Heterogeneity of Circulating Carcinoembryonic Antigen Analyzed by Sandwich-Enzyme Immunoassays with Different Specificities

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ABSTRACT

Nine monoclonal antibodies against carcinoembryonic antigen (CEA) were prepared and used to investigate the immunological and physicochemical heterogeneity of circulating CEA. Two of these, M221-73 and M272-11, recognized "CEA-distinctive" epitopes and they gave sandwich-enzyme immunoassays far less reactive with nonspecific cross-reacting antigen (NCA) and nonspecific cross-reacting antigen-2 (NCA-2). Sandwich-enzyme immunoassays selective for NCA-2 and NCA were also established using suitable monoclonal antibodies as competitive inhibitors against enzyme-labeled antibodies. The studies on the serum CEA levels determined by the "CEA-specific" assay indicated that CEA molecules recognized by M221-73 and M272-11 were generally found in the sera of both cancer patients and normal adults. CEA and related substances in sera were further analyzed by the sandwich-enzyme immunoassays after adsorption on M272-11-coupled immunosorbents and by gel filtration on an Ultrogel AcA-34 column. These studies revealed the presence of a CEA variant detected by the "NCA-2-selective" assay. The variant seemed to be closely related to NCA-2 because it lacked CEA-distinctive epitopes and had an apparent molecular weight similar to that of NCA-2. This variant appeared in the sera of some cancer patients and normal adults.

INTRODUCTION

CEA2 (1, 2) is one of the most extensively investigated human tumor-associated antigens. A glycoprotein containing 50–60% carbohydrates, CEA has shown some heterogeneity in its immunological and physicochemical properties (3, 4). In addition, a number of related substances, NCA (5), NCA-2 (6), and NFA (7, 8), have been isolated from normal tissues. The development of hybridoma technology (9) has provided potent reagents to characterize complex antigen systems such as CEA. There have been some reports that described monoclonal CEA antibodies unreactive or far less reactive with NCA-2 (10–12), NFA-2 (11), or meconium antigen (13). Some of these "CEA-specific" antibodies appeared to be reactive only with subpopulations of CEA molecules (11, 12, 14) and were applied to immunoassays with increased specificity for carcinomas (10, 12). However, the immunological and/or physicochemical heterogeneity of circulating CEA remains to be explored using monoclonal antibodies with well-defined specificities (15).

In the study reported here, we prepared nine monoclonal anti-CEA antibodies and developed sandwich-EIAs for CEA, NCA-2, and NCA. The latter two assays were set up by a newly developed technique using suitable monoclonal antibodies as competitive inhibitors against the enzyme-labeled ones. These assays were used to characterize CEA and related substances in sera. Our results suggest that a CEA variant lacking "CEA-distinctive" epitopes is present in the sera of some cancer patients and normal adults.

MATERIALS AND METHODS

CEA and Related Antigens. Two CEA preparations, CEA(I) and CEA(II), were isolated as follows. CEA(I) was extracted from a primary colon carcinoma with 0.15 M NaCl containing 1% Tween 20, and partially purified by affinity chromatography on Concanavalin A-Sepharose 4B (Pharmacia, Sweden) (16) and by gel filtration on an Ultrogel AcA-34 column chromatography. CEA(II) was extracted from the lung metastasis of a primary colorectal carcinoma with 1 M perchloric acid (17) and, purified by rabbit anti-CEA antibody (DAKO, Copenhagen, Denmark)-coupled Sepharose 4B immunosorbents, followed by Ultrogel AcA-34 column chromatography. CEA-2 and NCA were purified from meconium and normal lung, respectively, as described previously (18, 19). CEA(II) and the NCA-2 preparation gave single diffuse bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The NCA preparation was heterogeneous and eluted from an Ultrogel AcA-34 column in two peaks; the latter peak being major. Smith-degraded CEA was prepared with CEA(II) by one cycle of degradation (20). CEA(II) was used as a standard in sandwich-EIAs.

Monoclonal Antibodies. Splenocytes of the mice (BALB/c, female) immunized with either CEA(I) or CEA(II) were fused with P3-X63-Ag8-UI myeloma cells as described previously (21). Hybridomas secreting monoclonal antibodies M221-73, M272-11, and M215-11 were obtained with the spleens from the mice immunized with CEA(I), while the other hybridomas secreting monoclonal antibodies M603-29, M4103-37, M6104-55, M440-11, M429-34, and M641-3 were derived from the mice immunized with CEA(II). Class and subclass of M604-55 and M641-3 were IgG2a(s) and those of the other antibodies were IgG1(s).

Monoclonal antibodies were purified from ascites fluids by precipitation with ammonium sulfate, followed by DEAE-cellulose (Whatman D52; Kent, England) column chromatography. Purified antibodies were conjugated with HRP using two heterobifunctional reagents as described previously (22). The HRP/antibody molar ratios calculated from absorbance at 403 and 280 nm were in the range of 2.0 to 3.4 (23).

Sandwich-EIA. The binding properties of monoclonal antibodies were investigated by sandwich-EIAs. Purified monoclonal antibodies or rabbit anti-CEA antibody (DAKO) were immobilized on microtest plates (Nunc, Denmark) as described previously (24). CEA, NCA, or Smith-degraded CEA (150 μl, 0.1–200 ng/ml) in buffer B (0.02 M sodium phosphate buffer, pH 6.5, containing 10% heat-denatured newborn calf serum, and 0.002% merthiolate) were put in each well and incubated at room temperature for 16 h. After being washed with PBS, the plates were incubated at room temperature for 4 h with 150 μl of NCA-cross-reactive anti-CEA antibody-HRP conjugates (DAKO) at a dilution of 1:500 in buffer B. After they were washed with PBS, the bound enzyme activity was measured by incubating at room temperature for 20 min with 150 μl of a chromogen solution (0.1 M citrate buffer, pH 5.5, containing 0.2% o-phenylenediamine, 0.02% H2O2, and 0.002% merthiolate). The enzyme reaction was stopped by adding 100 μl of 1 M H2SO4 and absorbance at 492 nm was measured by Titertek Multiskan (Flow Lab., VA).

Similarly, to investigate the relation between the binding sites of monoclonal antibodies, sandwich-EIAs for CEA were carried out using various combinations of immobilized and enzyme-labeled monoclonal antibodies. In these assays, saturated amounts (0.3–2.4 μg/ml) of enzyme-labeled antibodies were used.
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**Sandwich-EIAs Selective for NCA-2 or NCA.** Sandwich-EIAs selective for NCA-2 or NCA were performed in the same way as described above with the exception that suitable monoclonal antibodies were used as competitive inhibitors for enzyme-labeled ones. In the "NCA-2-selective" assay, microtest plates coated with M6104-55 were incubated with 150 μl of standard solutions or samples to be tested in buffer B containing a final concentration of 0.33-33 μg/ml of M272-11. After the plates were washed with PBS, they were incubated with 150 μl of enzyme-labeled M603-29 in buffer B containing a final concentration of 0.33-33 μg/ml of M272-11. The bound enzyme activity was assayed as described above. Similarly, in the "NCA-selective" assay, the sandwich-EIA using immobilized M641-3 and enzyme-labeled M215-11 was carried out in the presence of a final concentration of 0.33-33 μg/ml of M440-11.

**Determination of CEA Levels in Sera.** Heat-denatured (56°C, 30 min) serum samples were diluted more than five times with buffer B and were subjected to a sandwich-EIA using immobilized M6104-55 and mixed enzyme-labeled antibodies of M221-73 and M272-11. In parallel with the assay, serum CEA levels of 12 cancer patients and 30 normal adults were also determined by Abbott CEA-EIA kits (Abbott Lab., North Chicago), including the patients of colorectal carcinoma (n = 5, CEA levels: 54-220 ng/ml), stomach carcinoma (n = 4, 2.3-12.4 ng/ml), and liver, gallbladder, and pancreas carcinomas (2.9-10.3 ng/ml).

Adsorption of CEA in Sera. Various samples (1 ml) including heat-denatured serum subjects in buffer B were treated overnight at 4°C with M272-11 immunosorbents (4 mg of M272-11 was coupled to 1 g of CNBr-activated Sepharose 4B). The samples contained less than 300 ng of CEA, and 100 μg of immobilized M272-11 was added. After the mixture was centrifuged twice at 1500 × g for 10 min, the supernatants were subjected to sandwich-EIAs of either immobilized M603-55 and enzyme-labeled M221-73 or immobilized M6104-55 and enzyme-labeled M603-29.

**Sandwich-EIAs Selective for NCA-2 or NCA.** Sandwich-EIAs selective for NCA or NCA were performed in the same way as described above. In group II antibodies, M215-11 and M641-3 strongly cross-reacted with NCA. These results suggested that group II and group III antibodies recognized "NCA-2-common" and NCA-common epitopes, respectively. Monoclonal antibodies were also analyzed for their reactivity with Smith-degradated CEA. The epitopes recognized by group I antibodies were relatively labile for Smith degradation. In detail, the two epitopes were damaged by periodate oxidation in the same degree, and the following acid hydrolysis further destroyed only the epitope recognized by M272-11 (data not shown).

To investigate the relation of the binding sites between these monoclonal antibodies, eight of them were conjugated with HRP and sandwich-EIAs were carried out using various combinations of immobilized and enzyme-labeled antibodies (Table 2). When the same antibody was used for both immobilized and enzyme-labeled antibodies, none of the assays could detect CEA, suggesting that the epitopes recognized by these monoclonal antibodies occurred only once on the CEA molecules. Two antibodies of group I, M221-73 and M272-11, could bind CEA simultaneously, indicating that their epitopes were different. The sensitivity for CEA was rather low in the assays of the following combinations: M272-11 and M603-29, M6104-55, and M440-11, which were not used in this study because of its high sensitivity and specificity. Systems IV strongly cross-reacted with NCA-2 since the assays consisted of two NCA-2-cross-reactive antibodies, M603-29 and M6104-55. Systems V and VI were designed to compare their sensitivities with that of the most sensitive one.

**RESULTS**

**Characterization of Monoclonal Antibodies.** Reactivity of monoclonal antibodies with CEA and related substances were summarized in Table 1. The binding profiles revealed three groups of antibodies as described previously (13). Group I antibodies, M221-73 and M272-11, barely cross-reacted with NCA-2 and NCA, indicating that they recognized "CEA-distinctive" epitopes. Group II antibodies had nearly the same reactivity with CEA and NCA-2, but showed less than 3% cross-reactivity with NCA. In group III antibodies, M215-11 and M641-3 strongly cross-reacted with NCA. These results suggested that group II and group III antibodies recognized "NCA-2-common" and NCA-common epitopes, respectively.

**Table 1 Reactivity of monoclonal antibodies**

<table>
<thead>
<tr>
<th>Group</th>
<th>Antibody</th>
<th>Isotype</th>
<th>CEA</th>
<th>NCA</th>
<th>Smith-CEA</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>M221-73</td>
<td>IgGl(s)</td>
<td>100</td>
<td>1.8</td>
<td>0.5</td>
</tr>
<tr>
<td>II</td>
<td>M272-11</td>
<td>IgGl(s)</td>
<td>100</td>
<td>3.6</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>M603-29</td>
<td>IgGl(s)</td>
<td>100</td>
<td>120</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>M4103-37</td>
<td>IgGl(s)</td>
<td>100</td>
<td>220</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>M603-55</td>
<td>IgGl2a(s)</td>
<td>100</td>
<td>120</td>
<td>3.0</td>
</tr>
<tr>
<td>III</td>
<td>M215-11</td>
<td>IgGl(s)</td>
<td>100</td>
<td>150</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>M429-34</td>
<td>IgGl(s)</td>
<td>100</td>
<td>160</td>
<td>7.4</td>
</tr>
<tr>
<td></td>
<td>M641-3</td>
<td>IgGl2a(s)</td>
<td>100</td>
<td>140</td>
<td>9.7</td>
</tr>
<tr>
<td></td>
<td>M641-3</td>
<td>IgGl2a(s)</td>
<td>100</td>
<td>100</td>
<td>10.0</td>
</tr>
<tr>
<td>Rabbit antibody</td>
<td>CEA</td>
<td>IgGl(s)</td>
<td>100</td>
<td>110</td>
<td>20.0</td>
</tr>
</tbody>
</table>

| * Monoclonal and polyclonal antibodies were immobilized on microtest plates and sandwich-EIAs were performed using NCA-cross-reactive anti-CEA antibody (rabbit-HRP conjugates as label). Reactivity was calculated from standard curves for CEA, NCA-2, NCA, and Smith-degradated CEA, and was expressed relative to CEA.
| * Smith-degradated CEA.
| Obtained from DAKO (Denmark).

**Table 2 Sensitivity of sandwich-EIAs for CEA using various combinations of immobilized and enzyme-labeled antibodies**

<table>
<thead>
<tr>
<th>Immobilized antibody</th>
<th>Enzyme-labeled antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>M221-73</td>
<td>M272-11</td>
</tr>
<tr>
<td>M603-29</td>
<td>M4103-37</td>
</tr>
<tr>
<td>M4103-37</td>
<td>M6104-55</td>
</tr>
<tr>
<td>M440-11</td>
<td>M215-11</td>
</tr>
<tr>
<td>M429-34</td>
<td>M641-3</td>
</tr>
</tbody>
</table>

| * Sensitivities: 99-50% (+++), 49-20% (++), 19-5% (+), 4-1% (±), and <1% (-) of that of the most sensitive assay (++++).
be selective for NCA-2 and NCA, respectively, as described below.

Sandwich-EIAs Selective for NCA-2 and NCA. M272-11 and M603-29 recognized CEA-distinctive and NCA-2-common epitopes, respectively. They were, however, subjected to steric hindrance or other restrictions, when bound to CEA simultaneously. A similar phenomenon was observed in the case of M440-11 (or M6104-55) and M215-11, which were directed against NCA-2-common and NCA-common epitopes, respectively. On the basis of these phenomena, sandwich-EIAs selective for NCA-2 and NCA were developed according to the principles shown in Fig. 1. The binding of enzyme-labeled M603-29 to CEA may be competitively inhibited by coexisting M272-11, while such inhibition may not occur in the case of NCA-2 since the epitope recognized by M272-11 is not present on the NCA-2 molecules (Fig. 1A). Similarly, coexisting M440-11 may competitively inhibit the binding of enzyme-labeled M215-11 to CEA and NCA-2 but not NCA, since the epitope recognized by M440-11 is shared by CEA and NCA-2 but not by NCA (Fig. 1B).

The results of the sandwich-EIA using immobilized M6104-55 and enzyme-labeled M603-29 are shown in Fig. 2A. In the presence of 3.3 µg/ml of M272-11, the sensitivity for CEA was lowered to approximately one-seventh of its previous level, while there was no effect on the dose-response curves for NCA-2. The resulting assay showed increased selectivity for NCA-2. Fig. 2B shows the effects of M440-11 on the sandwich-EIAs using immobilized M641-3 and enzyme-labeled M215-11. At a concentration of 3.3 µg/ml. M440-11 reduced the sensitivity for both CEA and NCA-2, which was three to four times less than that without the antibody, but did not elicit any changes in the dose-response curves for NCA. The inhibiting effects of M272-11 and M440-11 were observed at concentrations up to 10 and 33 µg/ml, respectively, without any further changes. These results demonstrated that the selectivity of the sandwich-EIAs could be improved by adding suitable monoclonal antibodies as competitive inhibitors for the enzyme-labeled ones. Furthermore, these results supported the observation that M272-11 and M440-11 did not cross-react with NCA-2 and NCA, respectively. The sensitivity and specificity of these assays are also summarized in Table 3.

Determination of CEA in Sera. Serum CEA levels of 12 cancer patients were determined with system II (a CEA-specific assay) and in parallel with Abbott CEA-EIA reported to be CEA specific (26). The values obtained by the two methods are similar. The regression equation and the correlation coefficient (r) were Y (system II) = 0.721X (Abbott kit) + 6.54 (ng/ml) and r = 0.980, respectively. Similarly, CEA levels in the sera of 30 healthy adults were determined with both assays. In normal male sera (n = 17), system II gave slightly lower CEA levels than did the Abbott kit: the mean values obtained were 2.24 ± 1.20 (SD) ng/ml in system II and 2.63 ± 0.97 (SD) ng/ml in the Abbott kit. In female sera (n = 13), the mean values obtained were almost equal: 1.88 ± 1.20 (SD) ng/ml in system II and 1.93 ± 1.05 (SD) ng/ml in the Abbott kit. The regression equation and the correlation coefficient calculated from the values of 30 normal adults were Y (system II) = 0.999X (Abbott kit) - 0.237 (ng/ml) and r = 0.879, respectively. The fairly good accordance between the two methods indicates that CEA molecules expressing the epitopes recognized by M221-73 and M272-11 are generally found in the sera of both cancer patients and normal adults.

Absorption of Serum CEA with M272-11. To investigate the heterogeneity of serum CEA, standard antigens or serum sam-

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**Table 3 Sensitivity and specificity of sandwich-EIAs**

<table>
<thead>
<tr>
<th>No</th>
<th>Immobilized antibody</th>
<th>Enzyme-labeled antibody</th>
<th>Inhibiting antibody</th>
<th>Detection limit (ng/ml)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>M272-11</td>
<td>M221-73</td>
<td></td>
<td>0.50&quot;</td>
<td>100  3.3  0.7</td>
</tr>
<tr>
<td>II</td>
<td>M6104-55</td>
<td>M221-73</td>
<td></td>
<td>0.15&quot;</td>
<td>100  3.6  0.7</td>
</tr>
<tr>
<td>III</td>
<td>M6104-55</td>
<td>M221-73</td>
<td></td>
<td>0.25&quot;</td>
<td>100  3.0  0.7</td>
</tr>
<tr>
<td>IV</td>
<td>M6104-55</td>
<td>M603-29</td>
<td></td>
<td>0.25&quot;</td>
<td>100  3.0  0.7</td>
</tr>
<tr>
<td>V</td>
<td>M6104-55</td>
<td>M603-29</td>
<td></td>
<td>0.40&quot;</td>
<td>30   100  4.4</td>
</tr>
<tr>
<td>VI</td>
<td>M641-3</td>
<td>M440-11</td>
<td></td>
<td>0.40&quot;</td>
<td>5.2  8.8  100</td>
</tr>
</tbody>
</table>

"A competitive inhibitor for an enzyme-labeled antibody.
*Specificity calculated from standard curves for CEA, NCA-2, and NCA.
Detection limits were defined as the least amounts of CEA that could be distinguished from zero within 99% confidence limits.
Detection limit for NCA-2.
Detection limit for NCA.
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Gel Filtration of Serum CEA. The perchloric acid extracts of the serum of a cancer patient in Table 4 (No. 1), which contained relatively higher amounts of CEA species unreactive with M272-11, were applied to the column and immunological activities were monitored with the assays of system II (a CEA-specific assay), system V (a NCA-2-selective assay), and system VI (a NCA-selective assay) (Fig. 3). Three different peaks were detected corresponding to the elution positions of standard CEA, NCA-2, and NCA, respectively. These results indicate that the serum contained not only CEA and NCA but also a CEA variant with an apparent molecular weight similar to that of NCA-2. Furthermore, this variant was detected by a NCA-2-selective assay and not by a CEA-specific assay suggesting that it lacked the CEA-distinctive epitopes recognized by M221-73 and M272-11. The variant was also found in relatively small amounts in extracts of sera of other cancer patients.

Similarly, the perchloric acid extracts of normal sera were examined in the same manner. The activities of CEA, NCA, and the variant lacking the CEA-distinctive epitopes were also found (data not shown). These results suggested that the immunological activity in the sera nonadsorbed with M272-11 in system IV (Table 4) was due to the low molecular weight variant of CEA.

DISCUSSION

The studies presented here demonstrate the immunological and physicochemical heterogeneity of circulating CEA analyzed by several sandwich-EIAs with different selectivities. We prepared nine monoclonal antibodies directed against eight distinctive epitopes. Among them, M221-73 and M272-11 recognized CEA-distinctive epitopes and yielded sandwich-EIAs far less reactive with NCA-2 and NCA (systems I–III). These CEA-distinctive epitopes were relatively labile for Smith degradation suggesting that carbohydrates may play some role in the immunological recognition. However, our results do not rule out the possibility that these antibodies recognized peptide moieties since Smith degradation also destroys amino acids to a certain extent (20). It may be that these antibodies, similar to antibodies to human chorionic gonadotropin-β C-terminal peptide (27), recognized the tertiary structures of peptide chains, which are significantly influenced by carbohydrate moieties attached to the peptide chains.

Interesting sandwich-EIAs selective for NCA-2 (system V) and NCA (system VI) were also developed using suitable monoclonal antibodies as competitive inhibitors against enzyme-labeled ones. These assays were based on the observation that the simultaneous binding of the two monoclonal antibodies to CEA were unexpectedly restricted by steric hindrance or other reasons. Similar phenomena have been found in monoclonal antibodies reactive or unreactive with NCA (13, 28), but further investigations have not been made.
The monoclonal antibodies directed against CEA-distinctive circulating levels of NCA-2 or NCA-2-like substances have not provided useful methods to elucidate biological interrelations variants lacking CEA-distinctive epitopes (data not shown). This study is equal to NCA-2, and whether the CEA variant demonstrated in this study because it lacked only an epitope recognized by a slightly smaller than NCA-2, it resembles the CEA variant in this study. While monoclonal anti-CEA antibodies unreactive or far less reactive with NCA-2 have been prepared and applied to immunooassays with improved specificity for carcinomas (10, 12), circulating levels of NCA-2 or NCA-2-like substances have not been explored extensively. The variant demonstrated in this study seems to be closely related to NCA-2. However, it remains to be further investigated whether the NCA-2-like substance in this study is equal to NCA-2, and whether the CEA variant represents in vivo degrading products of CEA or not. Our preliminary investigation revealed that spent culture media from two CEA-producing cell lines also contained the CEA variants lacking CEA-distinctive epitopes (data not shown). The monoclonal antibodies directed against CEA-distinctive epitopes and unique sandwich-EIAs described in this study may provide useful methods to elucidate biological interrelations between NCA-2 and CEA.

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