Human B-Cell Immune Response to the Polymorphic Epithelial Mucin

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Abstract

Human antibodies generated by Epstein-Barr virus immortalized B-cells from tumor-draining lymph nodes of an ovarian cancer patient were screened for reactivity in enzyme-linked immunosorbent assay with a synthetic peptide corresponding to the protein core of the polymorphic epithelial mucin. Epitopes within this region are in fact considered tumor specific since they are selectively exposed in tumor cells due to aberrant glycosylation. Human antibody BBS, thus selected, reacts in enzyme-linked immunosorbent assay and immunohistochemistry with polymorphic epithelial mucin-expressing tumor cells. This is the first demonstration of the existence of a B-cell immune response to selected epitopes of polymorphic epithelial mucin and, together with the cytotoxic T-cell response already demonstrated, constitutes the basis for the use of synthetic peptides as a vaccine in cancer patients.

Introduction

The PEM belongs to the family of highly glycosylated, high molecular weight glycoproteins present on the surface of many epithelial cells. This membrane anchor protein is a very polymorphic molecule due to the presence of a variable number of tandem repeats of a 60-nucleotide sequence (MUC-1). O-Linked carbohydrates attached to seryl and threonyl residues of the molecule contribute to the polymorphism observed. In tumor epithelial cells mostly of breast and ovarian origin the PEM molecule appears to be overexpressed and distributed along the entire cell surface and cytoplasm. The differences found in the antigenic profile between the normal and malignant mucin appear to be caused by the degree of glycosylation of the protein backbone that would allow and/or increase the exposition of antigenic determinants.

A number of MoAbs thus far generated and showing restricted reactivity versus epithelial tumor cells recognize epitopes within the protein core region of the molecule.

We present here evidence of a B-cell immune response to the PEM molecule in an ovarian cancer patient and the isolation and partial characterization of a human antibody against PEM obtained by immortalization of B-cells from human tumor-draining lymph nodes.

Materials and Methods

Synthetic peptides with the amino acid sequence PDTRPAGSTAP corresponding to the central portion of the tandem repeat of PEM were prepared by solid phase synthesis with an AB 430A (Applied Biosystem, Foster City, CA) using hydroxymethyl(phenoxymethyl) resin using N-9-fluorenylmethoxycarbonyl chemistry. Deprotection of the NH2-terminal N-9-fluorenylmethoxycarbonyl protecting group was performed using the appropriate scavengers. The crude peptide was recovered by ethyl ether precipitation from the above acidic mixture and purified by high performance liquid chromatography on a Vidad C18 column (Vidac, Esperia, CA). The amino acid sequence was then determined by amino acid analysis. The peptide was conjugated either with BSA or KLH in 10 mM phosphate-buffered saline using 1% glutaraldehyde. The reaction was stopped after 2 h and the conjugates were lyophilized following extensive dialysis.

Tumor-draining lymph nodes were derived from a 22-year-old patient with a well differentiated serous adenocarcinoma of the ovary (stage I International Federation of Gynaecology and Obstetrics). Two years before admission to the Department of Gynaecology, the patient underwent right ovariectomy for a benign serous adenoma of the ovary. On admission the serum level of CA125 was 28. The patient underwent total hysterectomy with left ovariectomy, total omentectomy, appendectomy, and radical paraaortic and pelvic lymphadenectomy. Overall, 109 lymph nodes were removed (70 in the pelvis and 39 in the paraaortic areas). Histologically all the lymph nodes were negative. A representative sample of three lymph nodes was immediately transported to the laboratory in sterile culture medium. At the time of this report, the twelfth postoperative month, the patient is clinically free of disease.

Single cell suspension of the specimen was performed within 3 h from surgery. B-cells were immortalized using a semipurified preparation of EBV from the B 95-8 marmoset transformed leukocyte EBV-producing cell line in culture medium supplemented with 0.5 µg/ml cyclosporine A. After 3 weeks immortalized B-cells were seeded in 24- and 96-tissue culture plates and the supernatant was tested for human antibody production by ELISA. Production of specific human antibody was assessed by ELISA using the synthetic peptide complexed to BSA or KLH and incubated at 1 µg/50 µl in carbonate buffer for 2 h in 96-well polystyrene plates (Linbro). Alternatively uncoupled PEM-corresponding peptide and an unrelated synthetic peptide (gonadotropin-releasing hormone) for negative control were attached to glutaraldehyde-activated microplates. The second antibody used was a peroxidase-conjugated F(ab)2 fragment anti-human Ig G + A + M. ELISA was performed on cell lines as described previously. The indirect immunoperoxidase assay was used on an acetone-fixed cytospin preparation of cells and on formalin-fixed paraffin-embedded tissue sections. For these experiments the same linker as that of the ELISA was used and optimal staining was obtained by prolonging the incubation period of the primary antibody to 24 h in a humidified chamber at 4°C. Negative controls consisted in omitting the primary antibody or adding an unrelated antibody of identical isotype during the first incubation. The positive control for PEM was the murine MoAb 436 that recognizes the RPAP amino acid sequence within the tandem repeat.
Results and Discussion

Mucins, in particular PEM, have been proposed as potential vaccine candidates mainly on the basis of the high immunogenicity of the molecule. Although specific T-cell responses have been found in humans, no B-cell response has yet been demonstrated.

The rationale of our study was to investigate whether a B-cell immune response to PEM was present in cancer patients. Lymphocytes from an ovarian cancer patient were EBV transformed and immunoglobulin-producing B-cell clones were tested in ELISA for specific antibody production for the PEM tandem repeat. A 12-amino acid synthetic peptide corresponding to the central amino acid sequence of the tandem repeat that composes the protein core of PEM was used as the target antigen. Several B-cell clones showing a variable reactivity in ELISA to the synthetic peptide were obtained. One of these B-cell clones, clone BB5, producing an IgM antibody which shows a high reactivity with the synthetic peptide, was cloned by cluster picking on 96-well plates. The results of the ELISA are given in Table I. BB5 antibody displayed high reactivity only versus the PEM-corresponding peptide alone and coupled with either BSA or KLH but results were negative with the unrelated synthetic peptide used as control.

In order to evaluate if the human antibody BB5 isolated was able to detect the PEM molecule as it is expressed on tumor cells, ELISA was performed on a number of established cell lines. Table 1 summarizes these results. Human antibody BB5 reacted with several established cell lines of different origin. In all experiments the murine MoAb 436 generated in our laboratory (7), the minimum binding site of which maps in the RPAP sequence of the tandem repeat, was used as the positive control. All the PEM-expressing breast and ovarian human cancer cell lines (MCF-7, ZR 75-1, T47-D, SK Br-3, OVCA 433) were positive for binding with the human antibody while the colon carcinoma HT-29, the epidermoid cancer A431, the melanoma A375 human cell lines and the fibroblast murine NIH-3T3 cell line showed no binding of the BB5 antibody. The distribution of the PEM antigenic determinant recognized by the BB5 human antibody was evaluated by immunohistochemistry staining of tumor cells. Fig. 1 shows the immunoperoxidase images obtained from a cytospin preparation of the MCF-7 breast carcinoma cell line (Fig. 1A) and a paraffin section of an infiltrating duct breast carcinoma with mucinous aspects (Fig. 1B). The immunostaining performed with BB5 has, in both samples, a heterogeneous distribution among the tumor cells. Membrane staining is mostly evident in the cell line whereas in the tumor tissue sample the antigen distribution has a more marked cytoplasmatic location.

The pattern of reactivity of the human antibody BB5 indicated by the results obtained demonstrates that this antibody reacts with the protein core portion of the PEM molecule. Nevertheless, it cannot be excluded, as for other murine MoAbs, that adjacent carbohydrate structures may influence the binding of the antibody. To this end it may be interesting to further define the minimum binding epitope of this human antibody. The reactivity of the antibody in both ELISA and immunoperoxidase assay with established PEM-expressing cell lines further demonstrates that the antigen recognized is the PEM molecule as it is mounted in the plasma membrane of the tumor cells tested. Interestingly ELISA conducted on plasma samples of the patient revealed measurable antipeptide titers in the pre- and postsurgery serum.
Several investigators have in the past attempted to generate human antibodies against the tumor phenotype (12-14). Difficulties were mostly encountered in the screening process due to high backgrounds obtained when using human material. The use of synthetic peptides corresponding to the deduced amino acid sequence from the cloned gene facilitates this task enormously by allowing the screening of several B-cell clones. In this case we chose a 12-amino acid sequence corresponding to the central part of the tandem repeat of PEM that includes the putative recognition site of previously isolated CTL clones from cancer patients. This screening method facilitates the selection of antibodies reacting with the protein moiety of the molecule as compared to the high number of human antibodies thus far described which appear to recognize mostly carbohydrate epitopes (12-14) and are often not the result of an elicited immune response. For the PEM molecule the fact that BB5 recognizes the protein portion of the molecule is of particular interest since it is now well documented that tumor cells express a modified PEM wich is aberrantly glycosylated. The altered glycosylation would allow the expression of tumor-specific protein epitopes that are usually buried in the normal cell plasma membrane (15).

A good deal of evidence supports the idea that tumors can elicit an immune response in vivo. Morphologically this has been described as the presence of mononuclear cell infiltration in tumors and/or sinus hyperplasia in the lymph nodes. Immunohistochemistry studies have also demonstrated the presence of tumor-associated antigenic determinants within these structures in breast and colon cancers (16, 17). In particular for breast cancer this has been associated with a favorable prognosis, although this matter is still controversial (18, 19).

The isolation of a human antibody specific for an antigenic determinant identified within the protein core sequence of PEM and selectively expressed on tumor cells finally demonstrates the presence of a humoral immune response in cancer patients. Future studies should be directed toward the evaluation of PEM-primed mononuclear cells in cancer patients for the possible correlation with prognosis and tumor burden. Moreover, these results strongly support the use of mucins and or synthetic derivates as a vaccine in cancer patients.

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References


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