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Monoclonal Antibodies to Carcinoembryonic Antigen Produced by Somatic Cell Fusion[1]

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ABSTRACT

Hybridoma cell lines secreting monoclonal antibodies to carcinoembryonic antigen (CEA) were generated by fusing mouse immune lymphocytes with the mouse myeloma variant cell line, NS-1. Antibody secreted by one cloned hybrid cell line could bind only a select portion of the CEA bound by the commercially available goat anti-CEA antiserum used in clinical assays. Radiolabeled CEA could be purified on a monoclonal antibody affinity column. Incorporation of this purified radiolabeled CEA in a double-antibody solid-phase assay with goat anti-CEA antiserum led to an approximately 2.5-fold increase in sensitivity of the assay. Genetically stable hybrid clones may be sources of virtually unlimited quantities of such antibodies which may have potential utility in improving the cancer specificity of clinical assays.

INTRODUCTION

In 1965, Gold and Freedman (4, 5) described CEA[6] as an antigen specific for adenocarcinoma of the human digestive tract and appendage organs of human fetuses between 2 and 6 months of gestation. Subsequently, Krupey *et al.* (10, 11) characterized CEA as a glycoprotein with a molecular weight of approximately 200,000 with β-electrophoretic mobility and a carbohydrate/protein ratio of approximately 2.5/1. In 1969, a radioimmunoassay for CEA in the sera of patients was described (20). Circulating levels as low as 2.5 ng/ml of serum were reported in 35 of 36 patients with adenocarcinoma of the colon, and "insignificant" levels were found in the sera of patients with nongastrointestinal cancers or benign diseases. These findings, plus the disappearance of CEA from the circulation of patients, following complete surgical removal of the tumor offered the promise of an early diagnostic procedure for patients with digestive system cancer. Studies from many laboratories have now established that whereas a positive CEA assay may be a good indicator of invasive colonic cancer, and particularly of metastases to the liver, CEA elevation occurs in less than 50% of patients with localized disease (Dukes' Stage A) (12, 22). Additionally, using assays with conventionally produced antisera (12, 21), CEA has been identified and measured in the circulation and body fluids of patients with numerous nongastrointestinal cancers, patients with gastrointestinal benign diseases, and normal individuals.

This lack of specificity has been attributed both to heterogeneity of purportedly purified CEA and to variable intramolecular antigenicity (3). It is also possible that some individual antigenic determinants identified in present CEA assays are associated with CEA molecules synthesized by normal gastrointestinal tissues and are only quantitatively distinct in the presence of cancer.

Efforts to improve the specificity of CEA assays by alternative approaches for purification of the antigen (15), absorption of antisera with CEA-like components (18), or synthesis of an antigenic moiety of CEA (2) have met with only partial success. The recent adaptation of cell hybridization techniques to the construction of hybrid cell lines producing monoclonal antibodies with desired reactivities has revolutionized the approach to production and utilization of immunospecific reagents (7). This procedure involves hybridizing splenic lymphocytes from immunized donors with cells from a myeloma adapted to growth in culture. Hybrid cell lines producing the desired antibody are identified and can be cloned. Each hybrid clone produces a single species of antibody specific for an individual antigenic determinant regardless of the complexity of the antigen. Thus, the presence or absence of individual antigenic determinants associated with CEA preparations might be identified clinically if monoclonal antibodies to CEA are available. This report describes the production of monoclonal antibodies to CEA by somatic cell fusion of mouse myeloma and mouse spleen cells and demonstrates the potential usefulness of one of these antibodies.

MATERIALS AND METHODS

Cell Fusion. A 1-month-old female BALB/c mouse was immunized by i.p. injection of 8 μg of purified CEA (a gift of Dr. Charles Todd, City of Hope, Duarte, Calif.) in complete Freund's adjuvant. One month later, the mouse was given an i.v. boost of 5 μg CEA in 0.15 M NaCl. Three days later, the mouse was sacrificed, and the spleen was used as a source of immune cells for fusion with the 8-azaguanine-resistant myeloma, P3-NS1/1-Ag4-1 (hereafter called NS-1), which synthesizes but does not secrete light chain (8). Cell fusion was carried out as described by Marshak-Rothstein *et al.* (13) with minor modifications. Approximately 1×10^8 spleen cells were combined with 1.3×10^7

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[6] The abbreviations used are: CEA, carcinoembryonic antigen; FCS, fetal calf serum; PBS, phosphate-buffered saline [0.1 M ($NaH_2 \cdot NaH$)PO_4/0.15 M NaCl, pH 7.4]; Z-gel, zirconyl phosphate gel; DAG, donkey anti-goat IgG; GAM, goat anti-mouse IgG; G-As, goat anti-CEA antiserum, PCA, perchloric acid.

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NS-1 cells in 30% polyethylene glycol (M.W. 1000) for 6 min at 37°. Following fusion, the cells were divided equally among 298 microtiter wells (Costar, Inc., Cambridge, Mass.). The fused cells were suspended in Dulbecco's modified Eagle's medium with high glucose (4.5 g/liter) supplemented with 15% irradiated FCS, 2 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2 mM glutamine, 100 units penicillin per ml, 100 μg streptomycin per ml, hypoxanthine/aminopterin/thymidine. After selection, cells were grown in supplemented Dulbecco's modified Eagle's medium without hypoxanthine/aminopterin/thymidine at 37° in a humid atmosphere containing 5% CO_2.

Cloning and Ascites Production. Hybrids were cloned twice at limiting dilution on confluent BALB/c 3T3 fibroblast feeder layers.

In order to obtain larger amounts of antibody, clones of hybrid cells were injected i.p. into BALB/c mice which had been primed with 0.5 ml tetramethylpentadecane (pristane). Animals were observed daily, and paracentesis was performed every 2 to 3 days after appearance of ascites (approximately 10 days after injection).

Antibody-screening Assay. Culture media from wells containing growing hybrid cells were tested for the presence of anti-CEA antibody in a sensitive radioimmunoassay (17) in which flexible microtiter plates (Dynatech Laboratories, Alexandria, Va.) were coated with partially purified CEA and incubated with culture medium. The bound immunoglobulin was then detected with affinity-purified ^{125}I-labeled goat anti-mouse F(ab')$_2$ antibody. Briefly, individual microwells were each coated with 50 μg CEA in 100 μl PBS at 20° for 2 hr. The unbound antigen was aspirated, and each well was filled with PBS containing 10% FCS for 30 min at 20°. After 3 washes with PBS containing 0.2% FCS, 50 μl of sample were added for 2 hr at 20°. The wells were again washed 3 times with PBS/0.2% FCS, and 100 μl ^{125}I-goat anti-mouse F(ab')$_2$ antibody (approximately 30,000 cpm; 0.25 mCi/mg) was added for 18 hr at 20°. The wells were washed 3 times with PBS/0.2% FCS, cut, placed in individual tubes, and counted in a Packard gamma well counter. Positive wells had at least 5 times the counts found in controls [using medium from hybrid cells producing anti-tetanus toxin antibody (23)].

The isotype of antibody-positive hybrid cultures was determined in the same manner using ^{125}I-labeled antibodies specific for mouse IgM, IgA, IgG1, or IgG2b (gifts from Dr. Meredith Mudgett-Hunter, Massachusetts General Hospital, Boston, Mass.).

Liquid-Phase Radioimmunoassay. The Roche Z-gel assay (6) was used initially for titration of anti-CEA antibody activity in the hybridoma culture media. A double-antibody solid-phase radioimmunoassay was used for subsequent titrations of anti-CEA antibody activity standard inhibition curves and for determination of relative affinity constants (K_a). For titration and K_a determinations, antibody was added to 1 ml of 0.0037 M disodium EDTA (containing 0.002% bovine serum albumin and 0.017% sodium azide) and incubated with purified ^{125}I-labeled CEA in the absence of unlabeled CEA for 18 hr at 20°. Labeled CEA which was bound to antibody was precipitated with a 2% suspension of either DAG or GAM immobilized on Kynar (polyvinylidene fluoride) floccules after an additional 10-min incubation at 20° (14). To calculate K_a, the method of Steward and Petty (19) was used. For inhibition curves, antibody was incubated with unlabeled purified CEA for 30 min at 45° prior to addition of labeled CEA and subsequent incubation (18 h, 20°) and precipitation with the appropriate Kynar-conjugated antibody. Reagents for these assays, including purified CEA standard, ^{125}I-purified CEA, goat anti-CEA antisera and conjugated Kynar suspensions were kindly provided by Dr. H. Hansen, Roche Laboratories, Inc., Nutley, N. J.

Immunoadsorption of CEA. For immunoadsorption studies, ascitic fluid monoclonal antibody was purified on a DEAE-Affi-Gel Blue column (Bio-Rad Laboratories, Richmond, Calif.). Ascitic fluid (32 mg protein in 1 ml fluid) was first dialyzed against 0.02 M K_2HPO_4 (pH 8.0) and then applied to the column and eluted in the same buffer. The eluted IgG (4.6 mg) was coupled to cyanogen bromide-activated Sepharose 4B (2 hr, 20°) after concentration to 1 mg/ml on a bed of polyethylene glycol (M.W. 6000) (16). Unreacted sites on the Sepharose were blocked by incubation (2 hr, 20°) with 1.0 M ethanolamine (pH 8.0).

Approximately 80 ng of ^{125}I-CEA (7.7 × 10^6 cpm) were applied to a monoclonal anti-CEA antibody (4 mg IgG)-Sepharose 4B column in 0.05 M PBS (pH 7.5). The specifically bound, labeled CEA was eluted in 3.0 M NH_4SCN (pH 7.0) and dialyzed against 0.01 M PBS. Unlabeled purified CEA was similarly adsorbed and eluted from a comparable affinity column.

RESULTS

Of the original 298 wells containing fused cells, 58 contained growing hybrid cells after 2 to 3 weeks. Although anti-CEA antibody production was detected by the screening assay in 9 of these cultures, only 6 remained positive following propagation of the hybrids. When culture media from these 6 lines were tested for ability to bind ^{125}I-CEA in the Z-gel assay, one showed relatively high binding above T_0 (background; no medium added), 3 showed moderate binding, and 2 showed no binding above T_0 (Table 1). There were no significant additive effects when lines 5C8, 1F5, and 4E8 were added to each other or to line 5E9 in the Z-gel assay (Chart 1).

Thus, it appeared that the uncloned hybrid cells of 5E9 might be producing an antibody or antibodies recognizing the same sites or antigen populations recognized by antibodies produced

Table 1

Binding of hybridoma antibodies in tissue culture supernatants

Cell line	Microtiter plate assay[a] [cpm ^{125}I-goat anti-mouse F(ab')$_2$ antibody]	Z-gel assay[b] (% of ^{125}I-CEA bound)
5E9	4257	35.7
5C8	4342	20.7
1F5	3813	15.1
4E8	3704	9.7
1D8	2222	0.6
5G5	3657	0
Control[c]	396	ND[d]

[a] ^{125}I-goat anti-mouse F(ab')$_2$ antibody (30,000 cpm) added.
[b] Maximum percentage of ^{125}I-CEA bound to antibody above background (T_0).
[c] Cell culture medium containing monoclonal antibody to tetanus toxin.
[d] ND, not done.

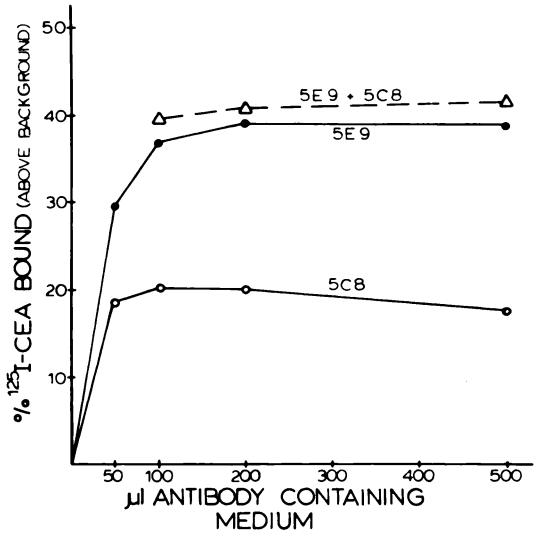

Chart 1. Representative titration curves of cultured media from hybridoma colonies showing maximum binding (5E9) and moderate binding (5C8) of radiolabeled CEA. No increase in binding was observed when equal amounts of 5E9 and 5C8 were added (total volume of combined media plotted).

by the uncloned hybrids 5C8, 1F5, and 4E8. It was also apparent that approximately 60% of the ^{125}I-CEA could not be bound no matter how much antibody was added to the assay system.

Line 5E9, which was selected for cloning, was found to be producing antibody of the IgG1 subclass. Cloning of 5E9 resulted in 10 lines which were passaged. Nine of the 10 lines produced antibody in the medium which could bind a maximum of approximately 40% of the ^{125}I-CEA. The tenth line produced no anti-CEA and was used as a negative control. Each of the 9 clones of 5E9 was cloned a second time, resulting in 25 single-cell clones, each producing a monoclonal antibody which could bind a maximum of approximately 30 to 40% of the ^{125}I-CEA from Roche.

Binding of Monoclonal Anti-CEA Antibody to ^{125}I-CEA. One of the 5E9 subclones, which was designated 1E9, was grown as a solid tumor in mice inoculated i.p. The accumulated ascites from these mice were pooled and used for subsequent studies.

The double-antibody solid-phase assay was used to titrate and compare the binding of 1E9 antibody to ^{125}I-CEA with that achieved using commercially available G-As. As shown in Table 2, 1E9 antibody could bind a maximum (above T_0) of 45.5% of the ^{125}I-CEA added while G-As bound 69.8%. In an attempt to increase the total binding of 1E9 antibody to the labeled CEA, the labeled CEA was adsorbed on an affinity column containing the monoclonal 1E9 antibody conjugated to cyanogen bromide-activated Sepharose 4B. The specifically adsorbed CEA, when eluted from the column, was apparently still bound to some 1E9 antibody which had leaked off the matrix creating a high background when precipitated with GAM. The "contaminating" antibody was eliminated by extraction of the specific ^{125}I-CEA eluate with 1.0 M PCA. After dialysis of the PCA supernatant against PBS, 1E9 antibody could bind a maximum of 60.5% of the labeled CEA. Although improved, this was still lower than the 77% bound by G-As. A portion of the nonadsorbable labeled CEA which passed through the affinity column without binding to the antibody could be bound by G-As but not by 1E9 antibody.

To determine the relationship of the species of ^{125}I-CEA bound by the monoclonal antibody and the G-As, an excess of G-As was incubated with immunoadsorbed and PCA-extracted (treated) labeled CEA, and the immune complexes were precipitated with DAG. The remaining unbound labeled CEA in the supernatant was then incubated with an excess of 1E9 antibody, and these complexes were precipitated with GAM. The reverse procedure was also carried out.

Table 2

Comparative maximum binding by 1E9 antibody and G-As to ^{125}I-CEA preparations using double-antibody solid-phase assay

	1E9		G-As	
^{125}I-CEA treatment[a]	T_0[b]	Maximum % ($-T_0$)[c]	T_0	Maximum % ($-T_0$)
No treatment	2.0	45.5	2.0	69.8
Immunoadsorbed	37.9	21.5	6.3	71.5
Immunoadsorbed + PCA	4.8	60.5	3.9	77.0
Nonadsorbable	11.5	1.5	8.7	18.5
Nonadsorbable + PCA	5.7	7.9	5.5	24.6

[a] See text for explanation of treatment.
[b] Percentage of ^{125}I-CEA precipitated nonspecifically in the absence of antibody.
[c] Maximum percentage of total ^{125}I-CEA bound at any dilution of antibody (background subtracted).

Table 3

Competitive binding of ^{125}I-CEA by 1E9 antibody and G-As using the double-antibody solid-phase assay

DAG- or GAM-conjugated Kynar suspensions were used for G-As or 1E9, respectively.

	% ^{125}I-CEA[a] bound − T_0[b]		
Anti-CEA	DAG	GAM	Total
G-As, then 1E9	71.2	→ 0.9[c]	72.1
1E9, then G-As	24.9	← 39.1[c]	63.0
G-As + 1E9[d]	73.3	35.9	
G-As → 1E9[e]	73.8	27.7	
1E9 → G-As[e]	72.9	36.9	

[a] ^{125}I-CEA was immunoadsorbed and PCA treated.
[b] In experiments where 2 antibodies were used, the T_0 control contained the antibody not precipitated by a Kynar conjugate.
[c] Arrows indicate sequence of specific precipitations.
[d] Antibodies added together to ^{125}I-CEA for incubation and complexes precipitated with DAG or GAM.
[e] Arrows indicate sequence of antibody addition. Each antibody was incubated for 18 hr at 20°.

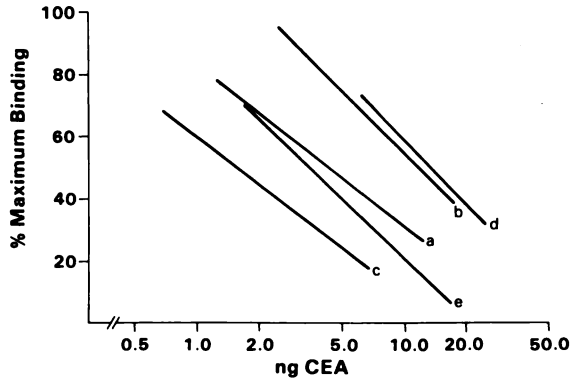

Chart 2. Comparative inhibition curves. Curves are drawn based on linear regression analysis and represent maximum percentage of binding of ^{125}I-CEA by antibody in the presence of increasing concentrations of unlabeled purified CEA.

$$\% \text{ of maximum binding} = \frac{X - T_0}{B_0 - T_0} \times 100$$

where X is experimental percentage bound, T_0 is background in absence of antibody, and B_0 is binding in absence of inhibitor. *Curves a* and *b*, inhibition curves when untreated CEA and ^{125}I-CEA were used with G-As and 1E9, respectively; *Curves c* and *d*, inhibition curves when untreated CEA and immunoadsorbed PCA-treated ^{125}I-CEA were used with G-As and 1E9, respectively; *Curves c* and *e*, inhibition curves when both unlabeled and labeled CEA, which had been immunoadsorbed and PCA-treated were used with G-As and 1E9, respectively. *Curve c* represents 2 curves since quantitation of the immunoadsorbed PCA-treated CEA was done using the untreated CEA as a standard.

Labeled CEA was also incubated with both G-As and 1E9 antibody simultaneously, and either DAG or GAM was used for specific precipitation as shown in Table 3. The goat antiserum bound all available labeled CEA if added first, and the monoclonal antibody could bind only 62% of the total bound (63%) by both antibodies. There was no apparent competition between the antibodies when both were added simultaneously; i.e., they still bound approximately the same amounts as when added first in the previous experiments. However, when G-As was incubated for 18 hr with treated labeled CEA, followed by addition of 1E9 antibody to the mixture and another 18-hr incubation period, and then the immune complexes were precipitated with DAG or GAM, less labeled CEA was available for binding by 1E9 (27.7%) than in the reverse experiment (36.9%) or when both antibodies were added simultaneously (35.9%).

CEA Inhibition Curves. Inhibition studies were undertaken

Table 4
Parameters of comparative inhibition curves

Curves shown in Chart 2[a]	CEA required for 50% inhibition[b] (ng)	Slope[c]
a	4.3	−51.6
b	12.0	−66.0
c	1.6	−51.5
d	13.0	−69.0
e	3.6	−63.4

[a] See legend to Chart 2 for description of inhibition *Curves a* to *e*.
[b] Concentration of unlabeled purified CEA required to achieve 50% inhibition of maximum binding.
[c] Slope was calculated by linear regression analysis and reflects decrease in percentage bound per log CEA.

to determine the ability of the monoclonal antibody to bind unlabeled CEA. The monoclonal 1E9 ascitic fluid was used at a 1/1 ($\times 10^6$) or 1/3 ($\times 10^6$) final dilution in the double-antibody solid-phase assay with treated or untreated ^{125}I-CEA, respectively. Similarly, Roche G-As was diluted 1/5 ($\times 10^5$) or 1/3 ($\times 10^5$). Chart 2 shows that when untreated CEA was used to inhibit the binding between untreated labeled CEA and G-AS (Chart 2, *Curve a*) or 1E9 (Chart 2, *Curve b*), 1E9 was about 2.8 times less sensitive to CEA inhibition than was G-As as determined by the concentration of CEA required for 50% inhibition (Table 4). Treatment of the radiolabeled CEA did not improve the inhibition sensitivity of 1E9 antibody for untreated unlabeled CEA but improved that of G-As by approximately 2.7-fold. Treatment of both the labeled and unlabeled CEA preparations significantly enhanced the inhibition sensitivity of 1E9 antibody for CEA (approximately 3.5-fold). Immunoadsorption and PCA treatment of either the labeled or unlabeled CEA did not result in any apparent changes in the immunological identity of the antigen preparations as recognized by the respective antibodies. This is shown by the similar slopes of inhibition for *Curves a* and *c* and *Curves b, d,* and *e* in Table 4.

Comparison of Binding Affinities. The apparent affinity constants (K_a) of the monoclonal antibody 1E9 and of the goat antibody to CEA were determined in the double-antibody solid-phase assay. To a limited amount of antibody (10 ng 1E9, 0.25 ng G-As), increasing amounts of immunoadsorbed PCA-treated ^{125}I-CEA (0.25 to 2.5 ng) were added to a final volume of 1.5 ml. After 18 hr at 20°, the complexes were precipitated with either GAM or DAG, and the amount above background of ^{125}I-CEA bound was determined. The results were calculated as described by Steward and Petty (19). The molecular weight of CEA was assumed to be 1.8×10^5. The apparent affinity constant of the monoclonal antibody was 1.0×10^8 liters/mol which was approximately one-twentieth of that observed for the goat antibody (1.9×10^9 liters/mol) but compared favorably with those reported by Accolla *et al.* (1) for 2 different anti-CEA producing hybridoma clones (1.4×10^8 and 1.1×10^7 liters/mol, respectively).

DISCUSSION

The fusion of mouse myeloma cells with splenocytes from a mouse immunized with purified CEA via an abbreviated immunization schedule yielded a number of hybrid cell lines which secreted antibodies of varying capacity to bind CEA. That this variation was probably not due to specificities for different antigenic molecules comprising the heterogeneous CEA preparation was shown by the fact that there were no added effects when the various antibodies were mixed (Chart 1). This could imply that the hybrid cell line 5E9 was polyclonal and could bind the entire spectrum of antigenic determinants or antigens which were identified by the other cell lines. Alternatively, differences in antibody avidity may have resulted in the inability to demonstrate additive binding as suggested by Koprowski *et al.* (9).

The selective binding of the commercially available radiolabeled CEA by antibodies secreted by the original 5E9 hybrid cell line (Chart 1) also occurred with the monoclonal antibody secreted by the subclone designated 1E9 (Table 2). Even after immunoadsorption and PCA extraction of the labeled CEA, the 1E9 antibody could bind no more than 79% of the same CEA bound by commercially available goat antiserum. Data presented in Table 2 could be interpreted to suggest the presence of at least 3 variants of CEA present within the initial labeled preparation and 2 variants, in equilibrium with each other, remaining after immunoadsorption and PCA extraction. For example, if the following equation is considered

$$CEA_{total} = (CEA_A \rightleftharpoons CEA_B) + CEA_C$$

G-As might bind all 3 variants in the initial preparation (100%) while 1E9 antibody might bind only CEA_A as 65% of the total (Table 2, Line 1). After immunoadsorption and PCA extraction, CEA_C is removed and CEA_A and CEA_B remain in equilibrium in a ratio of approximately 4/1, respectively (*i.e.,* 79 to 21%). Using this ratio, it can be estimated that the original untreated CEA preparation might have consisted of approximately 65% CEA_A, 16% CEA_B, and 19% CEA_C. Thus, treatment of labeled and unlabeled CEA could improve the binding and inhibition curves of 1E9 antibody but might not result in an inhibition sensitivity comparable to that for G-As because 2 variants (CEA_A and CEA_B) would always be present. Studies are under way to examine this possibility. The present studies do not rule out the regeneration of CEA_C or other species of CEA (a CEA_D or CEA_E) upon exposure of the affinity-purified material to PCA. The lower inhibition sensitivity of the 1E9 antibody for CEA limits its use in a conventional radioimmunoassay since the same degree of sensitivity as that commercially available with G-As cannot be obtained. However, the 1E9 antibody may be quite useful since it appears to have an increased selectivity for a specific CEA variant. When CEA adsorbed on a 1E9 antibody affinity column is inserted into the assay system, the inhibition sensitivity of the goat antiserum for treated or untreated CEA is enhanced without affecting the apparent immunological identity of the antigen as seen by the antiserum. If such an increase in sensitivity were accompanied by increased clinical specificity, clinical assays for CEA might be significantly improved.

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