Antibody to Carcinoembryonic Antigen in Hamsters Bearing GW-39 Human Tumors

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SUMMARY

The appearance of anti-carcinoembryonic antigen (CEA) activity in the sera of hamsters bearing i.m. heterografts of GW-39, a CEA-producing colonic tumor of human origin, was demonstrated in radioimmunoassay using radiiodinated CEA purified from GW-39. Column chromatography, sucrose gradient ultracentrifugation, and radioimmuno-electrophoresis of pooled sera from tumor-bearing hamsters showed that the CEA antibody was of the immunoglobulin M type. The CEA antibody in tumor sera contained two specificities that reacted with different determinants on the CEA. One specificity reacted with a blood group-related determinant, whereas the remaining specificity was against a unique site on the CEA. Sequential analysis of individual tumor-bearing hamsters showed that an increase in tumor growth paralleled the concomitant appearance of circulating CEA and highest antibody titer, suggesting that immunoglobulin M antibody, antigen, or complexes thereof influence tumor growth in this model.

INTRODUCTION

Gold and Freedman (7) have described an antigen specific for human adenocarcinomas arising from the endodermally derived gastrointestinal epithelium. It was called CEA of the human digestive system since it was also found in fetal gut, liver, and pancreas between 2 to 6 months of gestation but not in non-digestive tract cancers or adult normal tissues. CEA has been characterized as a glycoprotein (18) that is released from the tumor cell glycocalyx (8) and is found in the circulation of patients with intestinal cancer (26).

Goldenberg and Hansen (11) have recently shown the presence of CEA in a signet-ring cell carcinoma of the colon of human origin, GW-39 (13). Tumor cell suspensions were prepared in 0.01 M phosphate-buffered 0.15 M NaCl (pH 7.2) by pressing small pieces of non-necrotic tumor through a 60 mesh stainless steel screen. After sequential aspiration through 18- and 21-gauge needles, the suspension was centrifuged at 1000 rpm for 5 min. The pellet was resuspended 1:1 (v/v) in the above buffered NaCl and 0.2 ml was injected i.m. Pooled sera from tumor-bearing hamsters were obtained 6 to 8 weeks following implantation of tumor into both hind limbs, at which time the total mean tumor weight was 30.0 ± 2.96 g (S.E.). These pooled, tumor-bearing hamster sera were designated tumor sera. Although GW-39 tumors can be propagated in normal, unconditioned hamsters (9), 2 s.c. injections (2.5 mg each) of cortisone (Merck, Sharp, and Dohme, Inc., Westpoint, Pa.) were administered during the 1st week of tumor growth.

Hamster Immunization. Normal hamsters were immunized at weekly intervals with the s.c. injection of 10 to 25 μg of CEA, isolated from liver metastases of colonic adenocarcinomas (22), in an equal volume of complete Freund's adjuvant (Difco Laboratories, Inc., Mich.). A total of 125 μg of CEA was given to each animal. The animals were terminally bled 10 days after the last injection of CEA, and the sera obtained were designated hyperimmune sera.

Radioimmunoassay. The radioimmunoassay described by Hansen et al. (16) was used to measure the presence of CEA and CEA antibody. The presence of circulating CEA in tumor-bearing hamsters was determined in PCA extracts of 250-μl serum samples. CEA values were obtained from standard curves performed in PCA extracts of normal hamster serum. Quantitation of CEA activity in preparations

1 This investigation was supported, in part, by USPHS Grant CA-15799 from the National Cancer Institute through the National Large Bowel Cancer Project.
2 To whom requests for reprints should be addressed.
3 The abbreviations used are: CEA, carcinoembryonic antigen; PCA, perchloric acid.

Received March 4, 1975; accepted March 17, 1976.
other than serum was performed by direct assay without prior PCA extraction.

Purified CEA from GW-39 (F. J. Primus and H. J. Hansen, unpublished data) was radioiodinated by the procedure of Greenwood et al. (14) and was used to detect CEA antibody in hyperimmune and tumor sera. Radioactive CEA was separated from unbound \(^{125}\)I by filtration over a 2.6- x 90-cm Sepharose 6B column (Pharmacia, Piscataway, N. J.) and was stabilized in 10.0% normal hamster serum. Approximately 70% of the \(^{125}\)I-labeled CEA prepared in this manner reacted with specific goat anti-CEA serum (15) when tested in radioimmunoassay. For antibody assay, 25 \(\mu l\) of normal goat serum were added to 1.0 ml of 0.01 M or 0.1 M ammonium acetate (pH 6.25) which contained 0 to 100 \(\mu l\) of diluted or neat serum from experimental animals. Fifty \(\mu l\) of \(^{125}\)I-labeled CEA (about 3 ng) were then added and, after incubation for 30 min at 45\(^{\circ}\) or room temperature, 2 ml of zirconyl phosphate gel (pH 6.25) and 10 ml of 0.1 M ammonium acetate were added to terminate the reaction. The gel, which binds \(^{125}\)I-labeled CEA-antibody complexes but not free \(^{125}\)I-labeled CEA, was collected by centrifugation, washed with buffer, and centrifuged again prior to counting.

In inhibition studies, a constant amount of experimental hamster serum in 1.0 ml of buffer containing normal goat serum was first incubated for 30 min with 0 to 200 \(\mu l\) of unlabeled test material. Following the addition of 50 \(\mu l\) of \(^{125}\)I-labeled CEA, the tubes were incubated for an additional 30 min and were subsequently processed as described above.

**Column Chromatography.** For column chromatography, DEAE-cellulose (Whatman DE52, H. Reeve Angel & Co., Inc., Clifton, N. J.) was used in a 2.5- x 40-cm column, and the protein was eluted by a discontinuous ionic strength and pH gradient (2). Sephadex G-200 (Pharmacia) filtration was performed in a 2.5- x 90-cm column maintained at 4\(^{\circ}\) using 0.1 M Tris-HCl (pH 7.0):0.15 M NaCl as the eluting buffer. Samples, 1-ml from DEAE-cellulose and Sephadex G-200 column fractions were tested for antibody activity by radioimmunodiffusion.

**Ultracentrifugation.** A modification of the method of Stanworth et al. (25) was used for sucrose gradient centrifugation. In our procedure, 2.2 ml of 30% sucrose in 0.15 M phosphate-buffered 0.15 M NaCl (pH 6.8) were layered over 2.2 ml of 10% sucrose in the same solvent. A 0.5-ml serum sample was layered on top and centrifuged at 60,000 rpm for 6 hr in a Beckman Model L2-65B ultracentrifuge equipped with a SW 65L rotor. Fractions of 0.5 ml were collected from the bottom of the tube after needle puncture and were assayed for antibody activity in radioimmunodiffusion.

**Radioimmunoelectrophoresis.** Radioimmunoelectrophoresis was performed using the Pol-E-Film System (Pfizer Diagnostics, New York, N. Y.). Following the electrophoresis of 1.0-\(\mu l\) serum samples, precipitin bands were developed overnight at 37\(^{\circ}\). Radioactive antigen was then added to the troughs and, following a further incubation at 37\(^{\circ}\) overnight, the films were washed with 0.9% NaCl solution, stained, and exposed to Kodak RP-14 X-ray film. Rabbit antiserum to hamster serum and hamster IgG was pur-

**RESULTS**

**Demonstration of Humoral CEA Antibody in Radioimmunooassay.** Antibody against CEA was detected by radioimmunooassay following hyperimmunization of normal hamsters with purified CEA incorporated in complete Freund's adjuvant (Chart 1a). The activity of this antibody in radioimmunooassay was suppressed markedly when the ionic strength of the reaction mixture was increased, analogous to goat antisera that were raised against CEA given in a similar manner (16). Double immunodiffusion in agar plates of the hyperimmune sera gave a line of identity between GW-39 CEA, human colonic tumor CEA, and specific goat anti-CEA serum (Fig. 1). CEA binding activity was also found when undiluted sera from tumor-bearing hamsters were tested in RIA (Chart 1b). The total amount of CEA bound by both the hyperimmune and tumor sera varied between 65 and 75% of the radioactivity present in the serum. Since this percentage of binding of the radioiodinated CEA was similar to that bound by specific goat anti-CEA serum, it is unlikely that the hamster sera were primarily reacting with a radiolabeled contaminant in the CEA preparation. Optimal CEA antibody activity in tumor sera in radioimmunooassay was obtained at room temperature or 37\(^{\circ}\), whereas the antibody in hyperimmune sera was most active at 45\(^{\circ}\).

Unlike hyperimmune sera, the tumor sera did not precipitate CEA on gel diffusion, and there were no visible precipitin lines with a 0.9% NaCl solution or PCA extract of GW-39. The lower titer of CEA antibody of the latter in radioimmunooassay probably explains the inability to obtain precipitin activity in gel diffusion. However, the antibody activity in tumor sera was independent of the ionic strength under the conditions studied in radioimmunooassay. In fact, the latter was more active in 0.1 M than 0.01 M buffer (Chart 1b). This suggests that it reacts with a determinant(s) on the CEA molecule that is different from the ion-sensitive site detected by hyperimmune sera. More direct evidence for the latter will be presented below.

**Physical Properties of CEA Antibody in Tumor Sera.** Chart 2 shows the results obtained when 5 ml of pooled sera from tumor-bearing hamsters were chromatographed on DEAE-cellulose. Antibody activity eluted in 2 regions of the chromatogram, with the largest amount of activity appearing in the pH 4.6, 0.15 M fraction. A minor amount of activity eluted with the starting buffer. Following chromatography of 5 ml of pooled sera on Sephadex G-200, the majority of antibody activity was voided by this gel, whereas a much smaller amount of antibody activity was associated with the 2nd protein peak (Chart 3). In addition, sucrose gradient ultracentrifugation results agreed with those obtained

**Hemagglutination Inhibition.** Hemagglutination inhibition of human erythrocytes by specific reagent sera or lectins to blood group antigens A, B, O, Le(a), Le(b), M, and N was performed by a previously described technique (3).
Chart 1. Anti-CEA activity of hyperimmune (a) and tumor sera (b) in radioimmunoassay. Hyperimmune sera, 1:100 dilution; tumor sera and normal sera, undiluted; NH₄Ac, ammonium acetate buffer, pH 6.25.

Sephadex G-200. The tumor sera antibody activity concentrated in the bottom of the tube, the region where 19S antibodies are found when this gradient is used (25).

Confirmatory evidence that the primary tumor sera antibody was a 19S immunoglobulin was obtained by radioimmunoelectrophoresis. On autoradiography, prominent 19S bands developed when either rabbit antiserum to hamster serum or hamster IgG gave a 7S pattern (Fig. 2). These results indicate that an IgM antibody is primarily responsible for the anti-CEA activity in the sera of cortisone-conditioned hamsters bearing i.m. GW-39 tumors. Since cortisone treatment is known to influence immune responses, particularly thymus-mediated activities (21), it is possible that such conditioning accounts for the depressed levels of anti-CEA IgG observed in these animals. However, the antibody in the sera of unconditioned GW-39 tumor-bearing hamsters was found to have identical chromatographic and electrophoretic characteristics as that from cortisone-treated hamsters with GW-39 tumors.

Inhibition of Hyperimmune and Tumor Sera in Radioimmunoassay. Inhibition studies in radioimmunoassay were
used to determine the CEA specificity of the hyperimmune and tumor sera. The radiiodinated CEA (8A) that was used in radioimmunoassay was isolated from liver metastases of a colon adenocarcinoma (22) and was found to be identical to GW-39 in its reactivity with both sera. However, when these sera were tested at 35% binding of the labeled 8A, they demonstrated marked differences in their affinity for the 125I-labeled 8A. In order to obtain similar inhibition curves of both sera with unlabeled 8A, approximately an 8-fold increase in the amount of 8A (320 versus 40 ng dry weight) was needed to inhibit the tumor sera (Chart 4a).

As shown in Chart 4b, GW-39 CEA and 2 other preparations of CEA (PHB-2 and DRA-1) from 2 different individuals varied in their ability to inhibit the association of 125I-labeled 8A with the tumor sera. Approximately 55% inhibition was obtained with 320 ng of PHB-2 or DRA-1, whereas the same amount of GW-39 and 8A produced an 80 and 90% inhibition, respectively. By contrast, all of the CEA preparations were very similar in their ability to inhibit the hyperimmune sera, giving 75 to 90% inhibition at 40 ng. The slight variability in inhibition of the hyperimmune sera by the different CEA's is similar to that observed with specific goat anti-CEA serum and reflects, on a dry weight basis, the relative amount of immunoreactive CEA that is present in each.

Although the hyperimmune sera reacted with a CEA determinant that was common to and uniformly distributed among the CEA's studied, the failure of PHB-2 and DRA-1 to inhibit completely the tumor sera suggested the presence of a 2nd antibody specificity in the latter. A sialomucin that was isolated from GW-39 (F. J. Primus and H. J. Hansen, unpublished data) inhibited approximately 55% of the tumor sera at the same concentration used for 8A (Chart 4c). The same degree of inhibition was obtained with saliva from A, B, and O blood group individuals (Chart 4c), whereas both saliva and the GW-39 mucin caused only minimal inhibition of the hyperimmune sera. The presence of Le^® but not A, B, O, M, or N blood group antigenic activity was detected by hemagglutination inhibition in the GW-39 mucin. When equal amounts of the GW-39 mucin and 8A were mixed and tested for inhibition of the tumor sera in radioimmunoassay, the inhibition due to each was additive and yielded a curve that was similar to that obtained with 8A (Chart 4d). These results demonstrated that approximately 50% of the CEA antibody activity in the tumor sera was against a CEA determinant that was common to all CEAs studied. This common determinant is apparently different from the 1 detected by the hyperimmune sera, since the latter is only demonstrable at low ionic strength. The remaining CEA antibody activity in the tumor sera was against a CEA determinant that was expressed in 2 of the CEAs studied, GW-39 CEA and 8A, and that was common to elements appearing in saliva and the GW-39 mucin.

**Temporal Development of CEA and CEA Antibody.** Since the aforementioned results were obtained with a pool of sera from hamsters bearing relatively large tumors, it was of interest to study the CEA antibody activity during different stages of tumor growth. Hamsters were thus sacrificed at different time intervals following the i.m. implant of GW-39 into 1 hind limb. These animals also received 2 cortisone injections during the 1st week of tumor growth. Chart 5 shows the results obtained when 25 μl of serum from individual hamsters were tested in radioimmunoassay for CEA antibody. The highest antibody activity was observed at 6 weeks postimplantation of tumor, at which time the tumors weighed approximately 5 g. Subsequently, there was a decrease in the antibody titer that was coincident with a marked increase in tumor growth. By 12 weeks, the antibody activity had dropped to less than 50% of the activity detected at 6 weeks. When the serum from these animals was assayed for the presence of CEA, low levels of circulating CEA were first detected at 6 weeks (Chart 5). This same
quantity of circulating CEA was present at 8 weeks, but by 12 weeks it had increased approximately 9-fold. Thus, beginning at 6 weeks, both CEA antibody and CEA were found in the circulation of tumor-bearing hamsters.

**DISCUSSION**

The observations reported here establish that the CEA antibody activity in hamsters bearing i.m. GW-39 tumors is primarily an IgM antibody. In this study, we have examined pooled sera from tumor-bearing animals at a time in which near maximal anti-CEA activity was observed in radioimmunoassay. Column chromatography showed the presence of minimal CEA antibody of the IgG type. However, we cannot exclude the appearance of IgG and additional IgM antibodies against other GW-39 tumor antigens that do not cross-react with CEA. Their presence was not demonstrated in immunodiffusion, but further measurement is needed with other immunological assays to verify their absence.

The CEA antibody in tumor sera contained 2 specificities that reacted with different determinants on the CEA. The likelihood that 1 of these specificities reacts with a blood group-related determinant is strengthened by the demonstration of cross-reactive material in human saliva and in a sialomucin produced by GW-39 cells. Although "CEA-like" material has been found in saliva (20), both saliva and the
GW-39 sialomucin, when used at the same concentration that inhibited the tumor sera, failed to react with specific anti-CEA serum in radioimmunoassay. Depending on the genotype of the donor of the CEA, blood group A, B, and Lewis-activity has been demonstrated in the CEA (5, 17). The presence of Lewis-activity but not other blood group activity in the GW-39 sialomucin suggests the involvement of Lewis-in the inhibition of the tumor sera by GW-39 sialomucin. The lack of Lewis-on 2 of the 4 preparations of CEA studied would explain the partial inhibition that was obtained with each. The 2nd antibody specificity in the tumor sera was against a determinant that was apparently common to all the CEA preparations. The activity of this determinant was not appreciably altered by variation in ionic environment and suggests that it is different from the CEA-specific ion-sensitive site detected by goat and hamster antisera raised to CEA in adjuvant.

As measured in radioimmunoassay, the CEA antibody activity was maximal in hamsters with 5-g GW-39 tumors. An increase in tumor growth was accompanied by a decrease in radioimmunoassay antibody activity and by the appearance of circulating CEA. Gold (6) demonstrated an IgM autoantibody against CEA that was not detectable in patients who have extensive metastatic involvement. It was suggested that the tumor in these cases acted as an antibody sink (4). Likewise, growing GW-39 tumors may act as an antibody sink and remove circulating antibody. We have reported previously that i.m. GW-39 tumors larger than 5 g fail to accumulate preferentially radioiodinated goat anti-CEA IgG (23). The accumulation of hamster IgM antibody against CEA on the tumor cell surface may mask CEA sites that would otherwise be available to react with localizing goat anti-CEA IgG. Alternatively, since only free antibody was detectable in radioimmunoassay, the formation of circulating antibody-antigen complexes in hamsters with GW-39 tumors larger than 5 g could also explain the decreased CEA antibody activity in these animals.

It remains unclear whether the antibody response presently described in this xenogeneic tumor model is tumor associated or merely a reaction to foreign species antigen. The appearance of circulating CEA antibody and CEA may be more than fortuitous and suggests their possible influence on GW-39 tumor growth as described in other systems (1, 24). Since previous studies have presented contradictory evidence concerning the immunogenicity of CEA in humans with intestinal neoplasia (6, 19), further evaluation of the role of CEA and other colonic antigens (12) in the GW-39 tumor-host relationship may serve to increase our understanding of the biological significance of such tumor products.

ACKNOWLEDGMENTS

The authors express their appreciation to Dr. Maja Rozenberg for the hemagglutination inhibition analysis.

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