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HUMAN MENA PROTEIN, A SEREX-DEFINED ANTIGEN OVEREXPRESSED IN BREAST CANCER ELICITING BOTH HUMORAL AND CD8$^+$ T-CELL IMMUNE RESPONSE

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Screening of a cDNA expression library from a primary breast tumor with the autologous patient serum led to the isolation of 6 cDNA clones corresponding to 3 different genes, including a novel gene that maps to chromosome 1 and encodes the human homologue of mouse Mena (hMena, cDNA clone RMNY-BR-55), a protein of the Ena/VASP family involved in the regulation of cell motility and adhesion. A cancer-restricted antibody response against hMena was demonstrated, since 18/93 cancer patient sera, the majority (10/52) from breast cancer, showed anti-hMena-specific IgG, while no antibodies were present in healthy donors. When hMena protein expression was analyzed by Western blot and immunohistochemistry, the antigen was overexpressed in the majority of breast cancer cell lines and in 75% of primary breast tumor lesions evaluated. Furthermore, when HLA-A2-restricted peptides from the hMena sequence were used to stimulate CD8$^+$ T cells, an hMena-specific response was found in 9 out of 12 HLA-A2$^+$ breast cancer patients. In 4 patients, this cell-mediated immune response was concomitant with antibody response to hMena. Furthermore, an hMena-specific T-cell line was established from an HLA-A2$^+$ breast cancer patient whose primary tumor lesion overexpressed the hMena protein. The present findings highlight the emerging role that overexpression of cytoskeleton regulatory components may have in the induction of a specific antitumor immune response.

Key words: *tumor immunity; major histocompatibility complex; cytotoxic T lymphocyte*

The identification of the repertoire of molecules recognized by the immune system of cancer patients at different stages of the disease is of major biologic and clinical relevance,[1] as the immune system continuously shapes the immunogenic phenotype of the developing tumor[2] by a complex process recently referred to as cancer immunoediting.[3]

Genetic changes continuously occurring during tumor development and progression lead to a number of mutant and/or aberrantly expressed proteins, which can potentially function as tumor-associated antigens and elicit antitumor immune responses.[4] However, the dynamics and the consequences of these events have not yet been fully elucidated. The serologic analysis of cDNA expression libraries (SEREX) of human tumors has identified a broad spectrum of tumor proteins capable of eliciting a humoral immune response in tumor patients.[5] The majority of these SEREX-defined antigens do not show any detectable mutations and/or structural modifications. Although some tumor antigens show restricted expression in normal tissues, *i.e.*, cancer/testis (CT) and melanoma differentiation antigens, to date, the results indicate that the overexpression of normal proteins in the tumor may be of major significance in eliciting a tumor-specific humoral immunity.[6] The isolation of tumor antigens recognized by high-titer IgG implies CD4$^+$ and CD8$^+$ T-cell recognition, as extensively demonstrated for the C/T antigen NY-ESO-1 that induces a concomitant humoral and cellular immune response in a high proportion of NY-ESO-1$^+$ cancer-bearing patients.[7–10]

To identify and characterize new antigens in breast cancer, we applied the SEREX approach analyzing a primary breast tumor and the autologous serum of a long surviving patient. A novel gene was identified, which represents the human homologue (hMena) of murine Mena, encoding a protein of the enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) family that controls cell motility and cell-cell adhesion by regulating the actin cytoskeleton.[11,12] Here we report that hMena is overexpressed in breast cancer and is able to induce a cancer-restricted humoral response detectable in a high percentage of sera from breast cancer patients. Furthermore, 3 hMena HLA-A2 restricted epitopes were identified, being recognized by CD8$^+$ T lymphocytes from HLA-A2-positive breast cancer patients, half of whom also showed a concomitant specific IgG response. One of these epitopes (hMena-502) is naturally processed and presented by HLA-A2$^+$/hMena$^+$ tumor cell lines as demonstrated by functional analysis of a CD8$^+$ T-cell line generated from a breast cancer patient.

Abbreviations: APC, antigen-presenting cell; C/T, cancer/testis; CTL, cytotoxic T lymphocyte; ELISPOT, enzyme-linked immunospot assay; Ena/VASP, enabled/vasodilator-stimulated phosphoprotein; hMena, human Mena; IIF, indirect immunofluorescence; mAb, monoclonal antibody; MHC, major histocompatibility complex; NHM, normal human melanocyte; PBL, peripheral blood lymphocytes; SEREX, serologic analysis of cDNA expression libraries.

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The isolation of a new gene belonging to the Ena/VASP family such as hMena, encoding a protein overexpressed in breast cancer and inducing a cancer-restricted antibody and $CD8^+$ T-cell response, is consistent with the involvement of cytoskeleton regulatory proteins in breast oncogenesis[13] and with their emerging role as tumor antigens.[14]

MATERIAL AND METHODS

Cell lines, sera, peripheral blood lymphocytes and peptides

The MDAMB231, SKBr3, MDA-MB-361 and MCF7 breast cancer cell lines as well as the A549 lung tumor cell line were from American Type Culture Collection (ATCC; Rockville, MD). The MDAMB231cl2 was established by cloning the MDAMB231 cell line in soft agar, as previously described.[15] SAB and DAL cell lines were developed from the ascitic fluid of 2 breast cancer patients[16] and MAS from a primary melanoma lesion. Normal human epidermal melanocytes were purchased from Promo Cell (Heidelberg, Germany). EBV-transformed B-cell lines were established from the patients F.S.O. and D.P.N. and designed LCL F.S.O. and LCL D.P.N.

HLA-A2.1 human cell line 0.174 × CEM.T2 (T2) was used as antigen-presenting cell (APC) in the functional studies. Mycoplasma contamination was monitored by a mycoplasma detection kit (Boheringer Mannheim, Indianapolis, IN).

Following informed consent, blood samples were obtained from breast cancer patients at different times during the course of the disease and from patients bearing tumors of different histotypes. Peripheral blood lymphocytes (PBLs) and sera were collected from patients free of chemotherapy for at least 6 months. PBLs were separated from heparinized blood by centrifugation on a Lymphoprep (Nycomed Pharma, Oslo, Norway) gradient and tested for HLA-A2 expression by the use of the BB7.2 monoclonal antibody (mAb) and FACScan analysis.[17] Aliquots of PBLs were cryopreserved and thawed at the time of enzyme-linked immunospot assay (ELISPOT). Sera were stored at −20°C. Donor patients were from the surgery and oncology departments of the Regina Elena Cancer Institute (Rome, Italy). Peptides derived from the protein sequence of the SEREX-identified antigen hMena and chosen for the presence of HLA-A2-binding motifs were purchased from Sigma-Genosys (Cambridge, U.K.) and were > 95% pure by HPLC.

Serologic screening of a cDNA library from a primary breast cancer tissue and characterization of immunoreactive clones

A cDNA expression library was constructed from a primary breast cancer tissue using a λ-ZAP Express cDNA synthesis kit (Stratagene, La Jolla, CA). The specimen was obtained from a 48-year-old patient (F.S.O.) who underwent surgery in the Regina Elena Cancer Institute in 1995 for a ductal infiltrating carcinoma. To date, the patient is disease-free. To remove antibodies reacting with the vector system, F.S.O. serum (diluted at 1:10) was absorbed by passage through sepharose 4B columns coupled to *E. coli* Y1090 and bacteriophage infected *E. coli* BNN97 lysates (5 Prime 3 Prime, Boulder, CO). The cDNA library was screened by incubating overnight nitrocellulose membranes containing the phage plaques with autologous serum (collected at the time of surgery) diluted at 1:200 in TBS/1% BSA/0.02% NaN_3. After subtraction of phage plaques encoding human IgG, the filters were incubated with a 1:3,000 dilution of alkaline phosphatase-conjugated goat antihuman Fcγ secondary antibodies (Jackson Immunoresearch, West Grove, PA) and processed for 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate color development. Immunoreactive clones were isolated and subcloned. Phage cDNA clones were converted to pBK-CMV phagemid forms by *in vivo* excision. Plasmid cDNAs were extracted using a Wizard Plasmid DNA kit (Promega, Madison, WI) and the corresponding cDNA inserts were sequenced.

Allogeneic sera (1:200 dilution) from normal donors and cancer patients were preabsorbed to remove antivector antibodies and analyzed for their reactivity with SEREX-derived clones by the plaque immunoassay using nitrocellulose filters containing an equal number of seroreactive clones and negative control plaques (phage without cDNA insert, as background). Reactivity was considered positive when about half of the plaques were clearly colored compared to the background.

IgG titer against the RMNY-BR-55 clone product was evaluated in a number of representative positive sera by testing 7 different sera dilutions ranging from 1:25 to 1:1,600. The majority of negative samples were also tested using 4 lower sera dilutions ranging from 1:20 to 1:100 and similar results were obtained.

Northern blot analysis

Cancer cell line mRNA blots were purchased from Clontech Laboratories (Palo Alto, CA) and contained 2 μg of $poly(A)^+$ RNA per lane. Total RNA was isolated from 5 breast cancer cell lines (SBT, SKBr3, MDAMB231, DAL, MCF7) by guanidium thiocyanate (CsCl method) and 15 μg of each sample were transferred to Hybond N^+ nylon membranes (Amersham Biosciences, Little Chalfont, U.K.) after denaturing gel electrophoresis. Hybridization was carried out in the QuikHyb solution (Stratagene) for 1 hr at 68°C. As probe for Northern blot analysis, a 254 bp PCR product from the sequence of the cDNA clone RMNY-BR-55 was ^{32}P-labeled using the DECA-Prime II DNA labeling kit (Ambion, Austin, TX). After stringent washing, the filters were exposed to Biomax MS autoradiography films (Kodak, Rochester, NY) to detect hybridization signals.

Western blot analysis

Cells were lysed for 30 min at 4°C in 10% glycerol, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40 in PBS containing protease inhibitors. Lysates were centrifuged and supernatant was determined for protein quantification using BCA Protein Assay Reagent (Pierce, Rockford, IL). Normal heart and mammary gland tissue extracts (Protein Medley) were purchased from Clontech Laboratories. Thirty micrograms from cell lines or normal tissue extracts were separated on 10% polyacrilamide gel and transferred to nitrocellulose membrane (Amersham Biosciences). Blots were blocked for 1 hr with 3% skimmed milk in TBST and probed with anti-Mena mAb Clone 21 (BD Transduction Laboratories, Heidelberg, Germany) 1:250 or with 15 μg/ml of a rabbit polyclonal antibody (CKLK1) that we developed against the 20 amino acid C-ter peptide of hMena by the use of Sigma-Genosys service. The protein signals were detected by ECL kit (Amersham). For actin signal, blots were reprobed with 1 μg/ml antiactin mAb, clone AC-40 (Sigma-Aldrich, Milan, Italy).

Immunohistochemistry

Ig fraction (purified by ammonium sulfate precipitation) of CKLK1 rabbit antiserum to hMena was used in indirect immunofluorescence (IIF) at an appropriate dilution established by titration on SBT, DAL and MDAMB231 cells using the preimmune IgG as negative control. Normal tissues and tumor specimens were obtained following the guidelines of the ethics committee from patients without presurgical chemo- or radiotherapy undergoing surgical treatment at the Regina Elena Cancer Institute. Upon removal, tissues were snap-frozen in liquid nitrogen and stored at −80°C. Cryostat sections 4 μm thick were obtained and fixed in acetone for 10 min. Sections were either immediately used for IIF or stored at −80°C up to 6 months with no appreciable changes in immunoreactivity. Sections were also stained with 1% toluidine blue for morphologic evaluation. IIF was performed by standard procedure using an FITC-labeled $F(ab)_2$ goat antirabbit IgG antiserum (Cappel, West Chester, PA). Sections incubated with preimmune IgG were used as controls.

Anti-hMena IHC staining, using in parallel the CKLK1 IgG and the mouse monoclonal anti-Mena Clone 21, was carried out on 5 μm thick paraffin-embedded tissues. Sections were harvested on SuperFrost Plus slides (Menzel-Glaser, Braunschweig, Germany). The deparafinized and rehydrated sections were pretreated in a

thermostatic bath at 96°C for 40 min in citrate buffer pH 6 and incubated for 60 min at room temperature with primary CKLK1 IgG diluted 1:100. The reaction was visualized using an immunoperoxidase multilink Supersensitive Detection Kit (Biogenex, Menarini, Florence, Italy) and diaminobenzidine (Menarini) as chromogenic substrate. Sections were then slightly counterstained with Mayer hematoxylin and mounted in aqueous mounting medium (Glycergel, Dako, Milan, Italy).

ELISPOT assay

$CD8^+$ T cells isolated from PBL by the use of antibody-coated magnetic beads (Minimacs, Miltenyi Biotec, Auburn, CA) were tested in ELISPOT assays for IFN-γ production. Briefly, 5×10^4 $CD8^+$ T lymphocytes in 50 μl of medium were added to each well of 96-well plates coated with 50 μl of antihuman IFN-γ (murine mAb 1-D1K; Mabtech, Stockholm, Sweden) at a concentration of 10 μg/ml in PBS. Five $\times$ 10^4 T2 cells (in 50 μl of RPMI) pulsed overnight with the appropriate amount of peptide were lethally irradiated and added to each well. Cells were incubated at 37°C in 5% CO_2 in a water-saturated atmosphere for 16 hr. Captured IFN-γ was detected with mouse antihuman IFN-γ mAb (7-B6-1-biotin; Mabtech), avidin-biotin peroxidase complex (Vectastain-Elite kit, Vector, CA) and peroxidase staining using the AEC-Substrate-Solution (Sigma Chemical, St. Louis, MO). The numbers and areas of resulting spots were determined by the use of computer-assisted video image analysis (Axioplan 2, Zeiss, Thornwood, NY). To calculate the number of $CD8^+$ T cells responding to a particular peptide, the mean number of spots induced by T2 cells alone (background) was subtracted from the mean spot number induced by peptide-loaded T2 cells. For statistical evaluation, a *t*-test for unpaired samples was applied using the Inplot Software System (GraphPad Software, San Diego, CA). Values of $p < 0.05$ were considered significant.

Establishment of the hMena-502 D.P.N. cell line

$CD8^+$ T lymphocytes from the HLA-$A2^+$ D.P.N. patient (who underwent surgery for a ductal breast carcinoma) were separated from PBL by antibody-coated magnetic beads and seeded into 48-well plates at a concentration of 5×10^5 cells per well in RPMI-1640 medium supplemented with 10% human serum. As APCs, PBLs depleted of $CD8^+$ T cells were irradiated and incubated with 2.5 μg/ml β2-microglobulin and 40 μg/ml hMena-502 peptide for 2 hr at room temperature and added to the plates at a concentration of 1×10^6 per well. After 24 hr, IL-2 (Roche Diagnostics, Mannheim, Germany) and IL-7 (PeproTech, Rocky Hill, NJ; 2.5 ng/ml and 10 ng/ml, respectively) were added to the culture wells as previously reported.[18] Cells were restimulated after 7 days with 1 μg/ml of peptide, then weekly with T2 cells pulsed with 40 μg/ml of peptide, and tested in cytotoxicity assay as described.[16]

Immunofluorescence and cytofluorimetric analysis

The immunofluorescence on the hMena-502 D.P.N. cell line was performed on 5×10^5 cells by the use of Simultest CD3/$CD16^+$ CD56 and Simultest CD4/CD8, with Simultest Control γ_1/γ_{2a} as negative control, according to the manufacturer's protocol (Becton Dickinson, BD Biosciences, San Jose, CA). The percentage of positive cells was evaluated by FACS analysis (FACScan, Becton Dickinson).

Cytotoxicity assay

The cytotoxic activity of the hMena-502 D.P.N. cell line was determined using a conventional 4-hr ^{51}Cr release assay as previously described.[16] For peptide recognition, T2 cells were incubated overnight with 40 μg/ml peptide, washed, then labelled. Cytotoxicity assays were performed by incubating ^{51}Cr-labesled target cells with effector cells at different effector/target ratios ranging from 5:1 to 40:1. Blocking activity of mAbs was evaluated as previously described employing the anti-CD3 mAb (OKT3 IgG1, ATCC) or the antimajor histocompatibility complex (anti-MHC) class I W6/32 mAb (IgG2a).[19] The IgG1 mAb W6/800 and the IgG2a mAb W6/100[20] were employed as irrelevant antibodies. Controls included incubation of antibodies with targets to exclude antibody-dependent cell lysis. Cold-target competition assays were carried out in round-bottom plates as previously described.[16] Inhibition was performed using unlabeled T2 loaded with the hMena-502 peptide or with the HLA-A2-binding irrelevant peptide Flu-M1 (influenza matrix protein 58-66 GILGFVFTL) employed as negative control. Supernatants were harvested and radioactivity was determined using a gamma counter. Percent specific lysis was calculated as previously described.[16] The experiment was performed 3 times with similar results.

RESULTS

Screening of a breast tumor cDNA library with autologous serum

A cDNA expression library was constructed from the primary tumor of an HLA-A2-positive breast cancer patient. This tumor displayed HLA class I and HLA-A2 expression and marked intratumoral infiltration of $CD3^+$ T cells when tested by immunohistochemistry (data not shown) as previously reported.[21] The screening of the cDNA expression library with the autologous serum identified 6 immunoreactive cDNA clones that were deposited on the SEREX database (www.licr.org/SEREX.html).

The first clone, RMNY-BR-55, was 99% identical to the Genbank entry FLJ10773 (Genbank accession number NM_018212), which maps to chromosome 1q41. The 99 amino acid sequence of RMNY-BR-55 was 82% identical to both the C-terminal of murine and *Drosophila* orthologues of Mena, a protein involved in the control of cell motility and adhesion.[11,12] We obtained a full-length cDNA encoding RMNY-BR-55 from a breast tumor cell line (SBT) by RT-PCR using 2 oligonucleotides isolated by the comparison of mouse Mena sequence and human ESTs as primers (data not shown). The relative 591 amino acid protein sequence, whose C-ter is identical to RMNY-BR-55, showed 84% homology with mouse Mena. Henceforth, RMNY-BR-55 will be referred to as human Mena (hMena). The remaining 5 cDNA clones were derived from 2 different genes encoding products independently related to BRCA1 oncosuppressor gene[22] as BAP1[23] and BRAP2.[24]

Antibodies to SEREX-defined antigens in sera of healthy donors and cancer patients

To evaluate whether the antibody response to the 3 identified antigens is related to the presence of neoplastic disease, we tested a panel of sera by phage plaque immunoassay. The sera were obtained at the time of surgery from 52 unselected breast cancer patients, from 41 patients bearing tumors of different histogenesis and from 17 healthy donors.

The results of the screening identified 3 categories of serum reactivity with the SEREX-defined antigens (Table I). The immune response to hMena appeared to be cancer-restricted, since 18 of 93 (19%) cancer patients and none of 17 healthy donors showed the presence of specific anti-hMena IgG (Fisher's exact test, one-sided, $p = 0.037$). hMena was recognized by IgG in 10 out of 52 sera from breast cancer patients. However, the reactivity was not restricted to patients with breast cancer, as hMena was also recognized by IgG in the sera from 8 out of 41 patients with other types of neoplasia. No symptoms of paraneoplastic autoimmune

TABLE I – SEREX-IDENTIFIED BREAST CANCER ANTIGENS: REACTIVITY WITH SERA FROM TUMOR PATIENTS AND HEALTHY DONORS

	Total	Sera reacting with the cDNA clone products		
Sera		hMena	BAP1	BRAP2
Breast cancer patients	52	10	0	29
Tumor patients[a]	41	8	1	16
Healthy donors	17	0	0	9

[a]Patients bearing tumors of various histological origin.

diseases were observed in patients with anti-hMena antibodies. Anti-hMena IgG titer was evaluated in a number of representative positive cases by testing sera dilutions from 1:25 to 1:1,600. In all cases, antibody reactivity was detectable up to 1:200.

Serologic recognition of BAP1 was also cancer-related, but was only found in the autologous patient and in one ovarian cancer-bearing patient, while BRAP2 recognition was displayed either by sera from tumor patients or by sera from healthy donors. To evaluate the persistence of specific IgG response against the SEREX-identified antigens, we tested the autologous serum of F.S.O. patient collected 2, 3, or 5 years after the surgical removal of the tumor. The phage plaque immunoassay showed the persistence of immunoreactivity against the noncancer-related antigen BRAP2 in all the samples tested. By contrast, no reactivity was detected against BAP1 or hMena, as evaluated with serum dilutions ranging from 1:20 to 1:200.

Analysis of hMena expression by Northern and Western blot

The presence of anti-hMena antibodies in patient sera prompted us to study its tissue expression. By using a 254 bp PCR product derived from hMena as probe, Northern blots revealed several hybridization signals in 4 out of 5 breast cancer cell lines evaluated (Fig. 1*a*). In SBT cells, a major 5.0 kb band and 2 minor 4.0 and 3.5 kb bands were clearly evident. The clone obtained from the MDAMB231 cell line showed more intensive hybridization signals compared to the parental cell line. Among a panel of tumor cell lines of different histologic origin, A549 lung carcinoma and G361 melanoma showed a strong 5.0 kb hybridization signal (Fig. 1*b*). hMena expression was also found in normal tissues such as brain and heart but not in normal lung (not shown).

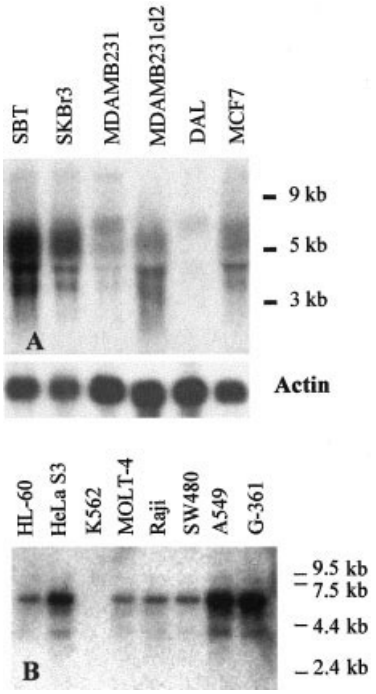

FIGURE 1 – Northern blot analysis of the hMena RNA expression in tumor cell lines. (*a*) Northern blot analysis using RNA (total RNA 15 μg/lane) purified from SBT, SKBr3, MDAMB231, DAL and MCF7 breast cancer cell lines and from MDAMB231cl2, a breast cancer cell line-derived clone hybridized with the 254 nt PCR-amplified sequence from the RMNY-BR-55 cDNA clone as probe. β-actin was used as an indicator of lane loading. (*b*) MTN Blot (Clontech, Palo Alto, CA) shows many mRNA hybridizing species in cervix carcinoma cell line HeLaS3, in lung cancer cell line A549 and in melanoma cell line G-361. By contrast, HL-60 promyelocytic leukemia, MOLT-4 lymphoblastic leukemia, Raji Burkitt's lymphoma and SW480 colorectal adenocarcinoma showed less intensive hybridization signals.

Western blot showed that normal mammary gland did not express the 90 kDa hMena, while a strong signal was displayed by 4 out of 5 breast cancer cell lines evaluated (Fig. 2). The hMena protein migrates with a molecular weight apparently higher than the predictive one, probably due to the proline-rich domain present in the sequence as reported for mouse Mena protein.[25] Overexpression of hMena was evident in the MAS melanoma cell line that we subsequently employed in the cytotoxicity assay, whereas normal human melanocytes (NHMs) showed very low hMena expression. No signal was detectable in the EBV-transformed B lymphocytes derived from patient F.S.O. Differently from results obtained by Northern blot, the extract from heart tissue showed a slight protein band. Western blot analysis was carried out employing 2 different anti-hMena antibodies and overlapping results were obtained.

Analysis of hMena expression by immunohistochemistry

As the immunohistochemical reactivity of the commercial anti-hMena mAb was unsatisfactory on frozen sections, the tissue

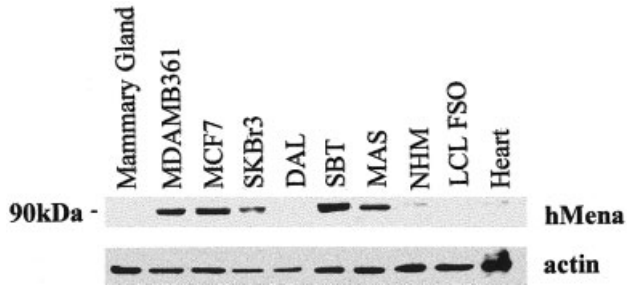

FIGURE 2 – Evaluation of hMena protein expression in human tumor and normal cells by Western blot. Thirty micrograms of lysates from normal human mammary gland (Clontech), MDAMB361, MCF7, SKBr3, DAL and SBT breast tumor cells, MAS melanoma cells, NHM normal human melanocyte, EBV-transformed B cells of patient F.S.O. (LCL F.S.O.) and normal human heart tissue (Clontech) were immunoblotted with a mouse anti-Mena mAb (1:250). As loading control, the same blots were probed with antiactin mAb (1 μg/ml).

TABLE II – IMMUNOHISTOCHEMICAL ANALYSIS OF hMENA EXPRESSION IN NORMAL HUMAN TISSUES AND IN PRIMARY BREAST CARCINOMAS

Normal tissues[a]	
Negative	Positive
Epidermis	Parotid: myoepithelium
Breast: lobular, ductal epithelium	Breast: myoepithelium
Thymus: epithelium	Colon-rectum: epithelium, muscolaris mucosae
Lung: alveoli, broncs	Bronchial glands: myoepithelium
Thyroid	Pancreas: acinar, ductal epithelium
Liver	Sweat glands: myoepithelium
Kidney	Endometrium: epithelium
Testis	Prostate: fibromuscolar stroma
Spleen	Placenta: vascular media
Lymph Nodes	
Heart	
Skeletal muscle	
Primary breast carcinomas	
Histotype	Immunoreactivity
Invasive ductal carcinoma	11[b]/14
Invasive lobular carcinoma	2/4
Tubular carcinomas	3/3
Medullary carcinomas	2/3
Total	18/24

The analysis was performed by the use of rabbit polyclonal CKLK1 Ab as reported in text.–[a]At least two individual tested.–[b]Fraction-positive.

distribution analysis was performed using the rabbit polyclonal antiserum CKLK1. In normal tissues, hMena expression was confined to myoepithelial cells, stroma and isolated epithelia (Table II). While ductal and lobular mammary epithelium were negative, detectable levels of hMena were observed in about 75% of breast primary tumors tested, even if the staining intensity was variable and on some occasions limited. Representative examples of hMena expression are shown in Figure 3.

$CD8^+$ T-cell response to SEREX-defined hMena antigen

In order to investigate the T-cell response against the hMena antigen, we synthesized 3 peptides from the hMena protein sequence that possess HLA-A2.1-binding motifs. These peptides were selected using the SYFPEITHI Epitope Prediction Database[26] and the HLA peptide-binding prediction analysis.[27] Peptides hMena-502 (TMNGSKSPV) and hMena-562 (ILDEMRKEL) are also present in the 99 amino acid predictive protein sequence of the RMNY-BR-55 clone, whereas hMena-443 (GLMEEMSAL) is present only in the hMena full-length protein. All the peptides bind HLA-A2 molecules, as evaluated by a previously described HLA-A2 stabilization assay on T2 cells.[28] Forty μg/ml was the optimal stabilizing peptide concentration. hMena-443 was the peptide with the highest HLA-A2 affinity (data not shown). The Her-2 neu peptide 689[29] with the appropriate HLA-A2-binding motifs and peptide F99 derived from *Parietaria officinalis* 1[28] lacking the HLA-A2-binding motifs were used as positive and negative control of the assay conditions, respectively.

The presence of $CD8^+$ T-cell precursors against these peptides was evaluated by IFN-γ ELISPOT in 12 HLA-A2-positive breast cancer patients. Nine patients were found to possess $CD8^+$ T-cell precursors against the hMena-562 peptide (Fig. 4). Two of these patients also reacted to the hMena-502 peptide. Only one patient showed IFN-γ spots in response to hMena-443 and to the other 2 hMena peptides. Three patients showed no reaction with any of the peptides. The cDNA library and the serum used to identify BAP1, BRAP2 and hMena were from T cells from patient F.S.O. Unfortunately, $CD8^+$ T cells from patients F.S.O. were only available 5 years after surgery. At this time, no T-lymphocyte response to the peptides was found (data not shown). As expected, $CD8^+$ T lymphocytes from HLA-A2-negative patients (3 cases) were unable to react against the selected peptides (data not shown).

In the majority of the assays, the HIV-1 peptide (reverse transcriptase HIV) was employed as internal negative control.[30] The assays were repeated twice and overlapping results were obtained.

Comparison between hMena tumor expression and humoral and $CD8^+$ T-cell–mediated immune response

Patients D.D.M., S.C.R., P.L.B. and P.L.N. overexpressing hMena on their primary tumors (as evaluated by immunohistochemistry) had specific anti-hMena IgG, and their $CD8^+$ T cells reacted against the hMena peptides. Patients D.A.D., S.L.M., R.N.Z. and S.M.Z. had no anti-hMena antibodies while they displayed $CD8^+$ T-cell response to hMena peptides and their primary tumors were positive for hMena expression. Patients A.R.T., M.R.S. and M.U.R. showed neither IgG nor $CD8^+$ T-cell reactivity to hMena and M.R.S. and M.U.R. tumor lesions were negative for hMena expression, whereas A.R.T. was positive (Table III).

$CD8^+$ T cells recognize $hMena^+$/$HLA\text{-}A2^+$ tumor cell lines in an HLA class I-restricted fashion

A T-cell line was established from the $HLA\text{-}A2^+$ patient D.P.N. by stimulating her $CD8^+$ T lymphocytes with the hMena-502 peptide as reported above. The specificity of this $CD3^+$ $CD8^+$, $CD16^-$, $CD56^-$ line (hMena-502 D.P.N.) was evaluated by a standard cytotoxicity assay on a panel of tumor cell lines either displaying or not HLA-A2 and hMena (Fig. 5*a*). The hMena-502 D.P.N. cell line lysed $HLA\text{-}A2^+$/$hMena^+$ target cells but not the $HLA\text{-}A2^-$ and/or $hMena^-$ targets. Moreover, the NK-sensitive K562 cells were also not lysed (Fig. 5*a*).

The lysis of MCF7 and MAS was abrogated by anti-CD3 mAb and partially inhibited by an anti-HLA class I mAb, whereas the irrelevant isotype control mAb did not interfere with the specific lysis (Fig. 5*a*). The EBV-transformed B cells from D.P.N. and F.S.O. patients were not lysed. The specificity of lysis was further confirmed by cold/target competition experiments (Fig. 5*b*). The addition of $HLA\text{-}A2^+$/$hMena^+$ MAS melanoma cells to the assay completely inhibited the lysis of MCF7 breast cancer cells, whereas the competition by cold MCF7 on MAS melanoma cells was significantly less marked. The inability of the K562 cell line to act as competitor confirmed the specificity of the inhibition.

The hMena-502 D.P.N. T-cell line recognized the hMena-502 peptide since it lysed T2 cells pulsed with the hMena-502 peptide but not unpulsed T2 cells, confirming the ELISPOT results (Fig. 5*c*). The specificity of MCF7 lysis by hMena-502 D.P.N. cells was further evaluated by adding an excess of cold T2 cells pulsed with the hMena-502 peptide or with the irrelevant Flu-M1 peptide. Lysis of MCF7 was significantly inhibited (73% of inhibition) by the cold T2 only when pulsed with the specific peptide. These data suggest that lytic activity of the CD3-dependent hMena-502 D.P.N. cell line rests on the recognition of hMena-502 peptide processed by MCF7 cancer cells in an HLA class I-restricted fashion.

DISCUSSION

On the trail toward new tumor antigens potentially able to elicit a protective immune response, we screened by SEREX a cDNA expression library obtained from the primary breast tumor of a long-surviving patient using the autologous serum. The primary tumor was selected on the basis of a dense intratumoral $CD3^+$ T-cell infiltrate, a phenotypic feature that correlates with the expression of HLA class I glycoproteins in a number of primary breast tumors.[21] Recently, clinicopathologic investigation has linked the presence of an intratumoral T-cell infiltrate with a favorable prognosis in ovarian cancer.[31]

By following this experimental approach, we identified 3 antigens displaying 3 different pattern of serologic reactivity, in agreement with data available in the SEREX database. The 2 BRCA1-related proteins, BRAP2 and BAP1, represented an autoantigen and a patient-specific antigen, respectively. Of particular interest is that the third antigen, hMena, showed a cancer-restricted specificity, since anti-hMena antibodies were found in 19% of cancer patients (the majority being affected by breast cancer) and in none of the healthy donors. The serum of the patient whose primary tumor was used to obtain the cDNA library was available at different times after removal of the primary tumor and in this patient the antibody response was maintained only against the autoantigen BRAP2. No antibodies were detectable against BAP1 and against hMena during the long follow-up of the patient. This suggests that, as in the NY-ESO-1 antigen,[32] the maintenance of detectable levels of serum IgG against the hMena protein in this patient is strictly dependent on the *in vivo* stimulation of the autologous tumor.

The evaluation of the T-cell response against SEREX antigens with a cancer-restricted seroreactivity, a major step in the definition of the molecules capable of eliciting a concomitant humoral and cellular immune response, is an important prerequisite in the identification of biologically and clinically relevant tumor antigens.[33] The role of hMena as an antigen able to induce a coordinated immune response is suggested by the analysis of T-cell reactivity. We identified herein 3 hMena T-cell epitopes. *Ex vivo* IFN-γ secretion by $CD8^+$ T lymphocytes of the 12 breast cancer patients studied demonstrated that the majority reacted with the hMena-562 peptide. This peptide did not show the highest HLA-A2 affinity among the 3 peptides evaluated (data not shown), indicating a lack of correlation between cytotoxic T lymphocyte (CTL) effector function and the extent of peptide-MHC interaction. This is in line with

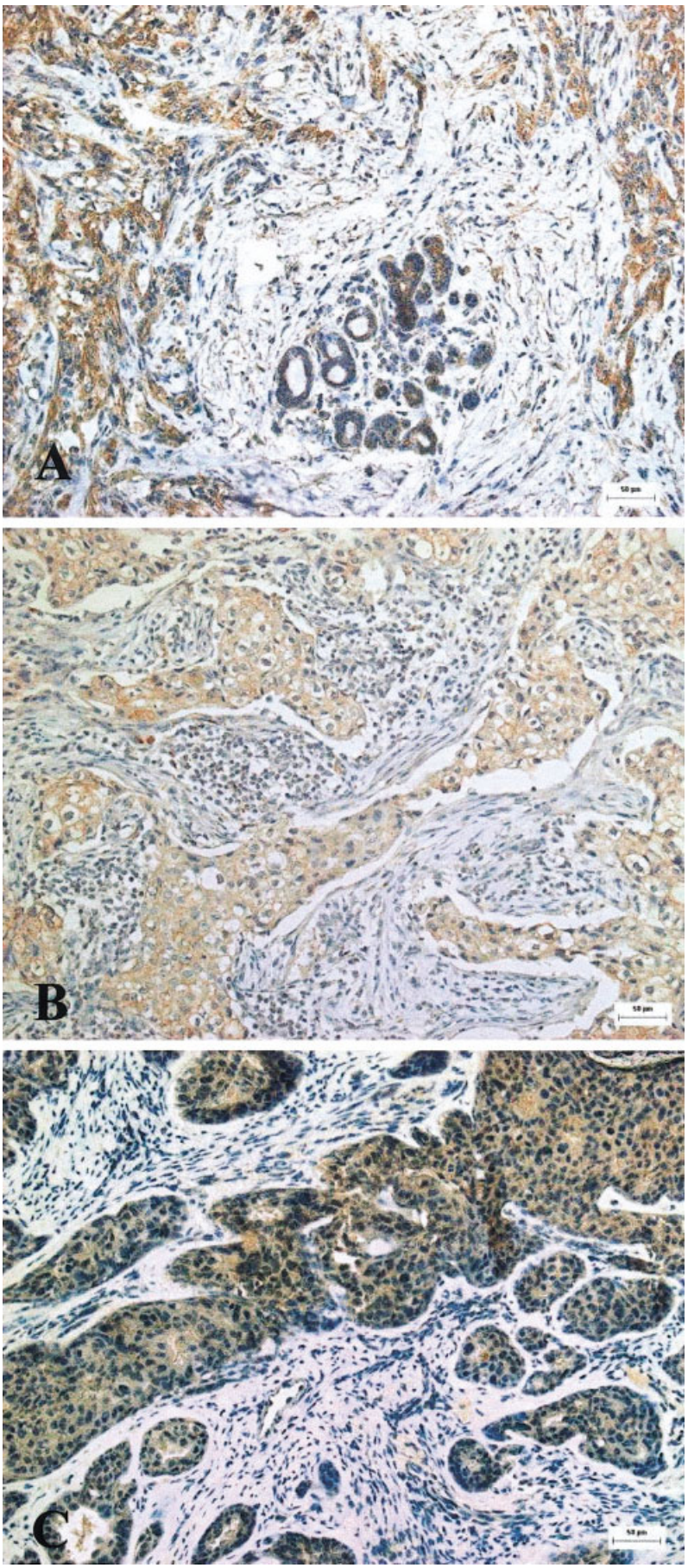

FIGURE 3 – Immunohistochemical analysis of hMena tumor expression. The expression of hMena was evaluated in paraffin-embedded tissue sections from an invasive ductal breast carcinoma (*a*). Staining of variable intensity is shown by the transformed cells while the residual glandular normal tissue is unstained. (*b*) Expression of hMena in the invasive ductal breast carcinoma from which the cDNA library for SEREX assay was developed. (*c*) Expression of hMena in the invasive ductal breast carcinoma of the patient from whom a $CD8^+$ CTL line specific to hMena peptide was generated. Scale bar = 50 μm.

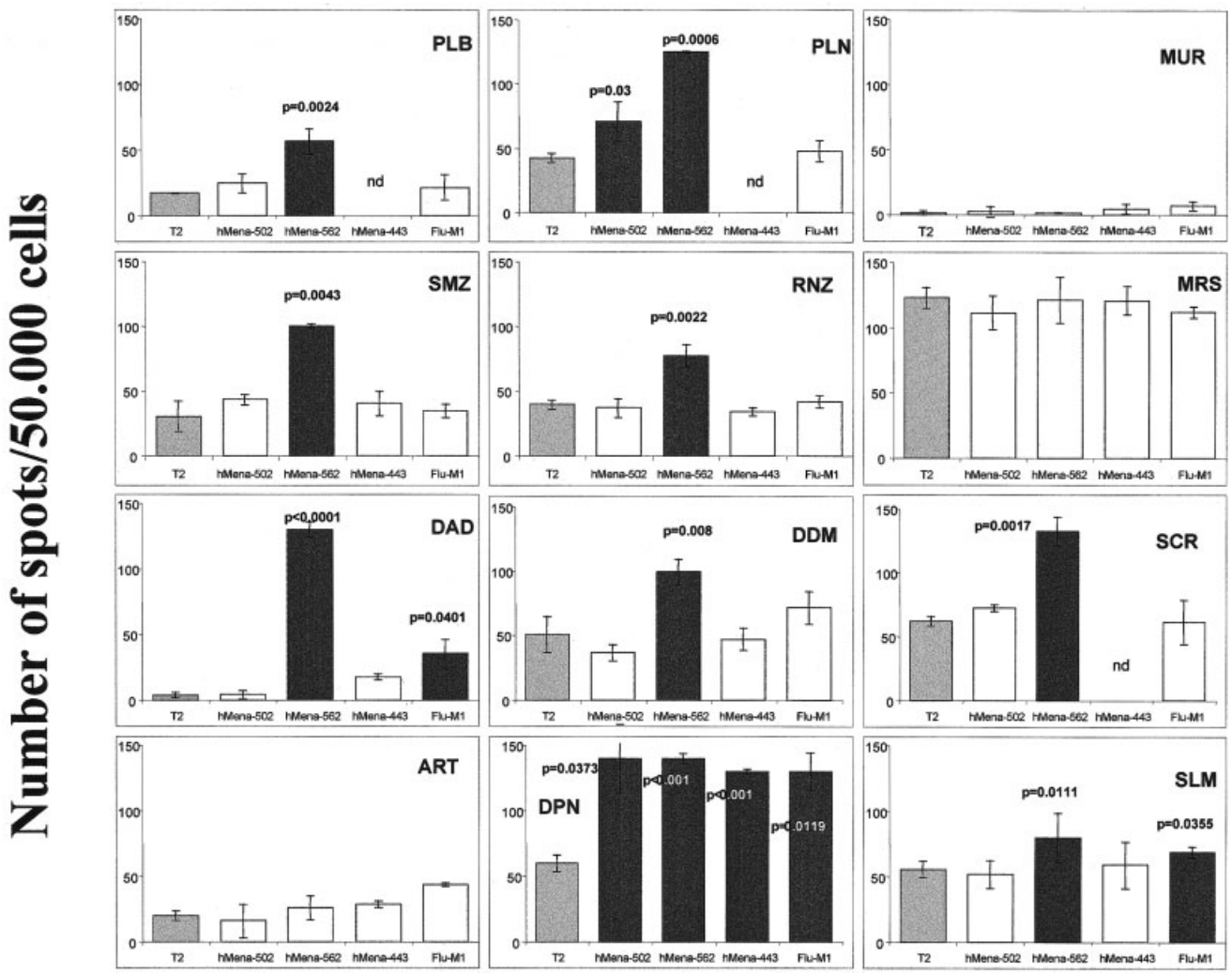

FIGURE 4 – $CD8^+$ T-cell reactivity of HLA-$A2^+$ breast cancer patients against hMena peptides by IFN-γ ELISPOT assays in 12 HLA-$A2^+$ breast cancer patients. Peptides are enumerated as reported in text. After a culture period of 16 hr, IFN-γ spots were developed according to text and enumerated by computer-assisted video image analysis. Each bar represents the mean spot number of triplicates with 5×10^4 $CD8^+$ T lymphocytes initially seeded per well. The *p*-value refers to the comparison between mean spot number ± SD induced by $CD8^+$ T cells incubated with T2 cells (T2, gray histograms) and $CD8^+$ T cells incubated with T2 pulsed with each peptide and was calculated applying the *t*-test for unpaired samples. Histograms are reported in black when the increase in spot number was significant ($p \leq 0.05$) and represent the presence of $CD8^+$ T-cell precursors *versus* the peptides under consideration. Flu-M1 (influenza matrix-protein peptide) was employed as control for the assay conditions.

results reported in different models demonstrating that immunogenicity depends on a number of different parameters, including the association and/or dissociation rate of TCR/peptide-MHC complex.[34,35] A high frequency (1:400–1:2,000) of *in vivo* primed $CD8^+$ T lymphocytes was demonstrated in breast cancer patients and half of them showed concomitant serologic and cell-mediated immune responses. The recognition of hMena peptides was confirmed by cytotoxicity assay on a panel of different tumor targets. Cold/hot competition assay demonstrated that the hMena-502 peptide is naturally processed in the breast cancer cell line that is specifically recognized and lysed in an HLA-A2-restricted and CD3-dependent manner by the $CD8^+$ CTL line. The specific T-cell response was also evident toward an HLA-$A2^+$ melanoma cell line overexpressing hMena at protein level, suggesting that this peptide can be naturally processed and presented also in tumors other than breast cancer.

Altogether, these functional studies suggested a particular hMena phenotype in breast tumors and our results on the hMena expression, at the RNA and protein level, strongly indicate that the

TABLE III – HUMORAL AND $CD8^+$ T-CELL-MEDIATED IMMUNE RESPONSE AGAINST hMENA IN 12 HLA-A2-POSITIVE BREAST CANCER PATIENTS

Patients	hMena tumor expression[a]	hMena IgG	IFN-γ spots against hMena peptides		
			hMena-502	hMena-562	hMena-443
D.D.M.	+	+	−	+	−
S.C.R.	+	+	−	+	−
P.L.B.	+	+	−	+	−
P.L.N.	+	+	+	+	−
D.A.D.	+	−	−	+	−
S.L.M.	+	−	−	+	−
R.N.Z.	+	−	−	+	−
S.M.Z.	+	−	−	+	−
D.P.N.	+	nd	+	+	+
A.R.T.	+	−	−	−	−
M.R.S.	−	−	−	−	−
M.U.R.	−	−	−	−	−

[a]Evaluated by immunohistochemistry on paraffin-embedded section as reported in text.

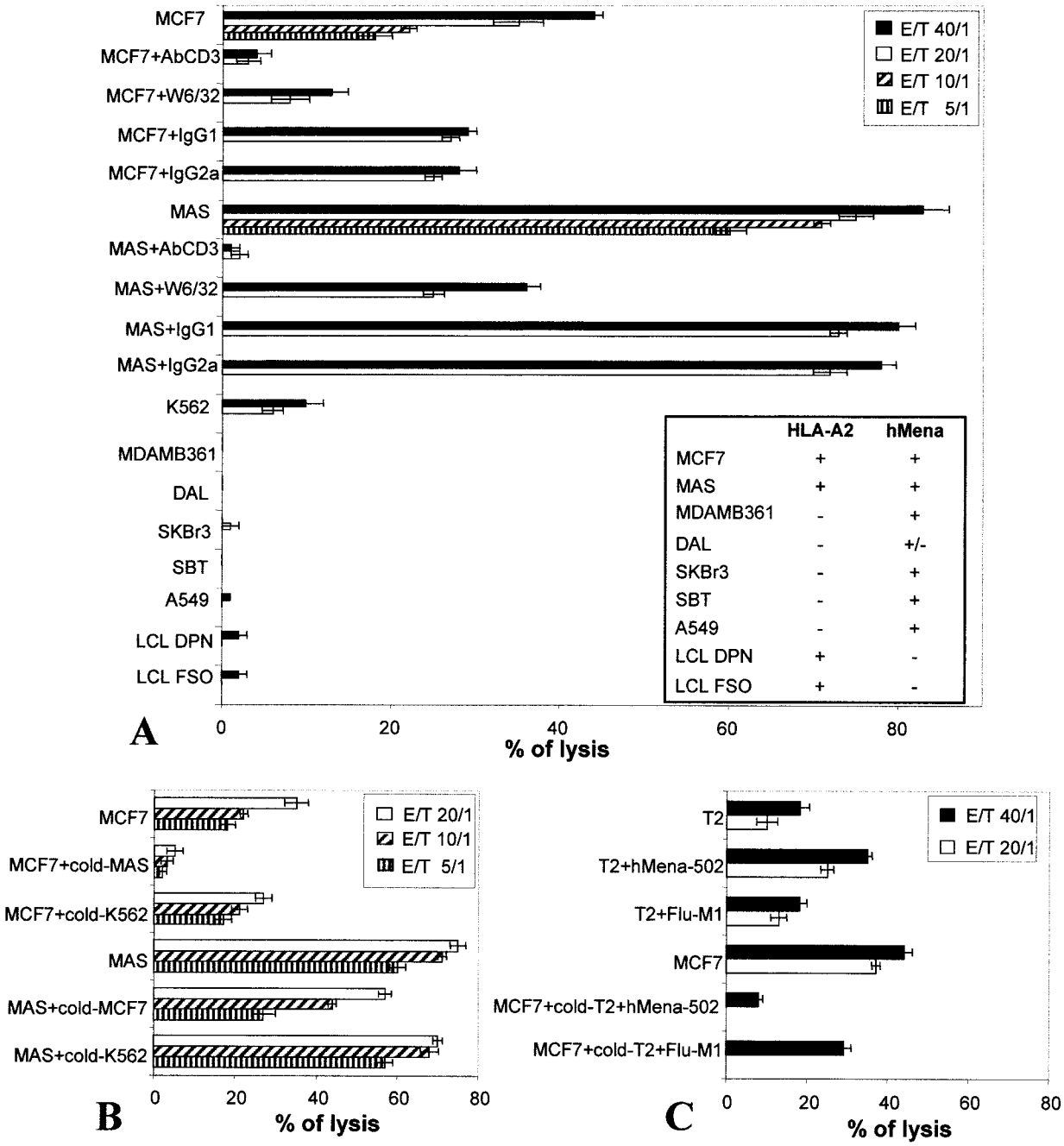

FIGURE 5 – Cytotoxic activity of hMena-502 D.P.N. CTL line. (*a*) Cytotoxicity of hMena-502 D.P.N. CTL line against tumor targets in a 4-hr ^{51}Cr release assay. The target cells tested were MCF7, an HLA-A2^{+}/hMena^{+} breast tumor cell line; MAS, an HLA-A2^{+}/hMena^{+} melanoma cell line; K562, an NK-sensitive erythroleukemia cell line; DAL, SKBr3, MDA-MB-361, SBT breast cancer cell lines; A549, lung cancer cell line; LCL D.P.N. and LCL F.S.O., EBV-transformed B-cell lines. E/T, effector target ratio. The HLA-A2 expression, evaluated by FACScan analysis using the BB7.2 mAb, and the hMena expression evaluated by Western blot analysis, of each cell line are reported in the box. The assay was performed in the presence of 10 μg/ml of anti-CD3 mAb (IgG1), anti-HLA class I mAb W6/32 (IgG2a), W6/800 IgG1 isotype control, W6/100 IgG2a isotype control. (*b*) Cold/target competition assay. The cytotoxicity of hMena-502 D.P.N. cell line was tested against MCF7 and MAS target cells in the absence or presence of the indicated unlabeled competitor cell lines at a 30:1 ratio. (*c*) Cytotoxic activity of hMena-502 D.P.N. line on T2 cells unpulsed or pulsed with the hMena-502 peptide. Cold target competition assay was carried out at E/T ratio of 40:1 on MCF7 in the presence of unlabeled hMena-502-pulsed T2 cells or T2 pulsed with an irrelevant peptide (Flu-M1). Results are representative of 3 independent experiments.

cancer-restricted seroreactivity and hMena immunogenicity may be related to a particular pattern of expression. Northern blot analysis revealed that hMena mRNA expression is neither breast cancer-restricted, being shared by cell lines of various malignancies, nor cancer-restricted, as it is detectable in some normal tissues. The analysis of protein expression confirmed the results of Northern blot in the tumor samples, whereas the hMena mRNA expression in heart tissue was not confirmed at protein level, as evaluated by immunohistochemistry. Expression of murine Mena has been described at the protein but not at the mRNA level and

has been shown in various normal tissues and cell types.[36] In addition, Mena protein undergoes downregulation during the heart development, and high levels of Mena are detected only in cultured neonatal, but not in adult cardiac myocytes.[36] Immunohistochemical analysis on normal tissues revealed that hMena protein is expressed in some epithelia, not including the acinar and ductal breast epithelium. On the other hand, hMena was highly expressed in myoepithelial cells of various tissues, including breast myoepithelium.

To our knowledge, no data on Mena expression in murine tumor models have been reported. A detailed analysis of breast tumor cell lines and primary breast tumors of different histologic origin, including the primary tumor used for the SEREX analysis, showed a clear hMena overexpression as compared to normal epithelia. It is interesting that the HLA-A2 patients showing a $CD8^+$ T-cell response against the hMena epitopes identified also overexpressed hMena in the primary tumors.

The finding that normal proteins controlling cytoskeleton may be immunogenic in cancer patients is not unprecedented. In medullary breast carcinoma, actin has been described as target of oligoclonal B cells.[37] Moreover, a number of cytoskeleton proteins differentially expressed in renal cancer and the normal counterpart and capable of inducing a humoral immune response have recently been identified in cancer patients by PROTEOMEX.[14]

In agreement with our results of hMena overexpression in primary breast tumors, the first proteomic analysis on breast cancer showed a great divergence between *in situ* breast cancer and matched normal tissue in the expression of a cluster of proteins controlling cytoskeleton dynamics.[13]

hMena is encoded by a novel human gene we identified, which, based on cDNA sequence analysis, represents the human homologue of the murine Mena gene. Mena is a member of the Ena/VASP, a family of proteins involved in the control of actin-cytoskeleton dynamics. These proteins are critical for cell motility[11,38] and epithelial cell scaffolding,[12] events frequently deregulated in transformed cells.[39,40] Since Ena/VASP proteins are involved in the control of cell shape and development, the detailed analysis of hMena expression in tumors of different aggressiveness and in preneoplastic lesions may establish a role of this protein in tumor progression. In this context, it is worth mentioning the recent finding that breast cell invasive potential correlates with the myoepithelial phenotype[41] and we report herein hMena expression in breast myoepithelium.

In view of the present finding, it will be relevant in future studies to correlate immune response to hMena with clinicopathologic parameters using different methodologic approaches, such as recombinant protein in ELISA assays and the SADA methodology, which, in block testing, allows the antibody reactivity of a large number of SEREX-defined antigens.[42]

To our knowledge, this is the first report describing a concomitant antibody and T-cell response directed against a cytoskeleton-regulatory protein. Thus, hMena is an attractive model for exploring the breast tumor progression and the kinetics of the correlated immune response, contributing to new insights in breast cancer biology management.

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