A Galactosidase Immunosorbent Test for Carcinoembryonic Antigen

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SUMMARY

A galactosidase immunosorbent test for carcinoembryonic antigen (CEA) is described in which the amount of galactosidase adsorbed to a cellulose disc is a hyperbolic function of CEA concentration. Thus, molecules with CEA-like activity can be characterized by mathematical analysis of data obtained from the galactosidase immunosorbent test. By such analysis, CEA-reactive molecules in normal human plasma were distinguished from normal cross-reacting antigen and from authentic CEA. Variation of the amount of antibody-enzyme conjugate used in the galactosidase immunosorbent test permitted CEA-reactive material in plasma of a patient with rectal carcinoma to be antigenically distinguished from the CEA-reactive material in urine of a patient with bladder carcinoma. The galactosidase immunosorbent test is a useful tool for analysis of CEA-reactive molecules.

INTRODUCTION

CEA is a glycoprotein found in fetal gut and in various endodermal carcinomas (4, 5). The CEA radioimmunoassays developed by Thompson et al. (16), LoGerfo et al. (6), and Egan et al. (2) have yielded important insights into the structure of CEA and the significance of CEA in adenocarcinoma of the large bowel and other neoplastic and nonneoplastic diseases (10, 15). While early findings indicated that elevated plasma CEA levels were specific for endodermal cancer (16), later studies showed that plasma CEA levels were elevated in nonendodermal cancer and even in some nonmalignant diseases (10, 15). It is possible that the apparent nonspecificity of CEA may in part result from the antigenic heterogeneity that exists among CEA molecules (1, 8, 11, 18). It is important, therefore, to analyze CEA molecules immunochemically in order to establish the basis for apparent antigenic heterogeneity. We have developed a galactosidase immunosorbent test (3, 19) which has immunochemical features useful for analyzing and comparing molecules with CEA activity.

MATERIALS AND METHODS

CEA Antisera. New Zealand rabbits were immunized by s.c. interscapular injections of 1 mg of CEA (BP114) dispersed in complete Freund’s adjuvant. The immunization was repeated 1 month later; after an additional 2 weeks the anti-CEA serum (Rabbit 100) was collected. Goat anti-CEA serum (No. 183) and CEA (BP114), prepared from hepatic metastases of a colon carcinoma, were kindly provided by Dr. H. Hansen.

Insolubilization of Rabbit Anti-CEA Immunoglobulin. Partially purified Ig fractions of rabbit anti-CEA sera were prepared by precipitation at 33% saturated (NH₄)₂SO₄. The precipitates were solubilized, dialyzed against 0.1 m NaHCO₃, and covalently coupled to discs (6.3 mm diameter) of Whatman No. 42 filter paper by the CNBr method (12).

Quantitation of CEA by Enzyme Assay. The galactosidase immunosorbent test for CEA is diagrammed in Chart 1. Rabbit anti-CEA immunoglobulin insolubilized on cellulose discs is incubated sequentially with samples, with goat anti-CEA serum, and with a covalent conjugate of rabbit anti-goat immunoglobulin with β-D-galactoside galactohydrolase. Antibody-enzyme conjugate bound to discs can be measured colorimetrically (13) or fluorimetrically (14).

Each sample to be assayed for CEA was added to a rabbit anti-CEA disc in Buffer 1, in Falcon polystyrene tubes (12 x 55 mm). Sample volume varied from 0.05 to 0.500 ml, and in all cases total volume of sample plus Buffer 1 equaled 1 ml. The tubes containing the discs and samples were stoppered with polypropylene caps and agitated overnight on a wrist action shaker at room temperature. Following overnight incubation, liquid was aspirated from the tubes and the discs were washed 4 times with 2 ml Buffer 2, using a Cornwall continuous-flow syringe. Goat-anti CEA serum (5 μl) was diluted with 83 ml of Buffer 1, and 1.0 ml of the diluted serum was added to each tube. The tubes were capped, incubated overnight, and washed 4 times as above. Antibody-enzyme conjugate was prepared as described elsewhere (21). The enzyme-conjugate was diluted with Buffer 1, and 20 enzyme units in 1 ml were added to each tube; in one experiment (Chart 3) 40 enzyme units were used, while in another experiment (Table 2) 200 enzyme units were used. The tubes were capped and incubated with agitation overnight. After overnight incubation, the discs were washed 5 times with Buffer 2. The final wash liquid was aspirated, and the discs were assayed for adsorbed galactosidase conjugate by colorimetric measurement of p-nitrophenylgalactopyranoside (Calbiochem, Los Angeles, Calif.) hydrolysis (13). For the assay of adsorbed conjugate,
Values for $Z_{\text{max}}$ were determined from the intercept of least-squares regression lines calculated for double reciprocal plots according to Equation A. SE. is standard error of $Z_{\text{max}}$ and $n$ is number of data points determining regression line. Data were obtained with 20 units of galactosidase conjugate.

<table>
<thead>
<tr>
<th>Sample material</th>
<th>Disease</th>
<th>$Z_{\text{max}}$</th>
<th>$n$</th>
<th>$Z_{\text{max}}$ CEA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>Normal</td>
<td>0.132 ± 0.007*</td>
<td>8</td>
<td>0.33</td>
</tr>
<tr>
<td>NCA A</td>
<td>Normal</td>
<td>0.216 ± 0.007</td>
<td>8</td>
<td>0.54</td>
</tr>
<tr>
<td>Urine</td>
<td>Cystitis</td>
<td>0.288 ± 0.015</td>
<td>8</td>
<td>0.72</td>
</tr>
<tr>
<td>Plasma</td>
<td>Rectal carcinoma</td>
<td>0.300 ± 0.006</td>
<td>8</td>
<td>0.75</td>
</tr>
<tr>
<td>Plasma</td>
<td>Breast carcinoma</td>
<td>0.301 ± 0.006</td>
<td>8</td>
<td>0.76</td>
</tr>
<tr>
<td>Urine</td>
<td>Bladder carcinoma</td>
<td>0.339 ± 0.011</td>
<td>6</td>
<td>0.85</td>
</tr>
<tr>
<td>Urine</td>
<td>Bladder carcinoma</td>
<td>0.372 ± 0.013</td>
<td>7</td>
<td>0.93</td>
</tr>
<tr>
<td>Purified CEA</td>
<td>Colon carcinoma</td>
<td>0.398 ± 0.022</td>
<td>6</td>
<td>1.00</td>
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</table>

* Mean ± S.E.
for normal plasma was significantly less ($p < 0.01$) than those for all other materials tested. The $Z_{\text{max}}$ value for NCA was greater than that for normal plasma ($p < 0.01$) but less than that for all other materials tested ($p < 0.01$). $Z_{\text{max}}$ values for materials from individuals with various neoplastic and nonneoplastic diseases were 0.72 to 0.93 times as large as $Z_{\text{max}}$ for authentic CEA. By the criterion of $Z_{\text{max}}$, therefore, the CEA-reactive material in normal plasma can be distinguished from NCA, from authentic CEA, and from the CEA-reactive materials from individuals with various neoplastic and nonneoplastic diseases. Thus, the parameter $Z_{\text{max}}$ in Equation A appears to be useful for characterizing molecules that react with anti-CEA.

Immunocchemical comparison of CEA-reactive materials was further facilitated by variation of the amounts of enzyme-conjugate used in the galactosidase immunosorbent test. As shown in Table 2, $Z_{\text{max}}$ for CEA-reactive molecules in plasma from a patient with rectal carcinoma increased 5.5-fold when the amounts of enzyme-conjugate were increased from 20 to 200 units. The corresponding change in $Z_{\text{max}}$ for CEA-reactive molecules in urine from a patient with bladder carcinoma was 8.2-fold. Thus, $Z_{\text{max}}$ for different populations of CEA-reactive materials displayed different dependence on the amount of conjugate used in the galactosidase immunosorbent test.

Inhibition of the galactosidase immunosorbent test by soluble RaCEA was used to compare molecules with CEA reactivity. To this end, normal plasma, NCA, and CEA were each incubated at room temperature with varying amounts of soluble RaCEA. After 4 hr, the anti-CEA immunosorbent discs were added and the galactosidase test for CEA was assayed either colorimetrically (13, 21) or fluorometrically (14). The use of immunosorbent in the assay eliminates the need to centrifuge a gel or immune precipitate. These features make the galactosidase test suitable for analysis of large numbers of samples, as is frequently required in quantitative immunochemistry.

In many cases, the relationship between bound galactosidase conjugate and total CEA concentration can be described by a rectangular hyperbola, as in Equation A. Numerical values for $K$ and $Z_{\text{max}}$ in Equation A can be obtained from double reciprocal plots of bound galactosidase conjugate versus total antigen concentration (Chart 2B). Although Equation A is a common form of adsorption iso-

### DISCUSSION

The galactosidase immunosorbent test for CEA described in this paper is a useful immunocchemical tool for analyzing molecules with CEA-like activity. Furthermore, the galactosidase immunosorbent assay utilizes enzyme-antibody conjugates which are stable for years, rather than unstable radioactive reagents. The galactosidase conjugate can be assayed either colorimetrically (13, 21) or fluorometrically (14). The use of immunosorbent in the assay eliminates the need to centrifuge a gel or immune precipitate. These features make the galactosidase test suitable for analysis of large numbers of samples, as is frequently required in quantitative immunochemistry.

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### Table 2

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<tr>
<th>Body fluid</th>
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<th>$Z_{\text{max}}$ with 20 units galactosidase conjugate</th>
<th>$Z_{\text{max}}$ with 200 units galactosidase conjugate</th>
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<tbody>
<tr>
<td>Plasma</td>
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<td>0.351 ± 0.007*</td>
<td>1.93 ± 0.04</td>
</tr>
<tr>
<td>Urine</td>
<td>Bladder carcinoma</td>
<td>0.427 ± 0.004</td>
<td>3.49 ± 0.24</td>
</tr>
</tbody>
</table>

* Mean ± S.E.

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**Galactosidase Immunosorbent Test for CEA**

**Chart 3.** Inhibition of galactosidase immunosorbent test by soluble RaCEA. A. Inhibition of the galactosidase immunosorbent test by soluble RaCEA. B. Inhibition of the galactosidase immunosorbent test by soluble RaCEA that had been previously absorbed with insolubilized NCA. Antigens used were normal plasma (○), NCA (△), and CEA (×), in amounts about equal to their respective values for $K$ in Equation A. The relative amount of galactosidase-conjugate bound per disc (ordinate) is the amount of conjugate bound in the presence of soluble RaCEA divided by the amount of conjugate bound in the absence of soluble RaCEA. Amount of soluble RaCEA (µg) used to inhibit each assay (abscissa) was calculated from absorbance at 280 nm, using an extinction coefficient (1 cm path length) of 1.4 per mg rabbit Ig per ml.

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therm (9), it is presented here as a phenomenological equation useful for data analysis.

The values for $Z_{\text{max}}$ obtained from double reciprocal plots and given in Table 1 show that the CEA-reactive molecules in normal plasma are different from CEA and from NCA. Therefore, much of the CEA-reactive material in normal plasma is cross-reacting antigen, other than NCA. Although there were statistically significant differences among values of $Z_{\text{max}}$ in various plasma and urine samples, the molecular and clinical significance of these differences is not yet understood. However, the data in Table 1 show that the galactosidase immunosorbent test is useful in detecting and analyzing differences among molecules that react with anti-CEA.

Varying the amounts of the reagents used in the galactosidase test further facilitates an immunochemical comparison of molecules with CEA-like activity. For example, the dependence of $Z_{\text{max}}$ on the amount of galactosidase conjugate used in the analysis was different for 2 materials studied (Table 2). Thus, differences between CEA-reactive material in rectal and bladder carcinoma were magnified by increasing total enzyme-conjugate used in the assay (Table 2). From mathematical models that we are now developing, it appears that the parameters obtained from galactosidase assay reflect antigenic valence and steric interactions among antigenic sites. Appropriate mathematical models should permit a molecular explanation for the differences in $Z_{\text{max}}$ values shown in Tables 1 and 2. However, some of the differences in $Z_{\text{max}}$ may be due to antigenic heterogeneity caused by nonspecificity of antisera used. For example, the unabsorbed anti-CEA sera used in the galactosidase immunosorbent test were reactive with NCA (Tables 1 and 3), a glycoprotein normally found in plasma, lung, and other tissues (7, 17). Adsorption of rabbit anti-CEA (Chart 3) and goat anti-CEA (Table 3) with insolubilized NCA prior to use should increase the immunospecificity of the galactosidase immunosorbent test. The immunospecificity of the galactosidase immunosorbent test may be further increased if CEA-reactive material in normal plasma can be identified, isolated, and used to absorb rabbit anti-CEA and goat anti-CEA. The comparisons of $Z_{\text{max}}$ given in Tables 1 and 2 were made with plasma and urine samples that were not extracted with perchloric acid, while the purified CEA had been extracted. It is thus possible that the higher value for $Z_{\text{max}}$ for purified CEA might be due to the extraction procedure.

The galactosidase immunosorbent test for CEA described in this paper is a useful tool to help characterize molecules with CEA-like activity, and ultimately it may facilitate clinical determination of CEA (20).

ACKNOWLEDGMENTS

We are indebted to Dr. Hans J. Hansen for his gift of goat (No. 183) anti-CEA serum and CEA (BP114). We thank Dr. J. Dyckman and Dr. Rudy K. Meiselman for clinical samples and specimens.

REFERENCES


Table 3  
Effect of absorption of GaCEAα on the galactosidase immunosorbent test

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Unabsorbed GaCEAα</th>
<th>Absorbed GaCEAα</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal plasma</td>
<td>0.102 ± 0.007a</td>
<td>0.044 ± 0.007</td>
</tr>
<tr>
<td>High-titered plasma</td>
<td>0.093 ± 0.008</td>
<td>0.077 ± 0.006</td>
</tr>
<tr>
<td>NCA</td>
<td>0.109 ± 0.014</td>
<td>0.002 ± 0.004</td>
</tr>
</tbody>
</table>

α GaCEA, goat antiserum to CEA.

Mean ± S.D. of duplicate measurements of antibody-enzyme conjugate activity adsorbed to a rabbit anti-CEA disc.
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