Improved Detection of the Early Stages of Colon Cancer by Determining Both Free Circulating and Immune Complex-bound Antigens Reactive with Monoclonal Antibody

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Abstract

The measurement of both immune complex-bound and free unbound tumor-associated antigen was evaluated independently on a panel of sera from colon cancer patients by radioimmunoassay (RIA). A monoclonal antibody (mAb 46.3) raised against secreted antigens from human colon cancer cells in vitro was utilized in the RIA. When circulating immune complexes alone were analyzed, the data demonstrated that 5 of 5 (100%) Dukes' A patients and 11 of 16 (69%) Dukes' B patients had elevated levels of immune complexes reactive with mAb 46.3. Analysis of free circulating antigens demonstrated elevated levels of mAb 46.3-reactive antigen present in 5 of 5 (100%) Dukes' A patients and 15 of 16 (95%) Dukes' B patients. However, by analyzing total reactivity, defined by combining results from RIA with free and immune complex-bound antigen, the sensitivity of detection for Dukes' B increased to 16 of 16 (100%). Total antigen levels in sera from patients with benign diseases (ulcerative colitis, Crohn's disease, adenoma) were not significantly different from normal controls. Analysis of both free and bound antigen in RIA is, therefore, a more sensitive indicator than RIA with immune complex alone. For the advanced stages of disease, only 1 of 5 (20%) Dukes' C and 0 of 5 (0%) Dukes' D sera were positive for reactive immune complexes. When the combined RIA was evaluated, 3 of 5 (60%) and 1 of 5 (20%) Dukes' C and D sera, respectively, were positive with mAb 46.3. Taken together, these results show that RIA with mAb 46.3 is a sensitive indicator for the early stages of colon cancer.

Introduction

Recognition of tumor antigens by host antibodies is the first line of defense in the immunological control of tumor growth. However, some tumors are able to escape this control when the binding of antigen to antibody leads to the formation of immune complexes that interfere with the ability of the host to react effectively against the tumor. Serum levels of CICs have been shown to be elevated in many diseases, including a number of cancers (reviewed in Ref. 1). In the past, CIC levels have been quantitated by nonspecific methods such as the complement-dependent Clq-binding assay (2, 3) or precipitation of total serum CIC with PEG 6000 (4). The lack of specificity, sensitivity, and predictive value of these nonspecific assays are major factors that have placed limitations on the clinical usefulness of CIC determinations for the detection of malignant disease.

Recently, we developed a RIA that circumvents many of the short-comings inherent in previous CIC assays (5). In this assay, antigens dissociated from CIC isolated from patient sera are immunoprecipitated with a specific rabbit pAb raised against secreted antigens from HCCs in vitro (HCC-pAb). Unlike previous methods, this assay is based on the levels of CIC containing a specific group of antigens. This approach provided a more sensitive detection of the early stage of colon cancer compared to assays analyzing free antigens such as carcinoembryonic antigen, α-fetoprotein, and Ca 19-9 (6).

To provide an unlimited source of antibody and to improve the specificity and reproducibility needed for clinical applications, 22 different mAbs were developed against antigens reactive with HCC-pAb. In this report, we have used RIA to independently analyze the levels of immune complexes and free antigens in patient sera showing reactivity with one of these mAbs (mAb 46.3). Our results show that RIA with mAb 46.3 is a sensitive indicator of colon cancer, especially the early stages, and that the combined results from RIA with immune complexes and free antigen are more sensitive for early stages of colon cancer than immune complexes alone.

Materials and Methods

Serum Samples. Blood obtained from patients prior to colon cancer surgery was the source of the serum samples provided by the Laboratory of Cancer Biology (Department of Surgery, Harvard Medical School, Boston, MA). Blood samples from patients with benign diseases were obtained prior to colonoscopy examination at Roger Williams Gastroenterology Clinic (Providence, RI). Two groups of 10 serum samples each were used as normal controls. The first group was obtained from colonoscopy patients with negative results and the second group was obtained from healthy individuals, 25-50 years old, and pooled for use in this study. Sera were stored at −70°C and thawed only once before use.

Production of mAb 46.3. The hybridoma secreting mAb 46.3 was constructed using immune spleen cells isolated from a mouse immunized with antigens shed by the DLD-1 human colon carcinoma cell line (American Type Culture Collection, Rockville, MD). DLD-1 antigens recognized by HCC-pAb were isolated from culture medium by immunoaffinity chromatography on HCC-pAb immobilized on Protein A-Aff-Gel and cross-linked with dimethyl pimelimidate (Pierce, Rockford, IL) (7). A female BALB/c mouse was immunized 5 times during a 5-month period with 100 μg of the purified DLD-1 antigens eluted from the HCC-pAb affinity column in 0.1 M citrate, pH 2.3. Immune spleen cells were fused with myeloma cells as described previously (8). The reactivity of mAbs secreted by hybridomas growing in selective medium was assessed by indirect immunofluorescence analysis on acetone-fixed frozen sections of normal colon and primary colon carcinomas. Hybridomas displaying reactivity with primary colon carcinoma were cloned by limiting dilution and positive cultures were recloned in soft agar.

Indirect Immunofluorescence Microscopy. Frozen sections 4–6 μm thick were mounted on glass slides, acetone fixed, and stained with primary mAb. Affinity purified, fluorescein-conjugated, goat anti-mouse immunoglobulin obtained from Sigma Chemical Co. was used as secondary antibody. Nonspecific staining was assessed by examining tissue sections stained with culture supernatants from P3x63Ag8 myeloma cells. Sections were examined...
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igm purification and radiolabeling. IgM 46.3, a monoclonal antibody of IgM subclass, was produced by a hybridoma grown in mice as an ascitic tumor. IgM was purified initially by euglobulin precipitation (9), followed by high pressure liquid chromatography on hydroxylapatite (Knauer Saulentech, Berlin, Germany) as described by Josic’ et al. (10). Purified IgM was then radiolabeled with 125I-labeled sodium (Amersham) using 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (Iodo-Gen iodination Reagent; Pierce). The unincorporated radiodiode was removed by applying the reaction mixture to an Excellulose GF-5 desalting column (Pierce).

preparation of free and bound antigen solutions and radioimmunoassay. Serum samples (75 μl) diluted in 75 μl of phosphate-buffered saline were added to 200 μl of protein A-agarose (Schleicher & Schuell, Keene, NH), incubated for 30 min at 4°C, and then centrifuged. The supernatant containing FrAg was collected for RIA. Bound serum immune complexes, eluted with 200 μl 0.2 M glycine-HCl, pH 3.0, were also collected for the assay. RIA, with minor modifications, was performed as described previously (5). Separate tubes were each prepared with 75-μl aliquots of FrAg, CIC, phosphate-buffered saline, and 0.2 M glycine-HCl. Following the addition of 75 μl (50,000 cpm) of 125I-labeled mAb 46.3, the pH was raised to approximately 7.4 with K2HPO4 and 1% bovine serum albumin (75 μl) was added to minimize nonspecific binding of radiolabeled mAb. Immune complexes formed during a 30-min incubation at room temperature were precipitated after the addition of 225 μl of 5% PEG. Precipitates containing newly formed ICs from FrAg or reforming IC from CIC were collected after incubation for 60 min on ice using a Millipore sample unit (Millipore, Bedford, MA) with 0.45-μm filters. Sample tubes were washed three times with 225 μl of 2.5% PEG and the pooled washes were filtered in the corresponding sample well. Following three additional washes with 225 μl of 2.5% PEG, filters were removed, placed in tubes, and counted in a LKB Clinigamma counter (LKB Instruments, Gaithersburg, MD). Counts obtained from duplicate samples varied by less than 5%. Values were routinely corrected for radiolabel decay.

results and discussion

A humoral response to human neoplasms suggests the presence of TAAAs which are distinct from those of normal tissue from which they are derived (11). The interaction between TAAAs and host antibodies ultimately leads to IC in the serum of cancer patients. In two previous studies we reported the analysis of CIC (bound antigen) utilizing a specific rabbit polyclonal antibody (5-6), designated herein as HCC-pAb. We showed that this HCC-pAb in a RIA was capable of detecting IC-associated antigens present in the early stages of colon cancer (Dukes’ A and B). In the present study, mAbs were raised against antigens purified from the culture medium of the DLD-1 carcinoma cell line using immobilized HCC-pAb. A total of 22 hybridomas secreting mAbs reactive with primary colon carcinomas or normal colon tissue were identified by indirect immunofluorescence. One of these, designated mAb 46.3, showed a much greater intensity of staining on acetone-fixed frozen sections of colonic tumor tissue when compared to its adjacent normal tissue (Fig. 1). In the present study, we have used the serum-based RIA for CIC (5, 6) to examine the ability of this mAb to detect early stages of colon cancer.

Using mAb 46.3 in RIA, levels of total antigens, FrAg, and CIC of 10 normal sera and 11 each of benign and malignant diseases were analyzed. Patients with benign diseases included those with ulcerative colitis, Crohn’s disease, and adenoma, whereas malignant diseases were represented by colon cancer patients with Dukes’ A, B, C, and D. The results demonstrate that the total antigen level for the 10 of 11 malignant sera were elevated when compared to those of the normal and benign groups (Fig. 2).

Similar results were obtained in a separate study which included 31 sera from patients with malignant disease. The results are summarized in Table 1. Pooled serum from 10 healthy individuals was used as a normal control. For Dukes’ A, CIC and FrAg from 5 of 5 patients showed elevated reactivity with mAb 46.3 when compared to normal controls. When both CIC and FrAg values were combined, total reactivity with mAb 46.3 for cancer patient serum samples was 1.5–3-fold greater than in the pooled sera from normal controls. For Dukes’ B, the reactivity of mAb 46.3 with CIC and FrAg was elevated in 11 of 16 and 15 of 16 patients, respectively. However, the 5 patients with normal CIC (patients 11, 17, 18, 20, and 21) all showed elevated reactivity with FrAg. Similarly, the single patient (patient 9) with normal FrAg levels displayed elevated reactivity in RIA with CIC. Consequently, when total reactivity was considered, all 16 Dukes’ B patients tested positive with mAb 46.3. Combining the results from the two RIA thus increased the sensitivity as measured by true positives for Dukes’ A and B patients from 76% for CIC alone (16 of 21), a sensitivity comparable to our previous results with HCC-pAb (5), to 100% for combined assays (Table 1). When the true positive rate for separate RIA with CIC and FrAg are both greater than 69%, which is the rate for the CIC RIA alone, then the combined RIA significantly increased the probability of detection (P = 0.003 for a binomial distribution).

Taken together, these results indicate that the total levels of reactive antigen, as defined by the sum of cpm from the RIA for both CIC and

Fig. 1. Indirect immunofluorescence (fluorescein isothiocyanate) analysis of primary human colon carcinoma. (A) Marginal section showing normal tissue on the left and tumor on the right. (B) Tumor tissue only. Intensity of staining of mAb 46.3 in tumor tissue is much greater than in normal tissue.
FrAg, a more sensitive for detecting elevated reactivity with mAb 46.3 in the early stages of colon cancer (Dukes' A and B) than RIA for CIC or FrAg alone. Variations in the immune responses of individual patients demand the analysis of both free antigen and CIC to increase the sensitivity of detection. This increased sensitivity may result from the correspondingly higher FrAg levels in patients with a weak immune response to antigens bearing the mAb 46.3 epitope. The addition of cpm from FrAg may also compensate for the decreased CIC reactivity in sera from patients with antibodies that effectively compete for the mAb 46.3 epitope during the reformation of immune complexes. Conversely, addition of cpm from CIC may offset low FrAg reactivity resulting from a predominant immune response to other epitopes on antigens recognized by mAb 46.3. This situation would increase the levels of CIC reactive with mAb 46.3 and the incorporation of mAb 46.3 during the reformation of IC. Epitope-specific responses of this nature have recently been shown to be the basis for the protective humoral immune response observed in a subpopulation of human immunodeficiency virus-infected mothers (12).

In more advanced stages of colon cancer, the sensitivities of both single and combined RIA results with mAb 46.3 were significantly lower. RIA values with CIC were positive for only 1 of 5 and 0 of 5 patients with Dukes' C and D stage colon cancers, respectively, a result in agreement with previous findings (5). Corresponding values for RIA with FrAg were 3 of 5 for both Dukes' C and Dukes' D. Combined reactivity from both assays increased the sensitivity of detection from 20 to 60% for Dukes' C but was not effective in increasing the sensitivity for Dukes' D.

The RIA in this study was adapted from a previously described assay with HCC-pAb; thus, it might not represent the optimal format for a mAb-based immunoassay. Since mAbs recognize only a single epitope, the ability of mAbs to precipitate antigen is less efficient than that of pAbs. Generally, mAbs are also less effective in competing in the reformation of immune complexes with antibodies present in patient sera. mAb 46.3, which belongs to the IgM subclass, may reflect another disadvantage in the present RIA. IgMs have a lower affinity for antigen than IgG and the pentameric structure enhances the tendency for aggregation and precipitation, thereby increasing the background levels in the RIA. This tendency could explain the positive CIC reactivity observed in normal controls which is unlikely due to immune complexes formed as a result of an immune response to antigens bearing the mAb 46.3 epitope. These and other problems could be avoided by capturing both FrAg and CIC with a polyclonal antibody against TAA carrying the mAb 46.3 epitope. It also might be possible to utilize functional single-chain antibodies produced in bacteria using expression vectors containing polymerase chain reaction-amplified IgM μ-chain transcripts from the 46.3 hybridoma for detection. Current efforts are directed toward the development of these antibodies.

Despite the current limitations, RIA with mAb 46.3 is capable of detecting significant differences in the reactivity of mAb 46.3 with normal sera and those from patients with Dukes' A and B stage colon carcinomas. The assay is significantly less efficient in detecting the more advanced stages (Dukes' C and D; Table 1). Possible epitope changes during the course of the disease may account for the decreased sensitivity of mAb 46.3 for the later stages of the disease.

In summary, RIA with mAb 46.3 detects 100% of the patients with early stage colon cancer (Dukes' A and B) but is significantly less efficient (40%) in detecting the more advanced stages (Dukes' C and D; Table 1). Possible epitope changes during the course of the disease may account for the inability of the mAb 46.3 to be effective in the RIA for FrAg and CIC in the late stages. In addition, the impaired immune response of patients with late stage disease may also contribute to the decrease in the levels of CIC in the serum. Sensitivity for Dukes' C and D might be enhanced by utilizing a battery of mAbs against circulating TAA, an approach that would mimic the original RIA with circulating TAA.

Fig. 2. RIA values of FrAg, CIC, and total antigens (FrAg + CIC) for 10 sera from colonoscopy patients with normal results; 11 sera from patients with benign diseases such as ulcerative colitis (UC), Crohn's disease (CD), and adenoma; and 11 sera from patients with colon carcinoma of Dukes' A, B, C, and D stages. *, mean value of total antigens from normal sera (samples 1–10); gray horizontal area, 99.9% confidence interval for Student's t test.
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#### Table 1 Summary of the RIA results for patients with Dukes' A, B, C, and D colon cancer

Numbers in parentheses are the diagnostic value which is defined as the percentage of patients with elevated FrAg, CIC, or total antigen (FrAg + CIC) levels to those who are pathologically confirmed with colon carcinoma. Dukes' A and B are considered early, whereas Dukes' C and D as considered advanced stages of colon cancer. *P* values of detection for RIA are shown as calculated by Student's *t* test.

<table>
<thead>
<tr>
<th>Dukes' stages</th>
<th>FrAg (%)</th>
<th><em>P</em></th>
<th>CIC (%)</th>
<th><em>P</em></th>
<th>FrAg + CIC (%)</th>
<th><em>P</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5/5 (100)</td>
<td>0.030</td>
<td>5/5 (100)</td>
<td>0.006</td>
<td>5/5 (100)</td>
<td>0.005</td>
</tr>
<tr>
<td>B</td>
<td>15/16 (94)</td>
<td>0.211</td>
<td>11/16 (69)</td>
<td>0.170</td>
<td>16/16 (100)</td>
<td>0.004</td>
</tr>
<tr>
<td>A + B</td>
<td>20/21 (95)</td>
<td>≤0.0005</td>
<td>16/21 (76)</td>
<td>0.009</td>
<td>21/21 (100)</td>
<td>≤0.0005</td>
</tr>
<tr>
<td>C</td>
<td>3/5 (60)</td>
<td>0.108</td>
<td>1/5 (20)</td>
<td>0.374</td>
<td>3/5 (60)</td>
<td>0.652</td>
</tr>
<tr>
<td>D</td>
<td>3/5 (60)</td>
<td>0.190</td>
<td>0/5 (0)</td>
<td>0.059</td>
<td>1/5 (20)</td>
<td>0.374</td>
</tr>
<tr>
<td>C + D</td>
<td>6/10 (60)</td>
<td>0.101</td>
<td>1/10 (10)</td>
<td>0.109</td>
<td>4/10 (40)</td>
<td>0.608</td>
</tr>
</tbody>
</table>

Fig. 3. RIA values of FrAg, CIC, and total antigens (FrAg + CIC) for Dukes' A, B, C, and D colon cancer patients, numbered from 1 to 31. A pool of 10 sera from healthy individuals is included as a normal control.

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might also be achieved by combining the mAb 46.3 RIA with other available assays for the detection of late stage colon cancer such as carcinoembryonic antigen. Our future studies are focused on identifying the reactive antigen of mAb 46.3. Further investigations are planned to determine the prognostic value of this RIA for colon cancer patients following resections.
statistical analysis of the data, and Peter Maimonis (Ciba-Corning, East Walpole, MA) for his helpful discussion.

References

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