Evaluation of a radioimmunoassay for carcinoembryonic antigen of the human digestive system

J. Serge LeBel, M.D.*
Department of Gastroenterology

Sharad D. Deodhar, M.D., Ph.D.
Department of Immunopathology

Charles H. Brown, M.D.
Department of Gastroenterology

Introduction

Few topics in cancer immunology have given rise to more controversies in the past few years than the detection of circulating tumor antigens. Gold et al made a significant contribution in this regard by demonstrating, isolating, and characterizing what appeared to be both a system- and tumor-specific neoantigen.[1-4] Apparently this antigen could be found in all entodermally derived adenocarcinomata of the gastrointestinal tract as well as in fetal gut in the first two trimesters of gestation, hence the name carcinoembryonic antigen (CEA). Using a radioimmunoassay technique,[5] they later demonstrated in a study of 200 cases, that circulating levels of this antigen as low as 2.5 ng/ml could be reproducibly detected. The antigen was present in the serum of 35 of 36 patients with adenocarcinoma of the colon and rectum, and disappeared from circulation following complete resection of the tumor mass. Moreover, this antigen could not be detected in the serum of normal individuals or

* *Fellow, Department of Gastroenterology.*
Presented in part at The Southern Medical Association Meeting, Dallas, Texas, Nov. 16, 1970 and in toto at The American Protologic Society Meeting in Las Vegas, Nevada, May 11, 1971.
This research was supported in part by grants from the American Cancer Society, Ohio Division, The Randall Foundation, and a donation from Mr. F. Ball.

patients with either benign or malignant disease in other systems.

Because of the diagnostic and prognostic significance of these findings we initially undertook the evaluation of Doctor Gold's assay using principal reagents he supplied without modifying his methodology. As is often the case with any new procedure, there were many techical problems, as a result of which only 60% of our initial samples (239 cases) were suitable for study.[6] In brief, the sensitivity of the assay was such that only 65% of adenocarcinomata of the colon and rectum and 60% of adenocarcinomata elsewhere in the gastrointestinal tract were detectable. However, there were also an unexpected number of positive results in cases of transmural colitis and/or ileitis (70%), mucosal colitis (33%), and diverticulitis (33%). Positive results were also observed in malignancies in five other systems (reticuloendothelial, breast, lung, kidney, cervix), in two benign chronic illnesses (chronic obstructive lung disease, diabetes mellitus), and in one out of 43 normal individuals. Although the number of cases in each of these nongastrointestinal categories was too small to make percentages meaningful, the spectrum of false positivity was impressive. Because of the difficulties encountered initially, technical modifications were introduced. A new antiserum of greater avidity was also provided. These changes resulted in increased stability, reproducibility, and sensitivity of the assay. The findings obtained with this modified assay are presented and discussed in this report.

Materials and method

The study was undertaken according to a double blind design. All samples were processed in duplicate. All cases either had a histologic diagnosis or, when surgery was not performed, had sufficient clinical and laboratory evidence to substantiate a final diagnosis. Quantitative limits of variability between duplicate samples were also established. Those cases which did not meet these criteria were excluded; 154 cases were considered acceptable. Twenty-one samples were processed independently by Doctor Gold's and our laboratories.

The CEA was labeled in the following manner. To 2 mCi of Na ^{125}I (Amersham-Searle, Chicago) were added, in succession: 100 μl of phosphate buffer (0.05 M, pH 7.5), 200 μg of CEA dissolved in 200 μl phosphate buffer, and 200 μg of chloramine-T in 20 μl phosphate buffer; the mixture was constantly stirred and the reaction allowed to proceed at room temperature for 2 minutes at which time it was terminated by the addition of 250 μg of sodium metabisulfite in 20 μl phosphate buffer. Labeled CEA was separated from the unreacted sodium iodide by chromatography on Sephadex G-100. One-milliliter fractions were collected in 1.0 ml 5% bovine serum albumin. The labelled CEA appeared immediately after the void volume. Specific activity did not vary by more than 10% from one labelling to the next. The labelled antigen was diluted in bulk (500 ml) immediately after labelling, so as to produce 50,000 CPM/500 ml. This solution was used for approximately 3 weeks and kept at 4C.

An equal volume of 2.0 M perchloric acid was added to each 5 ml aliquot of serum to be tested. The mixture was homogenized, then centrifuged at 3500 rpm, for 30 minutes at

4C. The supernate was dialyzed against cold running tap water for 24 hours and against ion-free water at 4C for an additional 24 hours. Following this, the samples were frozen and lyophilized to dryness. The powdered extract was dissolved in 500 μl of anti-CEA antiserum. Normal human serum diluted 1:10 with borate buffer (0.1 M, pH 8.4) was used to dilute the antiserum to 1:100,000. The antigen-antibody mixture was incubated at 37C for one hour and allowed to equilibrate at 4C overnight. The following morning, 500 μl of appropriately diluted labeled CEA (a ^{125}I-CEA concentration of approximately 40 ng/ml) was added to each tube which was then incubated at 37C for 3 hours. The antibody-bound CEA was separated from the free fraction by the addition of 1 ml cold saturated ammonium sulfate and 1 ml 50% saturated ammonium sulfate. The mixture was allowed to equilibrate at 4C for 1 hour, centrifuged at 4000 rpm for 30 minutes at 4C, and the activity of the supernate assessed in a gamma scintillation counter. Results were converted to ng/ml by comparison with a standard inhibition curve prepared simultaneously (*Fig. 1*). The latter was prepared by the addition of known amounts of unlabeled CEA to 5 ml aliquots of perchloric acid-extracted normal human serum. To separate positive from negative results a baseline level of 1 ng/ml was chosen.

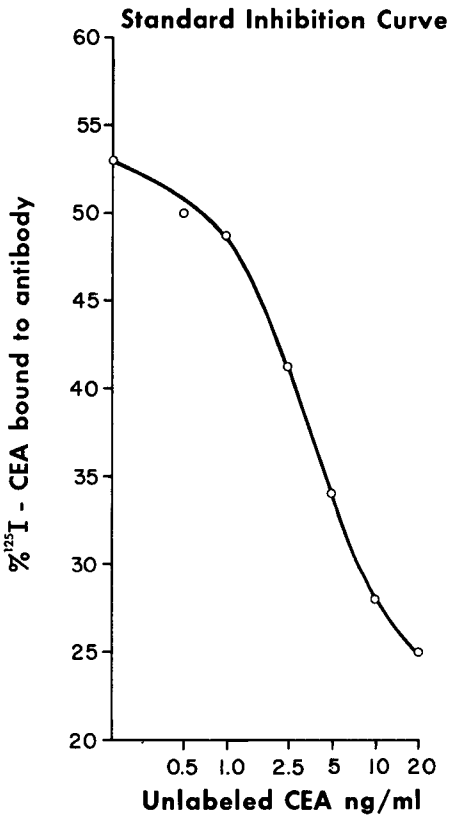

Fig. 1. Standard inhibition curve obtained by addition of cold CEA to a constant amount (approximately 20 ng) CEA-^{125}I, goat anti-CEA antiserum dilution of 1:100,000.

Results

The results of our study of 154 cases are outlined in *Table 1*. The detection rate for carcinoma of the colon and rectum was 82%, a significant improvement over our previous detection rate of 65%. There was an increase to 93% for other gastrointestinal malignancies. However, a similar increase in positive results was also noted in inflammatory bowel diseases with 77% positive in patients with transmural colitis and/or ileitis, 75% of those with ulcerative colitis, and 66% of cases of diverticulitis. There was no histologic evidence of malignancy in any of these inflammatory bowel diseases. The results obtained in benign noninflammatory gastrointestinal as well as benign and malignant nongastrointestinal diseases are also shown in *Table 1*, but the number of cases is probably too small to make percentages meaningful.

Table 1.—Results obtained with the CEA assay

	Total	Positive	Negative
Gastrointestinal	122		
Malignant			
Adenocarcinoma, colon and rectum	56	46	10
Adenocarcinoma, stomach	4	4	0
Adenocarcinoma, pancreas	9	8	1
Adenocarcinoma, biliary tract	2	2	0
Benign			
Transmural colitis and/or ileitis	18	14	4
Mucosal colitis, chronic	8	6	2
Diverticulitis coli	12	8	4
Diverticulosis coli	1	0	1
Villous adenoma, colon	1	0	1
Polyps, colon	3	1	2
Familial polyposis	1	0	1
Benign ulcer, cecum	1	1	0
Chronic active hepatitis	2	0	2
Sclerosing cholangitis	1	1	0
Chronic irritable bowel	2	2	0
Nutritional cirrhosis	1	1	0
Nongastrointestinal	32		
Normal healthy individuals	14	2	12
Malignant			
Hodgkin's lymphoma	1	0	1
Reticulum cell sarcoma	1	1	0
Reticulum cell sarcoma (stomach)	1	1	0
Dysgerminoma	1	0	1
Transitional cell carcinoma, urinary bladder	1	1	0
Benign			
Chronic obstructive pulmonary disease	2	2	0
Pulmonary alveolar proteinosis	1	1	0
Desquamative interstitial pneumonitis	1	1	0
Chronic renal insufficiency	2	2	0
Diabetes mellitus	2	2	0
Arteriosclerotic heart disease	1	0	1
Primary myocardial disease with chronic heart failure	1	1	0
Rheumatoid arthritis	2	2	0
Systemic lupus erythematosus	1	1	0

The results of 21 sera tested independently by the two laboratories are compared in *Table 2*. Perhaps the number of cases is too small to allow one to draw definitive conclusions. However the spectrum of positivity in both benign and malignant disorders is interesting. Again no histologic evidence of malignancy could be found in any of the inflammatory bowel diseases. Results obtained independently in both laboratories correlated well.

Discussion

We were satisfied that technically the changes made in the methodology improved the stability and reproducibility of the assay, and increased the rate of detection of gastrointestinal malignancies. However, these same im-

provements also brought about an increase in false positive results, particularly in inflammatory bowel diseases, thus confirming our previous findings.[6] There is a sufficiently broad spectrum of false positivity in this study of 154 cases (and previous 239 cases) to allow us to reach certain conclusions and postulate why this occurred. This spectrum extends from normal individuals (albeit only 3 of 57) to malignant and benign disease in a variety of systems.

Others have used the Gold assay and have observed similar problems with false positivity in certain situations. Moore et al in their studies[7, 8] reported a detection rate of 72% for carcinoma of the colon, 88% for carcinoma of the pancreas, and 39% for other gastrointestinal carcinomata. Of 41 patients with "colitis" only one had a positive titer, but was found to have a carcinoma at surgery; findings which are opposite to ours. However, there is one difference in the two groups of patients which we feel may be significant in terms of the results obtained; our patients were extremely ill and required surgery; most of their patients were from an out-patient group.[9] Moore et al also observed positive results in 5 of 22 patients with "diverticular disease" of the colon; in our study 8 of 12 cases of diverticulitis coli gave positive titers. The number of false positive results which they observed in nongastrointestinal malignancies, alcoholic cirrhosis, and pancreatic diseases, and in patients with chronic renal insufficiency is not inconsistent with our findings. Results of a preliminary study by Nugent et al[10] included a smaller number of cases which focused mainly on the gastrointestinal tract; positive results were noted in a spectrum of benign gastrointestinal diseases as well as carcinomata.

Table 2.—Comparison of cases assayed independently in Montreal and Cleveland

	Total	Montreal, no. positive	Cleveland, no. positive
Adenocarcinoma, colon and rectum	6	6	4 (1)*
Carcinoma in villous adenoma, colon	1	1	1
Adenocarcinoma, stomach	1	1	1
Adenocarcinoma, pancreas	4	4	3 (1)*
Reticulum cell sarcoma, stomach	1	1	1
Reticulum cell sarcoma, nongastrointestinal	1	0	1
Transmural colitis and/or ileitis	4	4	3 (1)*
Mucosal colitis, no malignancy	2	2	2
Systemic lupus	1	1	1

* Cases excluded for technical reasons in our laboratory.

There is now sufficient evidence to corroborate Gold's findings that there is such an entity as the carcinoembryonic antigen of the human digestive system which is both tumor- and system-specific,[11-14] and that it may result from antigenic reversion.[15] Data obtained with their original radioimmunoassay technique provided additional supportive evidence, as well as significant diagnostic and prognostic potential. However, neither the high detection rate nor the same specificity have been reported to date by other investigators who have used this assay.[6-8, 10]

An attempt was made to correlate size and/or staging of colorectal carcinomata with antigen titer, but none was apparent in our study, although Moore et al did report such a correlation.[8]

Furthermore we were unable to discriminate between malignant and inflammatory bowel diseases of the gastrointestinal tract on a quantitative basis. A significant degree of false positivity was observed in patients with chronic pulmonary and renal diseases, diabetes mellitus, and collagenoses. The fact that any combination of these chronic benign illnesses can and does occur in an age group where the incidence of malignancy is highest, could make the test difficult if not impossible to interpret. The diagnostic value of the radioimmunoassay for CEA in upper gastrointestinal malignancies or its prognostic value in following patients who have had their colonic malignancy resected has not been sufficiently explored in this study to warrant conclusions. Nevertheless it seems reasonable to assume that the assay could be valuable as an index of recurrent or metastatic disease if the test were positive before and negative after resection.

It is our impression that the present radioimmunoassay may not be measuring only CEA, but antigenically related substances as well. This by no means disproves the existence of CEA, but simply points out the limitations of the assay as a diagnostic tool in its present form. It has been shown that glycoproteins of both alpha and beta electrophoretic mobility are elevated in a wide variety of benign and malignant diseases.[16, 17] These sialoglycoproteins may interfere with antigen-antibody binding,[18] and thus potentially create problems in a radioimmunoassay system, particularly if they happen to share antigenic determinants. This may be one explanation for the wide variety of false positive results.

Whether more "gentle" preparative methods such as the method of Rosai et al[19] will prove more successful in the purification of tumor antigens needs further exploration. Other assay methods such as the zirconyl gel technique of Lo Gerfo et al,[20] the double antibody-triple isotope method of Egan et al,[21] or less sensitive methods such as the hemagglutination-inhibition technique of Lange et al[22] may in the end yield better results, but further experience is required.

Conclusions

We have reported 154 cases assayed for circulating CEA using a modified version of the assay employed by Gold et al. The gastrointestinal cancer detection rate was 82% for colorectal adenocarcinomata and 93% for other gastrointestinal adenocarcinomata. We found a sufficiently wide spectrum of false positivity to seriously limit the usefulness of this assay in its present form as a diagnostic tool for entodermally derived adenocarcinomata of the gastrointestinal tract.

Further modification of this assay, better antigen purification, more exhaustive absorption of antisera, or other methods of assay in the end may yield a test which is more suitable as a diagnostic aid.

Acknowledgments

We are indebted to Dr. Phil Gold for his advice in the preparation of this study, as well as the provision of antigen and antisera.

References

1. Gold P, Freedman SO: Demonstration of tumor specific antigens in human colonic carcinomata by immunological tolerance and absorption techniques. J Exp Med **121:** 439–462, 1965.
2. Gold P, Freedman SO: Specific carcinoembryonic antigens of the human digestive system. J Exp Med **122:** 467–481, 1965.
3. Krupey J, Gold P, Freedman SO: Purification and characterization of carcinoembryonic antigens of the human digestive system. Nature (Lond) **215:** 67–68, 1967.
4. Krupey J, Gold P, Freedman SO: Physicochemical studies of the carcinoembryonic antigens of the human digestive system. J Exp Med **128:** 387–398, 1968.
5. Thomson DMP, Krupey J, Freedman SO et al: The radioimmunoassay of carcinoembryonic antigen of the human digestive system. Proc Natl Acad Sci USA **64:** 161–167, 1969.
6. LeBel JS, Deodhar SD, Brown CH: Experience with a radioimmunoassay for CEA at the Cleveland Clinic. Presented at the Southern Medical Association Meeting, Nov. 16, 1970.
7. Moore TL, Kupchik HZ, Marcon N, et al: Carcinoembryonic antigen assay in cancer of the colon and pancreas and other digestive tract disorders. Am J Dig Dis **16:** 1–7, 1971.
8. Moore T, Dhar P, Zamcheck W, et al: Carcinoembryonic antigen (CEA) in diagnosis of digestive tract cancer (enlarged series). Proceedings of First Conference and Workshop on Embryonic and Fetal Antigens in Cancer, Oak Ridge National Laboratories (AEC), Oak Ridge Tennessee (In press).
9. Zamcheck W: Personal communication, Aug. 3, 1971.
10. Nugent FW, Hansen ER: Radioimmunoassay of carcinoembryonic antigen as a diagnostic test for cancer of the colon: a preliminary report. Lahey Clin Found Bull **20:** 85–88, 1971.
11. Von Kleist S, Burtin P: Isolation of a fetal antigen from human colonic tumors. Cancer Res **29:** 1961–1964, 1969.
12. Norland CC, Maass EG, Kirsner JB: Identification of colon carcinoma by immunofluorescent staining. Cancer **23:** 730–739, 1969.
13. Martin F, Martin MS: Demonstration of antigens related to colonic cancer in the human digestive system. Int J Cancer **6:** 352–360, 1970.
14. Kleinman MS, Harwell L, Turner MD: Studies of colonic carcinoma antigens. Gut **12:** 1–10, 1971.
15. Gold P: Antigenic reversion in human cancer. Ann Rev Med **22:** 85–94, 1971.
16. Greenspan EM, Lehman I, Groff MM, et al: A comparative study of the serum glycoproteins in patients with parenchymatous hepatic disease or metastatic neoplasia. Cancer **42:** 972–983, 1951.
17. Greenspan EM: Survey of clinical significance of serum mucoprotein level. Arch Intern Med **93:** 863–874, 1954.
18. Apffel CA, Peters JH: Tumors and serum glycoproteins. The symbodies. Progr Exp Tumor Res **12:** 1–54, 1969.
19. Rosai J, Tillack TW, Marchesi VT: A new method for the isolation of tumor-specific antigens from human neoplasm. Fed Proc **30:** 453, 1971.
20. Lo Gerfo P, Krupey J, Hansen HJ: Demonstration of an antigen common to several varieties of neoplasia. N Engl J Med **285:** 138–141, 1971.
21. Egan ML, Lautenschleger JT, Coligan JE, et al: Radioimmune assay of carcinoembryonic antigen. Immunochemistry **9:** 289–300, 1972.
22. Lange RD, Chernoff A, Collman RI: Preliminary report of clinical experience with a hemagglutination-inhibition test for carcinoembryonic antigen. Proceedings of the First Conference and Workshop on embryonic and fetal antigens in cancer, Oak Ridge National Laboratories (AEC) Oakridge, Tennessee (In press).