsbad, CA). DEAE-dextran-chloroquine transfection of COS-7 cells was performed as described previously (37). Briefly, 2 x 10^4 COS-7 cells were transfected with 150 ng of pcDNA3.1(-) containing the NY-BR-1 cDNA fragment and 150 ng of pcDNA3.1(-) containing the HLA-A2 cDNA. The transfected cells were incubated at 37°C for 48 h and tested in a CD8+ T cell stimulation assay after 24 h.

Immunoscreening of NY-BR-1 transfectants

COS-7 cells were transfected with the NY-BR-1 and HLA-A2 cDNA pcDNA3.1(-) constructs as described above and tested for their ability to stimulate the production of TNF-alpha by CD8+ selected presensitized T cells derived from patient NW1100. Briefly, 2500 CD8+ T cells in 100 µl RPMI medium supplemented with 10% human serum and recombinant human IL-2 (25 U/ml) were added to microwells containing COS-7 transfectants. After 24 h, the TNF-alpha content in 50 µl of supernatant was determined by testing the cytotoxicity against WEHI 164 clone 13 cells in an MTT colorimetric assay (37).

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