Humoral Immunity against a Tandem Repeat Epitope of Human Mucin MUC-1 in Sera from Breast, Pancreatic, and Colon Cancer Patients

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Abstract

Using synthetic peptides 60, 80, and 105 residues long, corresponding to 3, 4, and 5.25 tandem repeats of human mucin MUC-1 protein core, as antigens in a solid-phase enzyme-linked immunosorbent assay, we screened sera from 24 breast cancer patients, 10 colon cancer patients, and 12 pancreatic cancer patients, at various stages of disease, for the presence of mucin-specific antibodies. The 105-residue peptide was superior in allowing detection of high levels of anti-mucin antibodies in 10.9% of sera in each cancer group. Another 4.3% showed intermediate reactivity. Lower levels of detection were achieved with the 80-residue peptide, and no specific reactivity was detectable with the 60-residue peptide. Anti-mucin antibodies were previously undetectable when this assay was performed with purified whole mucin or short synthetic peptides. The presence or absence of antibody did not correlate with the levels of circulating mucin or stage of disease. One highly reactive serum sample was used to identify more precisely the epitope on the long synthetic peptide to which the reactivity was directed. The reactivity of this serum specific for the 105-residue peptide was blocked by a 9-residue peptide from the NH2-terminal region of the 20-residue tandem repeat containing the previously identified immunogenic epitope APDTRP. Another 9-residue mucin peptide, from the COOH-terminal region of the tandem repeat which does not contain the APDTRP epitope, had no effect. All the mucin-specific reactivity was found to be of the IgM isotype, indicating a helper T-cell-independent response, unusual for an antibody against a peptide epitope, but not unexpected for tandemly repeated epitopes.

Introduction

In a certain percentage of cancer patients tumor-specific humoral immune responses have been found directed against self-proteins that are either overexpressed, e.g., oncoproteins c-myc in colorectal cancer (1) and c-erbB-2/HER-2/neu in breast carcinoma (2, 3), or mutated, e.g., tumor suppressor protein p53 in lung cancer (4). While the therapeutic benefit of these antibodies is currently unknown, knowledge of potential antigens on tumors which can evoke tumor-specific immune responses is essential for exploring the immunotherapy of cancer as a clinical treatment.

Tumors of epithelial origin have been shown to express various mucin proteins on their surfaces. Human epithelial mucins are a family of high molecular weight glycoproteins with a large number of O-glycosylated tandem repeat domains which vary in number, length, and potential extent of O-glycosylation (5–10). Aberrant glycosylation of mucins by tumor cells has been shown to result in the differential expression of novel mucin epitopes that are associated with tumors (11–15). We have previously described tumor-reactive cytotoxic T-lymphocytes in breast (16), pancreatic (17), and ovarian (18) cancer patients, specific for the tandem repeat epitope on the polypeptide core of the mucin MUC-1. Until recently there was no evidence of a parallel mucin-specific humoral response. One explanation given for the failure to detect it was that the antibody may form antigen-antibody complexes with circulating mucin and thus would not be detectable. This was plausible inasmuch as patients with large tumor burdens are known to have increased levels of mucin in circulation (19). We considered an alternative possibility that the target antigens previously used to detect antibody reactivity, whole mucins purified from tumor cells or very short synthetic peptides corresponding to the polypeptide core sequences, may lack the precise immunogenic epitopes and thus would be suboptimal reagents. A recent report describing the isolation of EBV-immortalized B-cell clones from ovarian cancer patients which secreted mucin-specific antibody with reactivity against a specific epitope on the tandem repeat (21) confirmed the need for an optimal antigenic structure with which to look for such antibodies in the serum. We report here the first successful attempt to detect mucin muc-1-specific antibodies in sera of cancer patients. Our success was primarily due to the use of a chemically synthesized 105-residue peptide that contained 5.25 tandem repeats and closely mimicked the native structure of deglycosylated muc-1.

Materials and Methods

Patient Sera. Sera from patients with breast cancer or from normal donors were collected and provided by the Pittsburgh Cancer Institute Serum and Tissue Bank. Sera from pancreatic and colon cancer patients were collected at Duke University Medical Center, Durham, NC. Those tested in this study were selected for either elevated or normal levels of circulating mucin expressing the DUPAN-2 epitope (20).

Synthesis of Tandem Repeat Peptides. The peptides (20, 40, 60, 80, and 105 amino acids) corresponding to 1, 2, 3, 4, and 5.25 tandem repeats of muc-1 protein core were synthesized by a manual solid-phase synthesis using 9-fluorenylmethyloxycarbonyl NH2-terminal protected amino acids. Synthesis, purification, and characterization by mass spectroscopy of the products are described in detail elsewhere (10, 22). Briefly, peptides were synthesized using a manual Rapid Multiple Peptide Synthesizer apparatus from Dupont (Boston, MA). The expanding resins were divided after the synthesis of 30 amino acids was complete to allow sufficient volume for the growing peptide chains within the cartridge. The products of the synthesis were deprotected and cleaved from the resin support in concentrated trifluoroacetic acid in the presence of the suitable scavengers. The trifluoroacetic acid-soluble products were extracted sequentially in organic solvents and then transferred to water and lyophilized. The peptides were purified by conventional gel filtration and reverse-phase high pressure liquid chromatography. The shorter peptides,

The abbreviations used are: EBV, Epstein-Barr virus; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; ID, identification.

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ELISA for Detection of Mucin-Specific Antibodies. Detection of mucin peptide-specific antibody in sera was carried out by ELISA using synthetic peptides corresponding to 1, 2, 3, 4, or 5.25 tandem repeats of muc-1 protein core as follows. The peptide was dissolved in 0.05 M bicarbonate buffer (pH 9.6) at a concentration of 10 µg/ml. The solution (50 µl) was dispensed into 96-well plates and incubated overnight at room temperature. After removal of the peptide solution the plate was washed twice with PBS, pH 7.4. Next, the remaining protein-binding sites were blocked with a 1-h room temperature incubation of 100 µl of a 5% Carnation dry milk (Blotto) solution in each well and the solution was removed after 1 h. The plate-bound antigens were then reacted with antibodies in 50 µl of sera diluted appropriately with 5% Blotto. After a 1-h primary antibody incubation, the diluted serum was removed and the plate was washed 3 times with 200 µl of 0.2% Tween 20 in PBS. Next, the plate was incubated for 1 h with 50 µl of the secondary antibody consisting of goat anti-human polyvalent (IgM, IgA, IgG) antibody purchased from Sigma Chemical Co. (St. Louis, MO) and diluted either 500- or 1000-fold with 5% Blotto. After removal of the secondary antibody the plate was washed 3 times with 0.2% Tween 20 in PBS. Detection was accomplished using 100 µl of a 1.0-mg/ml solution of o-phenylenediamine in 0.05 M citrate buffer (pH 4.0) with 0.015% H₂O₂. The reaction was stopped after 1 h incubation in the dark with 50 µl of 2.5 M H₂SO₄ and the absorbance at 492 nm was measured.

The specificity of the antibody response was analyzed using 9-residue peptides that either contained the putative immunodominant epitope [p18—26, SAPDTRPAP] or did not [p11—19, APPAHGVTS]. The non-mucin control 12-residue peptide was derived from a small ribonuclease protein La sequence, amino acids 253—264. The putative immunodominant mucin epitope is formed by the sequence APDTRP. The diluted sera were incubated with different concentrations (0.01, 0.1, 1.0, 5.0, 10.0, and 15.0 mg/ml) of blocking peptides overnight at room temperature in PBS and 5% Blotto. Next, the ELISAs were performed as described above with the 105-residue peptide as antigen in order to detect inhibition of serum antibody reactivity by specific peptides.

Immunohistological Staining of Tumor Samples. Tumor samples were obtained from surgical specimens, embedded in O.C.T compound (Miles, Inc., Elkhart, IN) and stored frozen (−70°C), or embedded in paraffin. All tissue samples were supplied by the Pittsburgh Cancer Institute Tumor and Tissue Bank. Cryostat sections were fixed in acetone and then stained by an indirect immunoperoxidase technique using the Vectastain Elite ABC Kit (Vector Laboratories, Inc., Burlingame, CA). Primary tumor-reactive mucin core-specific antibodies SM3 and HMFG-2 (23, 24) were a gift from Dr. J. Taylor-Papadimitriou, London, United Kingdom, and antibody BC2 (25) was a gift from Dr. I. F. C. McKenzie, Melbourne, Australia.

Results

Anti-Mucin Antibody Reactivity Is Present in Sera from Cancer Patients. We screened sera from 24 patients with breast cancer, 10 patients with colon cancer, and 14 patients with pancreatic cancer by ELISA using the 105-residue-, 80-residue-, and 60-residue-long synthetic peptides corresponding to 5.25, 4, and 3 tandem repeats of MUC-1 protein core. The results are shown in Table 1. Two of 24 breast cancer sera (8.3%) (sample ID 1754 and 2672), two of 12 pancreatic cancer sera (16.2%) (sample ID 1893 and 1716), and 1 of 10 colon cancer sera (10%) (sample ID 1386) showed a strongly positive reaction. We have defined strongly positive as absorbance at 492 nm >3 times the absorbance obtained with the normal and nontumorous group in our ELISA. In addition, one other serum from a pancreatic cancer patient and one serum from a colon cancer patient are considered positive in the intermediate category (Table 1), due to their reactivity being 1.5—3 times higher than the absorbance level obtained for the control or the frankly negative cancer sera in the ELISA. Individual normal sera never showed positive reactivity and were pooled and used in many repeat ELISAs as a pooled negative sera control. Inasmuch as the majority of patient sera were also nonreactive they served as additional negative controls.

One explanation for the lack of detection of anti-mucin antibody in serum from patients with muc-1-positive tumors could be the presence of circulating mucin which could form immune complexes with the antibody, thereby preventing its reactivity. The serum samples from pancreatic and colon cancer patients were chosen because they had been previously characterized for the presence of circulating mucin. For example, patients 1893 and 1716 had elevated levels of circulating mucin, as detected by a competition radioimmunoassay using DUPAN-2 (19), and simultaneously made antibodies specific for muc-1. Conversely, serum from a colon cancer patient had high levels of anti-muc-1 antibodies and normal levels of circulating mucin.

In order to exclude the possibility that the absence of antibody may have been due to the absence of mucin or the absence of expressed mucin polypeptide core epitopes on the patient's tumor (an unlikely possibility inasmuch as mucin-negative breast tumors have not been reported), we performed immunohistological analysis on ten tumor samples, two from the two breast cancer patients with strongly positive reactivity in ELISA (sample ID 1754 and 2672) and eight from breast cancer patients who did not show anti-mucin reactivity. Table 2 shows that all tumor samples expressed mucin- and tumor-associated mucin polypeptide core epitopes as indicated by staining with the monoclonal antibodies BC-2, SM-3, and HMFG-2. Clearly, all these patients had the potential of being immunized with muc-1.

The level of detection of antibody reactivity in cancer patient serum was critically dependent on the choice of antigen used for detecting the antibodies. Fig. 1 shows that we were able to detect very high levels of anti-mucin antibodies using the 105-residue peptide, while much lower levels of antibodies were detected with the 80-residue peptide, and no antibodies were detected with the 60-residue peptide (Fig. 1). These dramatic differences emphasize the critical nature of the antigen used to detect the antibodies. We next examined what antibody isotype(s) were responsible for the anti-mucin reactivity. Fig. 2 shows that all anti-mucin reactivity in the positive sera was found in the IgM isotype.
Serum Anti-Mucin Reactivity Is Directed against APDTRP Epitope. Muc-1 specificity of the serum antibody reactivity was assessed using a blocking experiment in which the 20-, 40-, 80-, and 105-residue mucin tandem repeat peptides were preincubated with serum to test their ability to inhibit the reactivity and using the 105-residue peptide as the antigen bound to the plate. Fig. 2A shows that peptides corresponding to the tandem repeat domain of human mucin muc-1 are able to inhibit up to 80% of the serum reactivity in a concentration dependent manner. As expected, peptides containing more tandem repeats are able to maximally inhibit the reactivity at increasingly lower concentrations (Fig. 3A).

Fine specificity of the antibody reactivity was determined in a similar blocking experiment using 9-residue peptides which either contained APDTRP or did not contain this sequence and using the 105-residue peptide as the antigen bound to the plate (Fig. 3B). The mucin 20-amino acid tandem repeat domain has a sequence (PDTRPAPGSTAPPAHGVTSA)\textsubscript{80}. The peptide p18–26 (SAPDTRPAP) contains the putative immunodominant epitope, and not by peptide p11–19, excluding the immunodominant epitope. A 20-residue peptide (20-mer) was used as a positive control, and a non-mucin 12-residue peptide (cont. peptide) was used as a negative control. Serum 1754 was used for this experiment. All data are shown as means ± SD (bars) of three data points.

### Table 2. Breast tumor tissue staining with mucin-specific antibody

<table>
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<th>Sample ID no.</th>
<th>Disease stage</th>
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<th>Antibody&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>IV</td>
<td>+</td>
<td>BC2 SM3 HMPFG2</td>
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<tr>
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<td>II</td>
<td>+</td>
<td>++</td>
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<td>+</td>
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</table>

<sup>a</sup> Reactivity of serum is defined in Table 1.
<sup>b</sup> Definition of tumor reactivity is: ++ +, uniformly positive; ++, positive; +, weakly positive; −, negative.

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**Fig. 1.** Importance of a longer peptide for detecting anti-mucin antibody reactivity in patients’ sera. Two sera (nos. 1754 and 2672) which demonstrated positive ELISA reaction against the 105-residue peptide were tested against the 80- and 60-residue peptides. All data are shown as means ± SD (bars) of three data points.

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**Fig. 2.** Determination of the immunoglobulin isotype of the anti-mucin antibodies in sera from breast cancer patients. Two sera (nos. 1754 and 2672) which demonstrated positive reaction against the 105-residue peptide were used for this experiment.

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**Fig. 3.** Specificity of the serum reactivity for mucin polypeptide core tandem repeat sequences. (A) Serum reactivity against the 105-residue peptide is blocked by previous incubation with 20-, 40-, 80-, and 105-residue peptides. (B) Serum reactivity is blocked by previous incubation with a 9-residue peptide, p18–26, containing the immunodominant epitope, and not by peptide p11–19, excluding the immunodominant epitope. A 20-residue peptide (20-mer) was used as a positive control, and a non-mucin 12-residue peptide (cont. peptide) was used as a negative control. Serum 1754 was used for this experiment. All data are shown as means ± SD (bars) of three data points.
Discussion

Data presented in this paper show that patients with epithelial cell tumors of the breast, pancreas, and colon, all of which uniformly express mucin muc-1 glycoprotein on their surfaces, can mount a humoral immune response to this antigen. The only other evidence that anti-mucin antibodies exist in cancer patients had been provided previously by the isolation from a patient with ovarian cancer of an EBV-immortalized B-cell clone making a mucin-specific antibody (21). While this result suggested the potential of an antibody response it could not predict its significant presence in the serum, or the frequency with which it occurred. By creating a long synthetic peptide with five repeated epitopes and a native structure (10) we created a reagent capable of detecting anti-mucin antibodies in the serum as well.

The frequency of patients with anti-mucin responses which we found (8.3% in breast cancer, 16.7% in pancreatic cancer, and 10.0% in colon cancer) is similar to frequencies reported by others of humoral responses against tumor protooncogenes and oncogene suppressor genes. For example, 9% of breast cancer patients were found to have anti-p53 antibodies (26), and anti-c-myc antibodies were detected in 19% of breast cancer patients and in 16% of colon cancer patients (27).

Our findings, although limited to a relatively small number of patients, have importance for several specific reasons. We had shown previously that patients with these same mucin-expressing malignancies have T-cells which can be expanded in vitro and shown to be cytotoxic for the tumors and specific for an epitope on the mucin polypeptide core tandem repeat (16, 17). Data in this paper show that mucin tandem repeat is recognized by the B-cells as well. Moreover, by using several anti-mucin mouse monoclonal antibodies to block the T-cell reactivity we had narrowed the specific epitope recognized by the T-cells to the sequence APDTRPAP. This is a sequence which appears to be most immunogenic in the mouse, and most mouse antibodies specific for the mucin polypeptide core, rather than the carbohydrate epitopes, recognize a part or all of this sequence. This sequence is apparently immunodominant in the humans as well, inasmuch as not only T-cells, but, as we here show, the total anti-mucin serum antibody response as well appears directed against it.

The same epitope was recognized by the antibodies produced by the EBV-immortalized B-cells (21).

The predominant IgM isotype of the anti-mucin antibody is not surprising when the mucin tandem repeat structure is considered. Multiple identical epitopes are known to be capable of generating a T-independent B-cell response. Most such antigens are carbohydrate in nature, and here we show that proteins can have the same capacity, as long as they are tandemly repeated structures. Lack of other immunoglobulin-isotypes may be surprising, however, when one considers the existing and parallel mucin-specific T-cell response. We have shown previously that the T-cell response is directed against the native rather than processed and presented mucin epitope (16, 17). This may eliminate the possibility of cognate T-cell-B-cell interactions, during which the B-cells bind, take up, process, and present antigens to T-cells, which in turn provide cytokines necessary for isotype switching (28). Instead, both T-cells and B-cells can directly bind the mucin, and two parallel but not cooperative anti-mucin responses can develop (29). This may limit the overall effectiveness of the anti-mucin immune response against the tumor. It is attractive to speculate that influencing a more effective cognate T-cell help through carefully designed mucin vaccines may change the nature of this humoral immune response, potentially a very powerful one considering its tumor specificity. The potential destructive nature of an anti-mucin antibody, which could be harnessed for tumor destruction, can be shown in a recent report on detection of circulating anti-mucin antibodies in 5 of 19 patients with ulcerative colitis (30). The reactivity is directed to the same epitope on the mucin tandem repeat core expressed on this case in inflamed colonocytes. Moreover, it is associated with the sites of inflammation suggesting its pathogenic role. Most interestingly, the dominant isotype of the anti-mucin antibody in this destructive disease is not IgM but rather IgG.

References


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