ABSTRACT

Blood group determinants have been found on five carcinoembryonic antigen (CEA) preparations with a lectin and antibody-binding assay using polyethylene glycol 6000 to separate free from bound radiolabeled antigen. The assay described gives excellent sensitivity for the binding or inhibition of binding of various lectins or antibodies to CEA. One of the CEA preparations investigated has an Aα determinant, another has a B determinant, and all have H, Leα, Leβ, and MN blood group determinants. In addition, all of the preparations tested bound concanavalin A. These findings are consistent with the idea that incomplete or unexpected glycosylation patterns occur in glycoproteins produced by tumor cells. Since antibodies directed against blood group substances cross react with carbohydrate determinants on CEA, clinical determinations of CEA or anti-CEA levels in serum may be adversely affected.

INTRODUCTION

Studies on the carbohydrate determinants of glycoproteins and glycolipids suggest that there are considerable differences between normal and tumor tissues. In general, tumors appear to produce unusual or incompletely biosynthesized carbohydrate structures, perhaps as a result of either uncontrolled glycosyltransferase or glycosidase activities. Although it is unusual to find blood group activity in the colon, it has been observed by many laboratories that CEA, which was originally described by Gold and Freedman (19) and is found in 60 to 70% of colorectal tumors, may carry blood group determinants (2, 3, 5, 13, 21, 22). Although initially it was the intention of this work to determine whether or not antibodies against CEA are raised in colorectal cancer patients, it was found that the only antibodies binding CEA were anti-blood group antibodies. Previous evidence for blood group determinants on CEA was based on radioimmunoelectrophoresis (17), gel filtration radioimmunoassay (18), hemagglutination (22), immunodiffusion with lectins (28), and a Farr assay with antibodies or lectins (6, 21). These studies confirmed that CEA may possess A, B, H, Leα, and Leβ activities. Lectins specific for Blood Groups A, H, and T, as well as Con A and wheat germ agglutinin, have been shown to bind CEA (2, 3, 6, 7, 10, 17, 18, 21, 28).

In order to measure ng amounts of antibody or lectin binding to radiolabeled CEA, a new immunoassay using PEG precipitation was developed. This assay is characterized in this work using the binding properties of 9 lectins and 4 antibodies to CEA. The results of this work establish on a quantitative basis with a single technique the nature and distribution of blood group determinants in various CEA preparations.

MATERIALS AND METHODS

Six CEA preparations, coded 175, 10, 14, 23, BP160, and HF, were investigated. Five were prepared in this laboratory according to the method of Coligan et al. (8) from hepatic metastases of colon carcinomas, each obtained from an individual patient. One of these preparations (HF) was deglycosylated by treatment with hydrogen fluoride as described by Glassman et al. (16). The sixth CEA preparation (BP160) was kindly provided by Dr. H. J. Hansen, Nutley, N. J. The ABO blood group types were known in 3 of the 6 patients from whom CEA was purified: CEA 175 and 14 came from an A- and B-type patient; and CEA 23 came from an O-type patient.

Leα substance (Lot LS 104) was purchased from Ortho, Raritan, N. J. It contained Leα, 0.280 mg/ml, and Leβ, 0.277 mg/ml.

Anti-CEA was raised in goats as described by Egan et al. (14) and used without absorption. Human anti-A, and anti-B antisera as well as goat anti-Leα and anti-Leβ antisera (Lots A8630-1, B9313A4, LEA 161, and LEB 246, respectively), were obtained from Ortho.

Lectins with the following blood group and monosaccharide specificities were used: Dolichos biflorus (Aα, a-D-GalNAc), Bandeiraea simplicifolia (B, a-D-Gal), Lotus tetragonolobus and Ulmus europaeus (H, a-L-Fuc), Arachis hypogaea [T, β-o-Gal(1→3)α-GalNAc], Con A (−), Bauhinia purpurea (M+N, α-GalNAc), Salvia sclarea (Tn, −), and Iberis amara (M, −). The first 5 lectins were purchased from Sigma Chemical Co. (St. Louis, Mo.), the last 3 were from E-Y Laboratories (San Mateo, Calif.). PEG 6000 was purchased from Sigma.

MATERIALS AND METHODS

Radioiodination of CEA. Ten μg of the CEA preparations were labeled with 1 mCl [125I] by a modified chloramine-T method (14). Their specific activities were approximately 90 μCi/μg.

Binding and Inhibition Assays with Lectins. The binding of 0.5 ng of radiolabeled CEA to lectins in the presence or absence of the specific monosaccharides was performed in
duplicate in 210 µl of 0.075 M PBS, pH 7.2, containing bovine serum albumin, 1 mg/ml. Assays with Con A were performed in 0.1 M acetate buffer, pH 5.0, containing 1 mg/ml, and 0.1 mM with respect to CaCl2, MgCl2, and MnCl2. About 10,000 cpm of 57Co were used as a volume marker (15). The incubation was performed at 37° for 1 hr and at 4° for another 15 hr. The 125I-CEA lectin complexes were precipitated for 16 hr at 4° in 2 ml of a 10% PEG solution. The samples were centrifuged at 1500 x g for 4° for 30 min. The pellets were counted in a Beckman Gamma 300 counter with an efficiency of about 50%.

**Binding and Inhibition Assays with Antisera.** Anti-CEA, anti-Leα, and anti-Leβ antisera were diluted in human AB Leαβ serum (1:8 in PBS). Anti-A, and anti-B antisera were diluted in human AB Leαβ serum (1:2 in PBS). Otherwise, the assays were performed as described for the lectin assays.

**Absorption of Antisera.** Sera (500-µl aliquots) were absorbed with 300 µl of packed human erythrocytes for 12 hr at 4°. After centrifugation, the supernatants were used for the assay.

**RESULTS**

The optimal PEG 6000 concentration for the separation of free 125I-CEA from 125I-CEA bound to lectin or antibody was determined at a given pH and temperature by comparing 125I counts precipitated for CEA alone or CEA plus lectin or antibody at various PEG concentrations (11, 24). Chart 1 shows the results obtained for the precipitation of CEA by Con A or goat anti-CEA (in antibody excess) versus the percentage of CEA precipitated in the absence of lectin or antibody. Up to 90% of labeled CEA is precipitated in antibody excess with 10% PEG or greater. From these experiments, 10% PEG was chosen as the optimal concentration for precipitation of bound CEA. The titers of goat anti-CEA with each of the 5 CEA preparations were performed using the above assay conditions. Binding of radiolabeled CEA at an anti-CEA dilution of 1:125 varied from 60% for CEA BP160 to 90% for CEA 175. From 30 to 40% of the 125I-CEA 175 was bound by goat anti-CEA at a dilution of 1:2000; all others were at 1:1000 dilution.

Inhibition curves were performed with CEA 175, 10, 23, and BP160. The competition between cold and labeled antigen for binding with goat anti-CEA antisera proved to be comparable for each of the CEA preparations, as shown in Chart 2. The binding of 125I-CEA 175 to increasing amounts of the lectins, Dolichos, Bandeiraea, Bauhinia, Ulex, and Con A, is shown in Chart 3. The other lectins used did not bind to 125I-CEA 175 under the conditions of this assay. The values shown are the differences between the binding in the presence or absence of the specific inhibiting monosaccharides (0.1 M). The lectin binding of CEA in the presence of the specific monosaccharide inhibitor varied between 0 and 3%. Table 1 shows the net binding of 5 µg of the lectins for each of the 6 CEA preparations. These results show that CEA 175 carries an A determinant and CEA 10 carries a B determinant. All CEA preparations have H, MN, and Con A determinants. Two of them show a faint reaction with the anti-T lectin. The deglycosylated CEA shows binding to none of the lectins used. The lectin bindings to 125I-CEA 175 were inhibited by increasing concentrations of the specific monosaccharide. Chart 4 shows that 125I-CEA-Con A binding is inhibited by β-methylnanoside and that only fucose specifically inhibits the binding of the lotus lectin to CEA. The binding of Bauhinia to 125I-CEA 175 is inhibited by the specific monosaccharide GalNAc and, to a lesser extent, by GaL; GlcNAc does not inhibit the binding. GalNAc completely inhibits the binding of 125I-CEA 175 to Bandeiraea at a lower concentration than does Gal, which is supposedly the specific monosaccharide for this anti-B lectin (data not shown). Fucose does not inhibit this binding. This study demonstrates the utility of the PEG assay for determining the ability of various sugars to inhibit lectin binding to glycoproteins.

As expected from the lectin results, 125I-CEA 175 binds also to anti-A, antibodies as shown in Chart 5. Absorption with B or O RBC does not alter the binding, whereas absorption with A cells completely abolishes the binding ability. Similar results were obtained with 125I-CEA 10 and anti-B antisera before and after absorption, thus confirming the presence of B blood group determinants on CEA 10.
Carbohydrate Determinants on CEA

Table 1

<table>
<thead>
<tr>
<th>Lectins</th>
<th>CEA 175</th>
<th>CEA 10</th>
<th>CEA 14</th>
<th>CEA 23</th>
<th>CEA PB160</th>
<th>CEA HF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dolichos (A, α-D-GalNAc)</td>
<td>10.8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bandeiraea (B, α-Gal)</td>
<td>15.1</td>
<td>10.6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lotus (H, α-L-Fuc)</td>
<td>ND</td>
<td>16.9</td>
<td>15.3</td>
<td>18.7</td>
<td>14.7</td>
<td>0</td>
</tr>
<tr>
<td>Ulex (H, α-L-Fuc)</td>
<td>6.6</td>
<td>7.6</td>
<td>0</td>
<td>17.9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Iberis (M, −)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bauhinia (MN, α-GalNAc)</td>
<td>19.3</td>
<td>16.9</td>
<td>23.8</td>
<td>21.7</td>
<td>14.0</td>
<td>0</td>
</tr>
<tr>
<td>Salvia (Tn, −)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Arachis (T, β-o-Gal (1→3))</td>
<td>1.3</td>
<td>0</td>
<td>0</td>
<td>1.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>D-GalNAc</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Con A (−, β-D-Man)</td>
<td>35.4</td>
<td>17.3</td>
<td>25.7</td>
<td>18.0</td>
<td>3.80</td>
<td>0</td>
</tr>
</tbody>
</table>

All CEA preparations except the deglycosylated CEA showed binding to anti-Lea and anti-Leb antibodies. The binding of 125I-CEA 10 to both antisera is shown in Chart 6. The specificities of these reactions were proven for 3 of the native CEA’s by inhibiting the binding with Leab substance up to nearly 100%. Chart 7 shows the inhibition (at the dilution giving 30% binding) of anti-Lea antisemur to CEA 10 by increasing amounts of cold CEA 175, 10, 14, 23, and BP160. CEA 23 shows less inhibition.
Table 1. These results agree with those obtained for the lectins than do the rest of the CEA preparations tested. The data obtained with the anti-blood group antisera are summarized in Table 1. These results agree with those obtained for the lectins and show that Le^a and Le^b determinants are found on all CEA preparations tested.

DISCUSSION

The original intent of this work was to determine whether or not patients with high serum or plasma levels of CEA produced antibodies against CEA. Work in this area has been hampered by the lack of a sensitive assay, free of interfering substances, which can quantitate the levels of human immunoglobulin specific for CEA. Attempts to measure anti-CEA binding to CEA attached to Sepharose were unsuccessful because high background levels were encountered, and it was difficult to inhibit binding with ng levels of free, soluble CEA. Similar problems were encountered in attempts to show inhibition of the binding of CEA to lectins attached to solid supports. These assays, while useful in the \( \mu \)g to mg range, were difficult to use in the ng range. In addition, it became clear that various low-affinity antibodies in human serum, such as anti-blood group antibodies, were the major substances present which could bind CEA at the levels tested. Accordingly, we developed a new immunoassay for CEA which does not depend on a second antibody for precipitation, and which can detect substances which bind to CEA in the ng range. This work demonstrates the utility of this assay for measuring the binding or the inhibition of binding of lectins or antibodies to CEA. The assay is as sensitive as are conventional double-antibody assays and does not suffer the drawback of the effects of interfering substances in serum. It is reasonable to assume that this assay can be used to measure lectin or antibody binding to other glycoproteins and thus will allow one to determine whether or not the presence of blood group determinants on glycoproteins in cancer patients is an unusual phenomenon.

The results of this study show that all the CEA preparations studied bear blood group determinants. In the case where CEA was isolated from A-group patients (CEA 175 and 14), only one expressed this determinant. This result is similar to the findings by Holburn et al. (21), who studied several CEA preparations from A-group patients but found that only one expressed the A group. Gold and Gold (18) proposed that the Group A determinant in their CEA preparation was “A-like” since their preparation lacked the immunodeterminant sugar, GalNAc. An examination of the relative amounts of GalNAc present in the CEA preparations examined in this study shows little or no GalNAc in the CEA preparations lacking A-group determinants and a small, but reproducible, amount in CEA 175 which expresses Group A determinant. Previous studies in this laboratory have shown that GalNAc is a common contaminant in CEA and is usually due to the copurification of mucopolysaccharides with CEA. Mucopolysaccharides can be removed from CEA by Con A chromatography but, even then, certain CEA preparations still contain a small amount of GalNAc (25). Con A-purified CEA’s which contain GalNAc and which can be shown to be free of uronic acids (which are present in equimolar amounts to GalNAc in mucopolysaccharides) probably are CEA preparations which bear \textit{bona fide} Group A determinants. This finding is intriguing, since the majority of evidence indicates that CEA belongs to the class of glycoproteins which bears asparagine-linked oligosaccharides only (9).

Therefore, the presence of GalNAc is unusual and probably occurs only (but does not have to occur) in patients who express Group A determinants (12, 20, 23). The type of oligosaccharide structure which is responsible for A-group activity in porcine submaxillary mucin (4) is GalNAc\(\alpha\) 1 \(\rightarrow\) 3 (Fuc\(\alpha\)1 \(\rightarrow\) 2) Gal etc. It is interesting to speculate that an appropriate glycosyl transferase can convert terminal Gal residues in CEA into Blood Group A determinants.

CEA 10, which was purified from a patient whose blood group type was unavailable to us, showed strong-B group activity and no A-group activity. The immunodominant sugar in B-group active oligosaccharides is Gal 1 \(\rightarrow\) 3 linked to \(\beta\)-Gal. The presence of \(\alpha\)-linked Gal is unexpected in glycoproteins which have typical asparagine-linked oligosaccharides and, since CEA belongs to this class of glycoproteins, it is surprising to find B-group activity. It is likely then, as in the A-group situation stated above, that patients who express B-group determinants may modify their CEA glycoproteins to varying degrees with \(\alpha\)-linked Gal residues. This process may involve either adding \(\alpha\)-Gal to existing terminal Gal residues known to exist in CEA (13) or adding an oligosaccharide with B activity to CEA. There is no information at this time to decide which case is more likely.

All CEA preparations studied had H, Le^a, and Le^b activities. These activities are probably due to the presence of one or both of the following unexpected oligosaccharides in CEA in addition to those expected in asparagine-linked carbohydrate: Fuca\(\alpha\)1 \(\rightarrow\) 2 Gal \(\beta\)1 \(\rightarrow\) 3/4(NeuAc \(\alpha\)2 \(\rightarrow\)6)GlcNAc, and Fuc \(\alpha\)1 \(\rightarrow\)2Gal \(\beta\)1 \(\rightarrow\)3/4GlcNAc. These structures are precursors to oligosaccharides bearing A or B activities and may have either GlcNAc or GalNAc (4) but are usually attached to protein via O-glycosidic bonds to serine or threonine. Since CEA has little or no GalNAc and no evidence for serine or threonine-linked carbohydrate, the blood group determinants may be due to the addition of unexpected sugar residues to existing terminal sugars such as Gal or GlcNAc. In fact, Sodetz et al. (26) have converted an asparagine-linked carbohydrate unit to one bear-
ing A activity by the action of appropriate glycosyltransferases. Another possibility is that CEA contains trace amounts of serine-linked blood group determinants which are hard to detect chemically but are detected by antibodies or lectins. The data of Bali et al. (3) which demonstrate the conversion of H activity in CEA to A or B activity and the affinity purification of CEA on anti-H-Sepharose appears to rule out this latter possibility. Thus, it is likely that the glycosylation pattern of CEA is unusual in that blood group determinants are found on asparagine-linked carbohydrate units. This may be a result of irregular processing of the carbohydrate chains of CEA.

The occurrence of Bauhinia binding which is indicative of MN determinants in all CEA preparations tested is probably due to the occurrence of the group NeuAc α2→3 Gal, which has been previously shown to occur in CEA (13). The absence of Iberis binding which is indicative of M activity may reflect the involvement of protein determinants as suggested by Wasiowska et al. (27). The absence of T and St activity probably precludes structures such as Gal β1→3/4 GlcNAc/GalNAc or Gal β1→3/4 (NeuAcα2→6) GlcNAc/GalNAc, which are precursors to Leα or Leβ active groups.

Another aspect of this analysis is that lectins are able to bind only 10 to 40% of CEA molecules under the assay conditions described. Thus, it is likely that CEA molecules are not uniformly modified to contain blood group determinants or that their blood group activities are masked by other carbohydrate moieties. This latter finding has been demonstrated for certain glycoproteins by Aminoff et al. (1). Alternatively, the conditions for complete precipitation of lectin-CEA complexes may not have been reached using 10% PEG. In any case, the presence of blood group determinants on CEA is well documented, but the mechanism for these unusual conversions or additions is poorly understood. Further studies may establish whether or not the conversion or addition of carbohydrates with blood group activities to glycoproteins is unique to tumors. In addition, these studies prompt us to state that, in the majority of cases, the reported occurrence of natural antibodies to CEA is an artifact caused by the binding of anti-blood group immunglobulins from one patient to the radioabeled CEA of another.

REFERENCES

Sensitive Detection of Carbohydrate Determinants on Carcinoembryonic Antigen Preparations by Lectin and Antibody Binding Using Polyethylene Glycol

R. Pompecki, J. E. Shively and C. W. Todd


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