Epitopes of Carcinoembryonic Antigen Defined by Monoclonal Antibodies Prepared from Mice Immunized with Purified Carcinoembryonic Antigen or HCT-8R Cells

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ABSTRACT

A library of 18 monoclonal antibodies (MAbs) reactive with purified carcinoembryonic antigen (CEA) has been prepared. The specificity of these MAbs was tested and they have been separated into nine subgroups, each recognizing a different region of the CEA molecule. Seven MAbs from four of the groups also react with fragments of CEA obtained by chemical cleavage and the other groups react with both forms. The MAbs were tested for binding to fragments of CEA obtained by chemical cleavage and the groups of MAbs were found to react with different subsets of such fragments.

INTRODUCTION

CEA was first described in 1965 by Gold and Freedman as an antigen expressed in tumors of the human gastrointestinal tract (1) and in the fetal digestive system (2). Assays that measure the serum or plasma levels of CEA have been useful in assessing prognosis and monitoring the progress of cancer patients (3).

CEA is a large glycoprotein (M, 180,000) localized to the plasma membrane of cells (4) and consisting of a single polypeptide chain with about 40 N-linked oligosaccharide chains (5). The high content of sialic acid on the carbohydrate chains gives CEA an acidic isoelectric point (6, 7). Although the amino-terminal sequence of the intact molecule (8–10) and of several tryptic fragments (11) have been determined, the complete amino acid sequence has not yet been reported.

A preparation of immunologically pure CEA appears to be a collection of heterogeneous molecules when examined by various criteria (6, 12–15). CEA appears as a diffuse band on SDS-PAGE, even after neuraminidase digestion to remove sialic acid residues (16), and also exhibits a broad isoelectric point (6, 7).

The presence of CEA has been demonstrated in normal colon (18–20). Several immunologically cross-reacting substances have also been reported, e.g., NCA (21–23), BGP (24), meconium antigen (25, 26). It has been suggested that some epitopes on the CEA molecule are CEA specific while some are shared with other members of this family of glycoproteins (27, 28).

Many workers have found it expedient to use indirect immunological methods to define the structure of CEA. Sundblad et al. (27) demonstrated 10 CEA-specific determinants and six NCA-related determinants on CEA, using quantitative precipitin analysis with several rabbit anti-CEA antisera. Some antiserum reacted well with only a few of the total set of determinants. This exemplifies a major drawback in the use of conventional polyclonal antisera. It is difficult to define such reagents since, even after absorption with potentially cross-reacting substances, each antiserum contains an irreproducible set of antibodies binding to different subsets of the antigenic sites on the molecule.

When the technique for production of MAbs was introduced (29, 30), a more specific method was made available for the immunological study of proteins such as CEA. The first MAbs directed against CEA were reported by Accolla et al. in 1979 (31, 32), followed by MAbs made by other investigators (33–37), defining up to eight different epitopes (38).

The aim of the present project was to prepare a library of monoclonal antibodies and to use these as a tool to study the structure of CEA. A relatively large number of hybrid cells was selected for subcloning and characterization in order to generate a group of monoclonals looking at as wide range of epitopes as possible on the CEA molecule. These monoclonals were used in conjunction with four others developed previously in this laboratory (39) to compare the epitopes found on CEA, NCA, and denatured CEA, and to study the fragments generated from CEA by chemical cleavage.

MATERIALS AND METHODS

Hybridization Protocol. Male BALB/c mice were immunized i.p. three times at 1-month intervals with 100 µg of CEA in complete Freund's adjuvant. The CEA used for immunization was purified from liver metastases of human tumors by perchloric acid extraction (40). Three days before fusion a mouse was boosted i.v. with 100 μg CEA in PBS and the spleenocytes (4.6 x 10^8) cells) were fused at a 1:10 ratio with Sp2/0 myeloma cells (41) in log phase growth. The procedure used was an adaptation of the method of Geffter et al. (42). All cells were grown in RPMI 1640 supplemented with 10% fetal calf serum, 1 mM pyruvate, 2 mM glutamine, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 100 µg/ml each of streptomycin, penicillin, and amphotericin B.
acid, 100 units penicillin/ml, 100 μg streptomycin/ml (complete medium).

Initial Characterization. Tissue culture supernatants were screened for the presence of anti-CEA antibody using a liquid phase double antibody radioimmunoassay (40). A test supernatant (100 μl) was incubated at 4°C with 50 μl of a solution containing 50,000 cpm 125I-CEA [labeled by the chloramine-T method (43)] and 0.5 μl normal mouse serum. Rabbit anti-mouse antisemur (100 μl, diluted as necessary for optimal precipitation) was added after 1 h and the tubes were left to incubate for a further 1 h at 4°C. The tubes were then spun for 5 min in an Eppendorf centrifuge, the supernatants were removed, and the pellets were counted.

Wells were selected for further study on the basis of binding to 125I-CEA. Hybrids were subcloned twice in soft agar, and all subclones were tested for anti-CEA activity as above. The final clones were isolated using the MonoAb-ID kit (Zymed Laboratories, Burlingame, CA).

Cell-binding Assay. Supernatants from the final clones were tested for binding to HCT-8R cells. The cells were detached from the tissue according to the method of Brenner et al. (45).

Subgrouping of MAbs by Additive Binding. This was done using an inhibition assay, had an additivity index of 30. Thus two MAbs were considered to be in the same group when their additivity index was 30 or less.

Additivity of binding of a pair of MAbs is seen when the two MAbs represent a single radiolaabeled MAb from each of the groups as subdivided by additive binding assays. The procedure for the inhibition assay was as follows: wells of polyvinyl chloride microtiter plates were coated with CEA and postcoated with BSA, as outlined above. Unlabeled competing MAbs (50 μl of MAb concentrates) were added to each well immediately, followed by 100,000 cpm 125I-test MAb in 50 μl 5% BSA in PBS. The plates were incubated overnight at 4°C. The next day the wells were rinsed with PBS, separated, and counted.

In most cases the MAbs were labeled with 125I by the chloramine-T method (43). Bolton-Hunter labeling (50) was used to radioiodinate MAb F1.1.8 as it was inactivated by the chloramine-T method.

Reactivity of MAbs with NCA. The reactivity of the MAbs with NCA, purified according to the method of Krantz and Kranz (51), was tested using the liquid-phase radioimmunoassay described above for CEA, incubating 125I-NCA with serial dilutions of MAb concentrates for 18 h.

Solid-Phase Assay for Binding to Reduced and Alkylated CEA. Binding of MAbs to R/A CEA was tested using material prepared by the procedure of Krantz and Kranz (53). The R/A CEA was coated onto Removawell microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA) (10 ng/50 μl/well), and the plates were heated at 50°C until dry. The plates could then be stored at 4°C until used. Positive and negative control wells consisted of untreated CEA and BSA, respectively. All wells were postcoated with 1% BSA in PBS for 30 min to block nonspecific binding. They were incubated with serial dilutions of the test MAb for 18 h at 4°C, then washed three times with 200 μl of 0.05% Tween in PBS and incubated with 125I-goat anti-mouse IgG plus IgM (100,000 cpm/50 μl 5% BSA in PBS) for 2 h at 4°C. The plates were washed three times with 0.05% Tween in PBS, then separated and counted.

A matrix of n x n polyvinyl chloride microtiter wells (n, number of MAbs being tested) was coated with CEA (50 ng/100 μl) in citrate buffer (0.05 M sodium citrate:0.6 M NaCl:0.02% NaN₃, pH 5.5) for 4 h at room temperature. The wells were rinsed with PBS, then coated with 5% BSA:PBS for 2 h at room temperature. The coating buffer was removed and MAb concentrates (50 μl) were added singly or in pairs. Where 50 μl of a single MAb concentrate were placed in a well, 50 μl of 5% BSA in PBS were added to make the final volume equivalent to wells containing pairs of MAbs. Each MAb was used at a concentration that gave saturation binding in preliminary binding assays. The microtiter plates were incubated overnight at 4°C. After washing the plates with PBS, 125I-labeled goat anti-mouse IgG plus IgM (100,000 cpm/100 μl 5% BSA:TS) was added for 2 h at 4°C. The plates were washed with PBS and the wells were separated and counted. The wells were washed with PBS, and the wells were separated and counted.

Additivity of binding of a pair of MAbs is seen when the two MAbs recognize two distinct epitopes (49), and is indicated by the binding of more radiolaabeled second antibody than that bound by one MAb alone. An additivity index for each pair of MAbs (MAB1 and MAB2) was calculated using the following formula:

\[
\text{Additivity index} = \frac{2(A_{1}+A_{2})-1}{A_{1}+A_{2}} \times 100
\]

where \( A_{1} \) = cpm bound by MAB1 alone, \( A_{2} \) = cpm bound by MAB2 alone, and \( A_{12} \) = cpm bound by MAB1 and MAB2 simultaneously.

Subgrouping of MAbs Using a Solid-Phase Inhibition Assay. Groupings were determined by performing an inhibition assay using one representative radiolaabeled MAb from each of the groups as subdivided by additive binding assays. The procedure for the inhibition assay was as follows: wells of polyvinyl chloride microtiter plates were coated with CEA and postcoated with BSA, as outlined above. Unlabeled competing MAbs (50 μl of MAb concentrates) were added to each well immediately, followed by 100,000 cpm 125I-test MAb in 50 μl 5% BSA in PBS. The plates were incubated overnight at 4°C. The next day the wells were rinsed with PBS, separated, and counted.

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Solid-Phase Assay for Binding to Reduced and Alkylated CEA. Binding of MAbs to R/A CEA was tested using material prepared by the procedure of Krantz and Kranz (53). The R/A CEA was coated onto Removawell microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA) (10 ng/50 μl/well), and the plates were heated at 50°C until dry. The plates could then be stored at 4°C until used. Positive and negative control wells consisted of untreated CEA and BSA, respectively. All wells were postcoated with 1% BSA in PBS for 30 min to block nonspecific binding. They were incubated with serial dilutions of the test MAb for 18 h at 4°C, then washed three times with 200 μl of 0.05% Tween in PBS and incubated with 125I-goat anti-mouse IgG plus IgM (100,000 cpm/50 μl 5% BSA in PBS) for 2 h at 4°C. The plates were washed three times with 0.05% Tween in PBS, then separated and counted.
Smith-degraded CEA. CEA was treated by two cycles of Smith degradation (54), then dried onto Removawells at a concentration of 20 μg/well. Reactivity with the MAbs was assayed in the same manner as for R/A CEA. Amino acid analysis of Smith-degraded CEA was performed and compared with untreated CEA.

Reactivity with ABO Antigens. MAb A21.9.1 was tested for the ability to agglutinate erythrocytes from individuals of blood type A, B, or O. Serial dilutions of A21.9.1 were added to serial dilutions of the washed cells in round-bottomed microtiter wells. Typing antisera were used as positive controls; MAB P3-NS1-1-Ag4 was a negative control. The wells were checked after 30 min for cell agglutination.

Solid-Phase Fragment-binding Assay. Fragments of CEA were prepared by cleavage of the reduced molecule with 2-nitro-5-thiocyanobenzoic acid. The fragments were separated by reverse-phase HPLC as described previously (53), using a Proteosil 300 Octyl, 250 x 9.4 mm (Whatman) column, and using 0.1% trifluoroacetic acid instead of 0.1% phosphoric acid for elution of the column. The solvents were removed by evaporation.

The MAbs were tested against material contained in each of the eight peaks eluted from the HPLC. The fragments were individually coated onto Removawells and were assayed for reactivity with all the MAbs in the same manner outlined above for R/A CEA. Binding of a MAb to a fragment was considered to be positive when significantly more counts were bound by the fragment-containing wells than by corresponding BSA control wells.

RESULTS

Fusion. Fourteen of the MAbs used in this study were prepared in a single fusion using cells from a mouse immunized with purified CEA. After the fusion a majority of the wells showed growth of hybrid cells, and supernatants from each individual well were tested for the presence of MAbs that bound 125I-CEA in a liquid-phase double antibody assay. Of 219 wells, 66 yielded supernatants that were able to bind more than 10% of the radioliodinated CEA added in the assay (nonspecific background binding is 1.5%).

The CEA preparations used for MAb characterization were considered as pure by two criteria: radiolabeled CEA gave one band after SDS-PAGE, and only one band reacted with polyclonal rabbit anti-CEA after immunoblotting. 10% of the total label was bound in the initial screening assay due to the low MAb concentration in the tissue culture supernatants used for testing.

The cells from 14 wells were subcloned twice in soft agar and used in this study. The other four MAbs included in the study (b18.7.7, d2.14.6, d14.6.43, and b17.31) had been prepared previously from mice immunized with HCT-8R cells or purified CEA (39). A list of the MAbs and some of their characteristics are shown in Table 1. The majority of the MAbs are of the IgG1,κ isotype. One MAb is IgM,λ, one is IgG2α,κ, and one is IgA,κ.

MAb Groups. The MAbs were subgrouped according to two methods: additive binding and competitive inhibition. Table 2 shows the additivity indices for the MAbs, obtained from two experiments: a 15 x 15 matrix was used to test the 14 MAbs prepared above with MAb b18.7.7, and a 4 by 4 matrix was used to test MAbs b18.7.7, d2.14.6, d14.6.43, and b17.31. The additivity index (30) of MAbs b18.7.7 and d2.14.6, which were previously shown to cross-react with the same epitope, was used to set the limits of the assay. Thus values less than or equal to 30 indicate the pairs of MAbs that are binding to the same or overlapping epitopes. The only discrepancy in the groupings is the nonadditivity of C11.14.7 and b18.7.7.

The 14 MAbs from one fusion were thus placed into seven subgroups, each subgroup representing MAbs that bind to the same or closely located epitopes. The four other MAbs partition into three subgroups. One of these latter subgroups (containing b18.7.7 and d2.14.6) is the same as subgroup 6 (containing D6.17.17 and G22.12.2). Thus at least seven and possibly nine different epitopes are identified by this procedure.

The ability of each MAb to competitively inhibit binding of a radiolabeled MAb (one representative from each group, except group 9) was tested using a solid-phase assay. Chart 1 shows an example of the results obtained with 125I-MAb D6.17.7 of subgroup 6. Unlabeled D6.17.7, G22.12.2, b18.7.7, and d2.14.6 all significantly inhibit binding of the labeled MAb to solid-phase CEA, whereas the other MAbs and controls do not. Both horse and rabbit anti-CEA polyclonal sera inhibit binding of labeled D6.17.7 compared to the normal horse and normal rabbit sera which serve as negative controls.

In each case the groupings determined by the additive binding assay were confirmed. MAb d14.6.43, which was tentatively placed in its own group after additive binding, was not able to significantly inhibit binding of any of the other MAbs, indicating that it is unique in its epitope specificity. Binding of MAb b18.7.7 was not inhibited by MAb C11.14.7. Thus a minimum of nine different epitopes was confirmed. The MAbs in Tables 1 to 4 are all ordered in their appropriate groups.

Some of the MAbs were inactivated by certain radiolabeling methods. MAb F1.1.8 (group 7) and B22.6.14, C11.14.7, and D6.20.7 (group 5) were all inactivated by the chloramine-T label methods. MAb F1.1.8 (group 7) and B22.6.14, C11.14.7, and D6.20.7 (group 5) were all inactivated by the chloramine-T labeling method which iodinates tyrosines, whereas MAbs from other
CEA EPITOPES DEFINED BY MONOCLONAL ANTIBODIES

Chart 1. Inhibition of binding of [125I]-D6.17.7 to solid phase CEA. The radiolabeled MAb was added to CEA-coated microtiter plate wells concurrently with unlabeled MAbs, polyclonal antisera, or BSA. After an overnight incubation the wells were washed, separated, and counted. Each value is the mean of duplicate wells. P3, P3-NS1/1-Ag4; HaCEA, horse anti-CEA antiserum; NHS, normal horse serum; RaCEA, rabbit anti-CEA antiserum; NRS, normal rabbit serum.

groups were not affected. MAB F1.1.8 could be labeled using the Bolton-Hunter reagent which iodinates terminal amino groups, but again MABs B22.6.14 and D6.20.7 were inactivated. MAB B22.6.14 was also inactivated by biotinylation, another procedure which alters amino groups on the molecule. Radiolabeled B22.6.14 was obtained by internally labeling the hybridoma with [35S]methionine in serum-free medium, followed by ammonium sulfate precipitation to concentrate the MAB.

Reactivity with HCT-8R Cells. The MABs were each tested for reactivity against CEA on the surface of HCT-8R cells (see Table 1). MABs b18.7.7 and d2.14.8, prepared using HCT-8R cells as an immunogen, bind to the cells as expected. Of the MABs prepared using CEA as an immunogen, B22.6.14, C11.14.7, D6.20.7, E13.21.4, and E13.21.25 (group 5), F1.1.8 (group 7), and b17.31 (group 8) were found to be unreactive with cell surface CEA.

NCA Reactivity. The MABs were all tested for their ability to bind to NCA, an antigen cross-reactive with CEA found in colon tumor and normal tissue (for summary of results see Table 1). Seven of the 18 MABs react with iodinated NCA in a liquid-phase radioimmunoassay. All five MABs from groups 3 and 6 are positive, as well as one MAB from group 1 (A20.12.2) and one from group 4 (J22.4.4). The MABs from groups 2, 5, 7, 8, and 9, and one MAB each from groups 1 and 4 (D13.1.2 and B7.12.3, respectively), are all unreactive with NCA.

Conformational Determinants. Dot-blots were used to visually examine the binding of MABs to CEA denatured by SDS and reduced with 2-ME. By determining which MABs are directed against nonconformational determinants, one can select for MABs that will be able to detect CEA after SDS-PAGE and blotting. Fig. 1 shows dot blots done with MABs B7.8.5 (group 3) and D6.17.7 (group 6). A summary of the results for all the MABs is shown in Table 3. MABs B7.8.5 (group 3), B22.6.14, C11.14.7, D6.20.7, and E13.21.4 (group 5), F1.1.8, and B7.31 (group 8) all required higher concentrations of denatured CEA to demonstrate binding comparable to nondenatured CEA, whereas...
CEA EPITOPES DEFINED BY MONOCLONAL ANTIBODIES

Table 3

<table>
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<tr>
<th>Group</th>
<th>MAb</th>
<th>Reduced with 2-Me\textsuperscript{a}</th>
<th>Reduced and alkylated\textsuperscript{b}</th>
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<td>1</td>
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<tr>
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<td>A21.9.1</td>
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</tr>
<tr>
<td>3</td>
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</tr>
<tr>
<td>4</td>
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<td>5</td>
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<tr>
<td>9</td>
<td>d14.6.43</td>
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\textsuperscript{a} Reactivity of MAb with native and denatured CEA was compared using dot blots as outlined in "Materials and Methods" in the presence of SDS.

\textsuperscript{b} Reactivity of MAb with native and reduced and alkylated CEA was done using a solid-phase binding assay as described in "Materials and Methods."

\textsuperscript{c} ND, not done.

MAbs A20.12.2 and D13.1.2 (group 1), A21.9.1 (group 2), B7.12.3 and J22.4.4 (group 4), D6.17.7 and G22.12.2 (group 6) and F1.1.8 (group 7) all showed equivalent binding to both treated and untreated CEA.

A solid-phase assay was used to test the MAb’s for their ability to react with reduced and alkylated CEA. This form of denatured CEA is not the same as that prepared by reduction in the presence of detergent, and some MAb’s are able to distinguish between the two forms. The results are shown in Table 3. MAb’s A20.12.2 and D13.1.2 (group 1), B22.6.14, C11.14.7, D6.20.7, E13.21.4, and E13.21.25 (group 5), F1.1.8 (group 7), and b17.31 (group 8) are all unable to bind to R/A CEA. The MAb’s of groups 4 and 6 are able to bind both SDS- and 2-ME-denatured CEA. In contrast MAb’s B7.8.5 (group 3) and d14.6.43 (group 9) bind to reduced and alkylated CEA and react poorly with CEA reduced in the presence of SDS.

Carbohydrate Reactivity. The MAb’s were tested for reactivity against Smith-degraded CEA to investigate the involvement of carbohydrate in the antigenic sites. After two cycles the molecular weight of the deglycosylated CEA was estimated by SDS-PAGE to be 100,000 ± 10,000 daltons, with approximately 45% of the sugars removed.

Two amino acids which can be modified by Smith degradation are tyrosine and cysteine. Amino acid analysis of Smith-degraded CEA showed no detectable change in the number of tyrosine residues. After treatment 100% of the cysteines are present as oxidized cysteic acid, in contrast to 25% cysteic acid from untreated CEA. (This is either due to oxidation of disulfide bonds during Smith degradation itself or oxidation during the hydrolysis step of amino acid analysis by trace amounts of remaining periodate.)

MAb A21.9.1 was the only MAb unable to bind to deglycosylated CEA. If this loss of reactivity were due to oxidation of sulfhydryl groups during the process of Smith degradation, then the MAb would also not bind to CEA that had been reduced and alkylated. However, A21.9.1 does react with R/A CEA, indicating that this MAb is directed against a carbohydrate moiety on CEA.

The possibility that A21.9.1 was reacting with a carbohydrate epitope shared with blood group antigens was investigated. This MAb was able to agglutinate type B and type O RBC but not type A cells. Thus A21.9.1 is not recognizing carbohydrate specific to the ABO antigens but some other carbohydrate determinant on the erythrocyte cell surface.

Immunoblot. Since the MAb’s were able to bind to the surface of intact HCT-8R cells, immunoblots were done with purified membranes from these cells, as well as from other CEA-expressing tissues, to verify that the antigen recognized by the MAb’s was indeed CEA. Fig. 2 shows an immunoblot done using MAb b18.7.7. Purified CEA was run on the gel along with membranes prepared from both the HCT-8R cell line and from a primary colon adenocarcinoma. When the blot was stained, the two lanes containing membrane samples exhibited bands with the same mobility as did the lane with the purified CEA. A control blot using P3-NS1/1-Ag4 showed no staining.

Protein blotting was also used to ascertain reactivity of MAb’s with other tissues. Fig. 3 shows an immunoblot of purified CEA and of membranes prepared from primary adenocarcinoma of the bowel and breast tumor liver metastases. MAb A20.12.2 binds to CEA in the membranes from both tumor sources. The MAb does not react with any of the material in membranes prepared from normal liver (blot not shown).

Reactivity with CEA Fragments. Chart 2 shows binding of MAb’s B7.8.5 (group 3) and B7.12.3 (group 4) to the fragments prepared by cleavage of purified CEA with 2-nitro-5-thiocyanobenzoic acid, followed by separation of the cleavage products on reverse-phase HPLC. Table 4 summarizes the binding of all 18 MAb’s to the fragments. As expected, the groups of MAb’s bind to different subsets of the fragments. Fragments 7 and 8 bind all those MAb’s which are not directed against conformational determinants. Both fragments consist primarily of M, 30,000 material when run on SDS-PAGE; there is no intact CEA present in either fragment fraction (data not shown). This M, 30,000 immunoreactive fragment was previously described as yielding 55% inhibition of binding of CEA by a horse-anti-CEA polyclonal antiserum (53). Fragments 1 to 6, which are of low molecular weight on SDS-PAGE (less than 10,000 daltons), bind fewer MAb’s. Each of the fragments contains carbohydrate as indicated by the diffuse bands obtained after SDS-PAGE and by the ability of each of the fragments to bind various lectins.6

MAbs B22.6.14, C11.14.7, D6.20.7, and E13.21.4 (all from group 5), F1.1.8 (group 7), and b17.31 (group 8) do not bind to any of the fragments. These MAb’s are directed against conformational determinants (see above), and thus were not expected to be reactive with fragments generated from CEA.

Those MAb’s that are able to react with cleaved CEA recognize a variable number of fragments. The MAb’s from groups 3 and 6 bind to only the two large fragments (fragments 7 and 8). MAb

\textsuperscript{6} A. Haggarty, unpublished results.
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Table 4
Summary of monoclonal antibody binding to fragments of CEA

<table>
<thead>
<tr>
<th>Group</th>
<th>MAb</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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Chart 2. Reactivity of MAbs B7.8.5 and B7.12.3 with chemically cleaved fragments of CEA separated on HPLC. Serial dilutions (neat, 1:4, 1:16, and 1:64) of each MAb concentrate were used, the values shown in the chart are those from undiluted MAb. 1 to 8, fragments 1 through 8; CEA, untreated CEA.

d14.6.43 binds to four of the fragments (fragments 5 to 8). MAb A21.9.9 binds to all eight fragments.

There are three exceptions to the intragroup consistency of fragment binding. MAb E13.21.25 (group 5) binds to fragments 6–8, even though the other members of the group do not react with any of the fragments. This MAb was shown to be the only member of group 5 to be directed against a nonconformational determinant (see Table 3). It is probably directed against a linear epitope close to or overlapping the conformational determinant recognized by the rest of the group.

Groups 1 and 4, each containing two MAbs, are the other exceptions to the homogeneity of group binding. D13.1.2 binds to more fragments than does A20.12.2, (fragments 5,6,7,8 versus fragments 7 and 8) while B7.12.3 binds more fragments than does J22.4.4 (fragments 1–8 versus fragments 4–8). This pattern of fragment binding corresponds to the ability of these four MAbs to bind to NCA (see above). Within these two groups, those MAbs which bind fewer fragments are also able to bind NCA.

Chart 3 shows a schematic representation of those epitopes on the CEA fragments recognized by the MAbs described in this paper.

DISCUSSION

A set of 14 MAbs directed against CEA has been prepared from a single fusion using purified CEA for immunization. These were used in conjunction with four other MAbs (prepared using either CEA or HCT-8R cells as immunogen) to analyze the epitopes on the CEA molecule.

These 18 monoclonal reagents, each with anti-CEA activity, demonstrated in both liquid and solid-phase binding assays, were initially subgrouped by using a modification of the additive binding method of Friguet et al. (49). Since each MAb is tested at a saturating amount, the additivity of two reagents binding concurrently implies reactivity with sterically distinct epitopes. By assaying each MAb in pairwise fashion with every other MAb, 16 of the 18 anti-CEA reagents could be separated into seven definite subgroups and the two remaining MAbs were tentatively assigned into their own unique groups. In order to confirm these assignments a direct competitive solid-phase assay was utilized. One member of a group was radiolabeled and the ability of each
of the MAbs to inhibit its binding to CEA was tested. These procedures in all cases confirmed the existence of nine sterically distinct groups of "paratopes," in that the members of one group were able to compete with the labeled member of that group, while members of other groups did not demonstrate this activity. The use of the additive binding assay to make initial group assignments allows the efficient use of direct competition systems without the need to radiolabel each monoclonal reagent individually.

The characteristics of the MAb(s) in each group can be used to define the nature of the different epitopes on CEA. Epitope 1, recognized by MAbs A20.12.2 and D13.1.2, is probably situated close to, but not inclusive of, a disulfide bond. Reduction alone does not destroy the epitope but reduction and alkylation does, possibly because the addition of alkyl group(s) to the binding site may prevent MAb attachment. However it is also possible that reduction and alkylation simply denature a conformational binding site more completely than does reduction alone.

This epitope can be subdivided into two sites which overlap or are sterically close together: epitopes 1a and 1b recognized by MAbs A20.12.2 and D13.1.2, respectively. The nonidentity of these two sites is revealed by differences in MAb binding to NCA and to CEA fragments. Fragments 5 and 6 contain a determinant not shared with NCA (epitope 1a), whereas fragments 7 and 8 contain both this determinant and a nearby sequence that is found on both CEA and NCA (epitope 1b).

Epitope 2, recognized by MAb A21.9.1, is found on CEA but not on NCA. This nonconformational determinant is found on all eight fragments, indicating that it is probably a repeating epitope. It seems to be carbohydrate in nature, for this epitope is not found on CEA that has undergone Smith degradation (it is the only epitope described by this collection of MAbs with that characteristic). It is not part of the ABO blood group antigen system.

Epitope 3, binding MAb B7.8.5, is shared by both CEA and NCA. It may be sensitive to the effects of the ionic detergent SDS since it disappears after reduction in the presence of SDS but not after reduction and alkylation. The negatively charged SDS used would neutralize positively charged regions of the molecule, including any that are accessible for recognition by MAbs. The epitope is found only on the two larger fragments 7 and 8.

Epitope 4, a nonconformational, noncarbohydrate determinant, can be subdivided into two overlapping sites, 4a and 4b, recognized by MAbs B7.12.3 and J22.4.4, respectively. The former is found on CEA but not on NCA, whereas the latter is shared by both antigens. Epitope 4a is found on all eight fragments and may be a linear amino acid sequence that repeats throughout the molecule. Epitope 4b is found on fewer fragments (fragments 4 to 8), so that fragments 1, 2, and 3 contain sequences not shared with NCA.

Epitope 5 is recognized by five MAbs. Four of the MAbs (B22.6.14, C11.14.7, D6.20.7, and E13.21.4) appear to be directed against a common conformation-dependent epitope (epitope 5a) destroyed by both reduction or by reduction and alkylation. Epitope 5a is not found on any fragments, nor on the surface of HCT-8R cells. This nonreactivity suggests that the epitope is cryptic when CEA is on the cell surface, but is accessible after the molecule is extracted from the membrane by perchloric acid.

Epitope 5b, bound by MAb E13.21.25, overlaps or is located close to the epitope described above. This determinant is destroyed by reduction and alkylation but not by reduction. It can be detected on four of the chemically cleaved fragments (fragments 5 to 8), while the site recognized by the other MAbs of group 5 is not present on any fragment. When CEA was prepared for chemical cleavage it was reduced but not alkylated, a procedure which alters epitope 5a but not epitope 5b. This absence of an alkylation step may explain why E13.21.25 is the only member of group 5 with fragment-binding activity.

Epitope 6 is recognized by four MAbs (D6.17.7, G22.12.2, b18.7.7, and d2.14.6) derived from separate fusions using two different immunogens (purified CEA versus HCT-8R cells). This nonconformation-dependent determinant is shared by both CEA and NCA. Since the epitope is found only on the two large fragments 7 and 8 it is probably not repetitive. Using immunoblotting, the determinant can be demonstrated in membranes from both a primary colon adenocarcinoma and a liver metastasis of a breast tumor, with the same relative mobility as purified CEA. It was not detected in a parallel blot done with normal liver membranes. This epitope cannot be considered as truly tumor specific, however, because of its presence on NCA, a component of normal bowel as well as of other tumor and normal tissues.

Epitope 7 (bound by MAb F1.1.8) is found on purified CEA but not on the surface of the CEA-expressing HCT-8R cell line; this nonreactivity indicates that the epitope may be cryptic on the cell surface. The determinant is not altered by reduction in the presence of detergent but is destroyed by reduction and alkylation, indicating either that there is an alkylation site where the MAb binds, or that alkylation after reduction alters the conformation of the molecule more severely than does reduction alone.

Epitope 8 (MAb b17.31) is dependent upon the conformation of CEA for recognition. This epitope is not present on reduced and alkylated CEA, nor on any of the fragments, nor on the surface of HCT-8R cells. In these respects this determinant is similar to epitope 5, but, since its ability to react with b17.31 is not affected by the MAbs of group 5, it is unique.

Epitope 9 (bound by MAb d14.6.43), may require ionic interactions with the MAb for binding in the same manner as outlined for epitope 3, since reduction in the presence of SDS abrogates MAb binding. However, the epitope defined by d14.6.43 is not the same as that defined by B7.8.5, since the former is found on fragments 5 through 8 versus only fragments 7 and 8 for the latter.

It is perhaps noteworthy that there seem to be a limited number of antigenic determinants on the CEA molecule. The anti-CEA MAbs prepared by other investigators have defined a maximum of eight different epitopes on the CEA molecule (38), and polyclonal antisera have defined a maximum of 10 CEA-specific epitopes (27). The set of 18 monoclonals described in this paper defines nine sterically different sites as determined by binding studies to the intact molecule. Twelve different epitopes, including seven which are CEA specific, can be defined when data on binding to NCA, denatured CEA, and CEA fragments are considered as well. It is possible that certain regions of the molecule are more immunogenic than others, as has been found with H-2 antigens (55). This would explain why certain subgroups (i.e., subgroups 4 and 6), contain several MAbs directed against the same determinant, even when the mice used for the fusions were immunized with different antigens (purified CEA or HCT-
CEA EPITOPES DEFINED BY MONOCLONAL ANTIBODIES

When chemical cleavage at the 12 cysteines of CEA is complete one expects 13 fragments to be generated. After HPLC purification two large fragments of $M_r$ 30,000 and six smaller ones were separated. It is possible that fewer fragments than expected were separated by HPLC because of the presence of repetitive sequences, resulting in coelution of homologous fragments from different regions of the molecule.

Some of the MAbs (i.e., A21.9.1 and B7.12.3) seem to be directed toward such repetitive epitopes since they are able to bind to all of the fragments. When repetitive binding sites are found in a glycoprotein it seems logical to assume that they are due to carbohydrate side chains because these are usually the most common repetitive elements. Indeed the site recognized by A21.9.1 is most likely carbohydrate in nature since it is destroyed by Smith degradation of the CEA molecule and is not affected by reduction.

In contrast to A21.9.1, the site recognized by B7.12.3, although not affected by reduction as one would expect for a carbohydrate determinant, is also not affected significantly by Smith degradation. Other investigators have concluded that the carbohydrate moiety is not the most immunogenic part of the CEA molecule (5, 37, 38, 54, 56), so MAbs such as B7.12.3, which are reactive with repetitive sequences, may be interacting with the protein backbone. However, it may also be reacting with inner carbohydrate residues that were not removed by Smith degradation.

Hedin et al. (36) produced anti-CEA MAbs directed against six conformation-dependent protein determinants (the MAbs reacted well with Smith-degraded CEA but poorly with reduced and alkylated CEA). One of the MAbs was able to combine with CEA in a molar ratio of 2:1 to 4:1 and could precipitate CEA in immunodiffusion when used alone. Another MAb combined with CEA in a molar ratio of 2:1. Thus two of the epitopes appear to occur twice or more, and the authors concluded that CEA could possibly contain two or more regions of sequence homology.

Another study which supports the existence of repeating determinants in the protein moiety of CEA was done by Sundblad et al. (57). When tryptic and chymotryptic peptides of CEA were separated fewer peptides were found than anticipated (12 tryptic peptides found versus 43 expected). They hypothesized that this could be due to regions with similar structures within the protein.

When Shively et al. (11) sequenced the amino-terminal regions of seven tryptic peptides purified from digested CEA, they found that three of the peptide peaks were not completely pure, and gave a minor sequence as well as a major sequence. They concluded that the contaminating peptides were unique and were copurified with other peptides. They postulated that the tryptic cleavage sites of CEA vary in their accessibility to the enzyme, so that some peptides would be generated in low amounts. However, it is possible that the peptides which copurified on ion-exchange HPLC share repetitive sequences in regions other than those which were sequenced.

An alternative explanation exists for the ability of certain MAbs to bind to more than one fragment. If the CEA molecule contains a core, resistant to enzymatic or chemical cleavage, surrounded by sites with variable resistance to cleavage, then the digestion of CEA would result in the production of fragments of varying sizes containing a common portion. Such resistance to cleavage may be due to the numerous oligosaccharide chains found on CEA that may hinder complete digestion. When Shively et al. digested CEA with trypsin they found that in order to get complete digestion it was necessary to treat CEA with Triton-X as well as reducing and alkylating the disulfide bonds. If the detergent was omitted only a few high molecular weight digestion products were obtained. Since the CEA used in this study was reduced but not alkylated and no detergents were used, it may be that some fragments were not completely cleaved.

Seven of the MAbs used in this study are reactive with both NCA and CEA. The ability of anti-CEA MAbs to bind to determinants on cross-reacting antigens has been studied by a number of workers. Anti-CEA MAbs have been prepared that also react with epitopes found on: NCA (58); meconium antigen (14); BGP I (59); NCA and meconium antigen (14); NCA and BGP I (59); and NCA-2 (60). It is not surprising that some epitopes are shared between CEA and NCA since partial sequence homology between these two molecules has already been demonstrated. When the sequences of 30 amino-terminal residues of the two molecules were compared they were found to be identical except for one amino acid difference (61). Sequence homology with the NH$_2$-terminus of CEA is also seen with other CEA-related proteins (62, 63).

Certain regions of the CEA molecule are CEA specific as determined in this study. Fragments 5 and 6 contain three epitopes recognizable by MAbs D13.1.2, E13.21.25, and D14.6.43 that are not found on NCA. Fragments 1, 2, and 3 each contain two CEA-specific epitopes detectable with MAbs A21.9.1 and B7.12.3; these epitopes may be repetitive since they are found on all the fragments.

In order to define the epitopes on CEA completely one needs to look at a wide range of characteristics: epitopes shared with cross-reacting antigens; epitopes present in different tissues; epitopes present on denatured, deglycosylated, and/or fragmented CEA. It is also advantageous to use a large number of MAbs, since minor differences between epitopes will then be apparent, such as those detected with the MAbs from groups 1, 4, and 5.

The absolute specificity of a MAb for CEA can only be determined by exhaustively investigating all of the potentially cross-reacting antigens. Some investigators have checked for cross-reactivity of their anti-CEA MAbs with NCA alone or in combination with other cross-reacting antigens such as BGP or meconium antigen. Other investigators, interested in tissue specificity, tested their MAbs against normal tissues such as liver or granulocytes (which are known to express CEA) to see if their MAb is specific for tumor tissue. The reactivity of representative MAbs from eight of the groups described was tested against a wide range of tissue samples, and an increase in specificity was observed with some of the reagents. The results will be reported in detail elsewhere (64). The development of CEA-specific MAbs will be useful in clinical assays and may reduce the incidence of false positive results that are presently encountered, in addition to yielding information on the interrelationships of this family of related glycoproteins.

REFERENCES

2. Gold, P., and Freedman, S. O. Specific carcinoembryonic antigens of the...


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**Fig. 1.** Dot-bLOTS of MAbs B7.8.5 and D6.17.7 against denatured and untreated CEA. D, CEA denatured with SDS and 2-ME; N, native untreated CEA.

**Fig. 2.** Protein blot using MAb A20.12.2. A 5-20% gradient polyacrylamide gel was used. Lane A, purified CEA; lane B, membranes from a primary colon adenocarcinoma; lane C, membranes from a liver metastasis of breast tumor.

**Fig. 3.** Protein blot using MAb b18.7.7. A 5-20% gradient polyacrylamide gel was used. Lane A, purified CEA; lane B, membranes from HCT-8R cells; lane C, membranes from a primary colon adenocarcinoma.
Epitopes of Carcinoembryonic Antigen Defined by Monoclonal Antibodies Prepared from Mice Immunized with Purified Carcinoembryonic Antigen or HCT-8R Cells

Allison Haggarty, Christine Legler, Mark J. Krantz, et al.


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