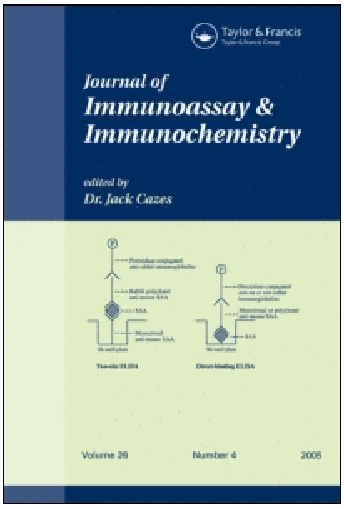

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CAPTURE AND DETECTION OF CARCINOEMBRYONIC ANTIGEN ON ANTIBODY COATED BEADS USED IN ENZYME IMMUNOASSAY

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ABSTRACT

We have evaluated antibody coated beads for capture and detection of carcinoembryonic antigen (CEA). Assay parameters of time, temperature, buffer molarity, specificity of antibody on the bead and reagent addition sequence have been studied. Optimal assay kinetics occurred at a temperature of 45°C and a buffer molarity of 0.1M or above. The type and quantity of antibody on the bead surface were also critical to optimal CEA detection. Beads coated with baboon or goat anti-CEA antibody were able to capture a higher percentage of CEA than monoclonal mouse anti-CEA antibody or guinea pig anti-CEA antibody. The sequence of addition of CEA, anti-CEA antibody coated bead, and anti-CEA-horse radish peroxidase conjugate was important for optimal CEA detection. Formation of an immune complex of CEA with the anti-CEA horse radish peroxidase conjugate prior to capture of the CEA on an antibody bead resulted in the optimal detection of CEA.

INTRODUCTION

Carcinoembryonic antigen (CEA) was initially described by Gold and Freedman (1) as a specific tumor marker for adenocarcinoma of the colon and rectum. However, elevated blood levels of CEA also occur in other forms of malignancy and in

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nonmalignant chronic inflammatory conditions (pancreatitis, ulcerative colitis, cirrhosis, emphysema, etc.) (2). Measurement of CEA blood levels is of value as a monitor of metastatic disease activity in a variety of types of carcinoma (3-6).

The first assay developed for CEA was a liquid phase antibody radioimmunoassay (RIA) developed by Thomson, et al. (7). Since then there have been a number of other liquid phase (2, 8-12) and solid phase antibody (13-18) assays advanced. More recently, a solid phase enzyme linked immunoassay (EIA) for CEA has become available (19). Differences in measurement of CEA values have been observed when identical samples were analyzed by RIA versus EIA methodology (20). This has led us to examine the parameters of the EIA which influence capture and detection of CEA.

Nonstandard abbreviations: CEA, carcinoembryonic antigen; OPD orthophenylendiamine; BSA, bovine serum albumin; NBP, normal baboon plasma; Anti-CEA-HPO, goat anti-CEA antibody-horseradish peroxidase conjugate; Tris-BSA buffer, 0.1 mol/L Tris HCl, pH 7.5, containing 1 gm/L of BSA and 0.5 gm/L of thimerosal; Ammonium acetate buffer, 0.01 mol/L ammonium acetate, pH 6.75, containing 0.001 mol/L sodium azide; Phosphate-BSA buffer, sodium phosphate (0.0 to 0.8 mol/L), pH 6.5, containing 1 gm/L of BSA and 0.5 gm/L of thimerosal.

MATERIALS AND METHODS

EIA Assay Reagents

Guinea pig anti-CEA antibody coated polystyrene beads (gift from Abbott Laboratories Inc. Chicago IL). Mouse monoclonal Anti-

CEA antibody coated polystyrene beads, goat anti-CEA-HPO, OPD ^{125}I-CEA, and cold CEA (gift from Hoffman-La Roche, Inc., Nutley, NJ).

<u>Anti-CEA Antibody Affinity Purification for use in Polystyrene Bead Coating Experiments</u>

Antibody to CEA was raised in the common baboon (Papio) (21) and in goats by monthly intradermal injection of 50 ug of purified CEA given in incomplete Freund's adjuvent. The CEA was purified from a hepatic metastasis of colonic carcinoma (21). This same CEA was utilized for preparation of a sepharose-4B-CEA affinity column. The affinity column had a binding capacity for anti-CEA antibody of approximately 5 mg. Aliquoits of immune serum (goat or baboon) were added to the column in sufficient quantity to saturate the affinity column's antibody binding capacity. Unbound protein was removed by washing the column with saline. The bound anti-CEA antibody was eluted from the column with 6 mol/L guanidine HCl, pH 7.0, (Sigma Chemical Co., St. Louis, MO). The eluted antibody was separated from the guanidine by passage through a Sephadex G-50 buffer exchange column (Pharmacia Fine Chemicals Inc., Piscataway, NJ). Trace albumin and possible protease contaminants in the antibody preparation were removed by passage of the affinity purified antibody through a CM-Affi Blue column (Bio-Rad Laboratories, Inc., Richmond, CA).

The biological activity of the affinity purified antibody was assessed by its binding capacity of ^{125}I-CEA. Affinity purified antibody (baboon and goat) at a concentration of 1 O.D. unit at

280 nm was diluted 1/2000 in ammonium acetate buffer containing 1 gm/L BSA. The diluted antibody was assayed for binding to 1 ng of ^{125}I-CEA in 6.5 ml of ammonium acetate buffer. For both affinity antibody preparations approximately 50% specific binding of ^{125}I-CEA occurred with addition of 50 ul of the antibody dilution.

Procedure for Coating Polystyrene Beads with Affinity Purified Antibody

The affinity purified goat and baboon anti-CEA antibody were adsorbed to the surface of polystyrene beads (6.5 mm dia., specular finish: Precision Plastic Ball Co., Chicago, IL). by the following method. Each stock antibody solution was buffer exchanged into 0.05 mol/L sodium bicarbonate buffer, pH 8.2, using a Sephadex G-50 buffer exchange column. The antibody concentration for the bead coating procedure was set at 50 mg/L for the affinity purified goat antibody and 25, 50, and 100 mg/L for the affinity purified baboon antibody; (experiments performed on the baboon antibody coated beads, except for the measurement of CEA capture capacity, utilized beads coated with baboon antibody at 50 mg/L). For the antibody coating reaction Ehrleneymer flasks were each filled with 400 beads then washed 3x with distilled H_2O. Added to the flask was 50 ml of the respective affinity purified antibody solution. The flasks were gently agitated to remove any trapped air then left at room temperature overnight. The antibody solution was decanted and the beads washed 3x with distilled H_2O. The flasks were then filled with 100 ml of 1 mol/L sodium chloride and 0.05 mol/L sodium phosphate buffer, pH 7.5, agitated for 15 minutes at room temperature, the buffer solution was decanted, the beads washed 3x with distilled H_2O, then 100 ml of Tris-BSA buffer was added to each flask and the flasks incubated at 37°C for three days. After this incubation the beads were washed once with 1 mol/L sodium chloride, then resuspended in Tris-BSA buffer and stored at 4°C.

Beads coated with BSA instead of antibody were prepared in an identical fashion to the above procedure. The antibody coating step was omitted and a 50 mg/L BSA solution was substituted in this part of the procedure.

<u>Measurement of the Effect of Buffer Molarity on the Capacity of Antibody Coated Beads to Capture CEA</u>

A constant quantity (1ng) of ^{125}I-CEA was prepared in 250 ul aliquots of phosphate-BSA buffer at seven different molarities of the phosphate ion (0.00, 0.001, 0.01, 0.2, 0.4, 0.8 mol/L). The capacity of antibody coated beads (goat, baboon, guinea pig, and mouse monoclonal) to capture the ^{125}I-CEA in each of the different molarity buffers was tested in the following manner: Each respective antibody coated bead was added to test tubes (in dublicate) containing 250 ul of each molarity of phosphate buffer solution. Assay incubation time points were set up between one hour and twenty five hours for each buffer molarity. Assay tubes were incubated at 37°C for the respective time points, the beads were then washed 3x with distilled H_2O, then the beads were transferred to clean test tubes and the amount of ^{125}I-CEA bound to each bead determined by counting in a gamma counter (Packard-

Prias Auto-Gamma Counter). Results were recorded as the percentage of ^{125}I-CEA bound. BSA-coated beads were also tested to determine the degree of non-specific binding of ^{125}I-CEA to the beads.

Measurement of the Effect of Temperature on the Capacity of Antibody Coated Beads to Capture CEA

Antibody coated beads (goat, baboon guinea pig, and mouse monoclonal) were added to test tubes containing 1 ng of ^{125}I-CEA in 0.13 mol/L phosphate-BSA buffer (250 ul). The assay tubes were incubated for one to twenty four hours at 24°C, 37°C, and 45°C respectively. The beads were then washed 3x with distilled H_2O, transferred to clean test tubes and the percentage of ^{125}I-CEA bound to each bead determined by gamma counting.

Determination of the Effect of Washing Beads on the Retention of Bound CEA

The effect of distilled water washing on the retention of ^{125}I-CEA bound to antibody coated beads (goat, baboon and mouse monoclonal) was tested as follows: to sets of twelve test tubes was added 1 ng of ^{125}I-CEA in 0.13 mol/L phosphate-BSA buffer (250 ul) followed by each respective bead type; the tubes were incubated overnight at 37°C, then four tubes were washed with 4 ml once and aspirated; four tubes were washed and aspirated three times; and four tubes were washed and aspirated fifteen times. All beads were then transferred to clean test tubes and counted by gamma counting to measure the amount of ^{125}I-CEA retained on each bead.

Procedure for the Measurement of CEA Capture Capacity by Antibody Coated Beads

The binding capacity for CEA of antibody coated beads (goat, baboon, guinea pig, and mouse monoclonal) was tested by determining each beads capability to bind ^{125}I-CEA in the prescence of increasing quantities of cold CEA. The assay was performed in the following manner: each type of antibody coated bead was added to test tubes containing 1 ng of ^{125}I-CEA in 200 ul of 0.15 mol/L phosphate-BSA buffer plus 50 ul of NBP which had been spiked with cold CEA from 0 to 2000 ng. The assay tubes were incubated overnight at 37°C, then the beads were washed 3x with distilled H_2O. The beads were transferred to clean test tubes and counted by gamma counting to measure the amount of ^{125}I-CEA bound to each bead. The total amount of CEA captured by each bead at each concentration of added cold CEA was calculated from the percentage of ^{125}I-CEA bound in the presence of cold CEA multiplied by the amount of cold CEA added: (^{125}I-CEA bound with cold CEA present / ^{125}I-CEA bound without cold CEA present) X ng of cold CEA added equals ng CEA captured. A table was constructed of the amount of cold CEA added versus the total ng of CEA captured. The plateau for the total ng of CEA captured with increasing amounts of cold CEA added was defined at the "capture capacity" of CEA for each bead type. This data was also subjected to a Scatchard analysis of B/F versus ng CEA bound.

Procedure for Determination of the Effect of the Sequence of Addition of CEA and Anti-CEA-HPO Conjugate to Antibody Coated Beads with Regard to Optimizing CEA Capture and Enzymatic Detection

In order to analyze the effect of sequence addition of CEA and anti-CEA-HPO conjugate in the capture of CEA and enzymatic detection of captured CEA on antibody coated beads three separate sequences of reagent addition were examined. Sequence A - initial incubation overnight at 37°C of 200 ul of goat anti-CEA-HPO conjugate with 100 ul of ^{125}I-CEA in ammonium acetate buffer. The ^{125}I-CEA was prepared as serial dilutions at concentration from 10 to 1.25 ug/L so that the 100 ul aliquots contained 1.0, 0.5, 0.25, and 0.125 ng of ^{125}I-CEA respectively. After this first incubation step an antibody coated bead was added to each assay tube followed by 200 ul of 0.2 mol/L of phosphate-BSA buffer. A second overnight incubation at 37°C was then performed.

In Sequence B antibody coated beads were incubated overnight at 37°C with 100 ul of ^{125}I-CEA (range 1.0 to 0.125 ng of ^{125}I-CEA) in ammonium acetate buffer plus 200 ul of phosphate-BSA buffer. Added directly to each assay tube after this first incubation step was 200 ul of goat anti-CEA-HPO conjugate solution then a second overnight incubation at 37°C was performed.

The third type of incubation sequence tested was the simultaneous addition of ^{125}I-CEA (range 1.0 to 0.125ng of ^{125}I-CEA in a 100ul volume) plus 200ul of goat anti-CEA-HPO conjugate; plus 200ul of 0.2 mol/L phosphate-BSA buffer and an antibody coated bead. The reaction mixture was incubated overnight at $37^{o}C$. This simultaneous reaction sequence was tested for goat baboon and mouse antibody coated beads.

At the completion of the assay reaction, the antibody coated beads, in all three assays, were washed 3x with distilled H_2O then counted by gamma counting in clean assay tubes to determine the amount of ^{125}I-CEA bound to each bead. Next enzymatic detection of the bound CEA was performed by addition of 0.5 ml of OPD substrate solution to each assay tube. The OPD substrate solution was prepared by addition of one tablet of OPD (22 mg OPD) to 5 ml of substrate buffer (gift of Hoffmann-La Roche) plus 0.5 ml of 1 N HCl. This volume of OPD substrate solution was prepared fresh for every ten assay tubes to be tested. The enzymatic reaction was stopped after thirty miuntes of incubation at room temperature by the addition of 2 ml of 1 N HCl containing 8 gm/L of sodium bisulfite. The presence of sodium bisulfite in the 1 N HCl solution stabilized the OPD color product. The supernatants were decanted into disposable plastic cuvettes (Chasma Scientific Co., Brighton, MA) and the absorbance read at 492 nm on a Coleman Model 46 Spectrophotometer.

RESULTS

Effect of Buffer Molarity on Capture of CEA

The effect of buffer molarity on ^{125}I-CEA binding to anti-CEA antibody coated beads was investigated for goat, baboon , guinea pig, and mouse monoclonal anti-CEA antibodies. Each type of

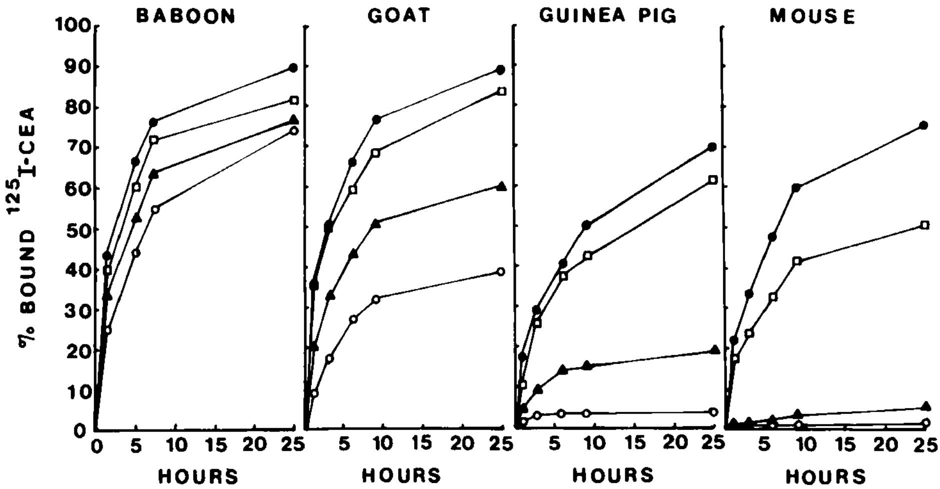

Figure 1. Effect of buffer molarity on the percentage capture ^{125}I-CEA with time for four different anti-CEA coated beads (see methods for description of beads and assay conditions). The antibody coated beads were tested in phosphate buffer molarities of: 0.00 mol/L = (○); 0.001 mol/L = (▲); 0.01 mol/L = (□); and 0.1, 0.2, 0.4, and 0.8 mol/L = (●).

antibody coated bead was incubated for one to twenty-five hours at 37°C in phosphate-BSA buffer with the phosphate ion concentration being varied from 0.00 to 0.8 mol/L (Figure 1). The percentage of capture of ^{125}I-CEA (^{125}I-CEA bound / ^{125}I-CEA added) for each type of antibody coated bead was dependent on both the incubation time and the buffer molarity. After 25 hours of incubation the goat and baboon antibody coated beads had captured approximately 90% of the added ^{125}I-CEA while the mouse antibody coated beads had captured approximately 75% and the guinea pig antibody coated beads approximately 70%; provided the assay buffer molarity was

0.1 mol/L or above. When the phosphate buffer ionic concentration was tested between 0.00 and 0.01 mol/L there was a marked decrease in ^{125}I-CEA captured by mouse and guinea pig antibody coated beads (Figure 1). In contrast, baboon antibody coated beads showed the least ion sensitive effect and were still able to capture approximately 75% of the added ^{125}I-CEA in the 0.00 mol/L phosphate-BSA buffer.

In contrast to anti-CEA antibody coated beads, when BSA-coated beads were tested for capacity to bind ^{125}I-CEA in both low and high ionic strength buffers no specific binding was observed. Less than 1% of added counts bound to the beads.

In order to test whether the 0.00 mol/L phosphate-BSA buffer was denaturing antibody on the bead surface and thus preventing ^{125}I-CEA capture we pre-incubated the mouse antibody coated beads in 0.00 mol/L, 0.05 mol/L, and 0.2 mol/L phosphate-BSA buffer solution for 24 hours then washed the beads x3 within Tris-BSA buffer (assay bead storage buffer) then exposed the beads to ^{125}I-CEA in 0.1 mol/L phosphate-BSA buffer for 25 hours. The percentage of binding of ^{125}I-CEA was essentially identical for all three pre-incubation procedures on the mouse monoclonal antibody coated beads indicating no antibody denaturation effect by buffers of different molarity (Table 1).

Effect of Temperature on Capture of CEA by Antibody Coated Beads

The four types of anti-CEA antibody coated beads (goat, baboon, guinea pig, and mouse monoclonal) were tested for percentage of ^{125}I-CEA binding of a 72 hour period at incubation

TABLE 1

Effect of a 24 Hour Preincubation of Mouse Antibody Coated Beads in Buffers of Various Ionic Strength on the ^{125}I-CEA Capture Capacity

Preincubation Buffer Ionic Strength	Capture Buffer Ionic Strength	% Bound ^{125}I-CEA
0.00 M Phosphate	0.1 M Phosphate	64.0%
0.05 M Phosphate	0.1 M Phosphate	62.5%
0.20 M Phosphate	0.1 M Phosphate	62.8%

temperatures of 24°C, 37°C, and 45°C (Figure 2). Capture of ^{125}I-CEA occurred most rapidly for all bead types at 45°C. Ninety percent of maximal capture at 45°C was attained in approximately 6 hours for baboon antibody coated beads, 8 hours for goat antibody coated beads, 9 hours for mouse monoclonal antibody coated beads, and greater than 24 hours for the guinea pig antibody coated beads. Reaction time at 37°C was slightly slower than at 45°C, while at 24°C it was markedly slower. However, even at 24°C the maximal binding capacity of each type of antibody coated bead for ^{125}I-CEA was reached by 72 hours of incubation time (Figure 2).

Effect of Bead Washing on Retention of Captured CEA

Washing goat, baboon, and mouse monoclonal antibody coated beads with distilled H_2O after ^{125}I-CEA had been bound to the beads was tested for 1x versus 3x versus 15x washing cycles (see methods). The percentage of ^{125}I-CEA bound did not appear to be appreciably decreased by the increased washing cycles (Table 2)

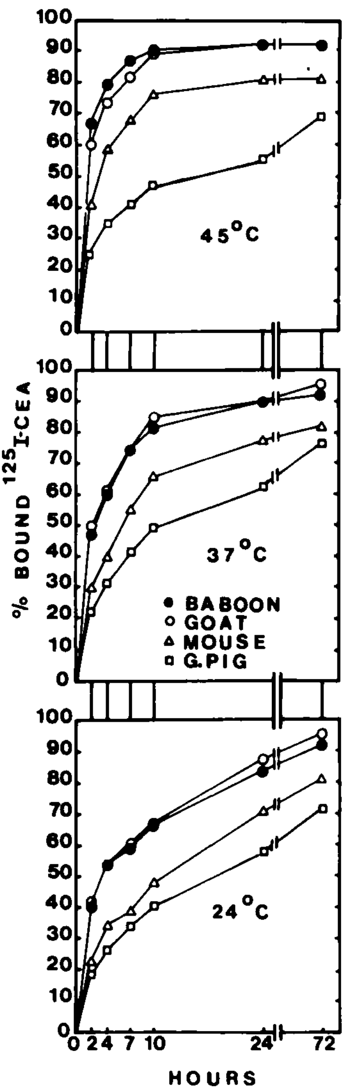

Figure 2. Effect of incubation temperature on the percentage capture of ^{125}I-CEA with time for four different anti-CEA coated beads (see methods for description of beads and assay conditions).

TABLE 2

Effect of Washing on Bead Captured CEA

Bead Type	% ^{125}I-CEA Captured		
	1x Wash	3x Wash	15x Wash
Goat	79.8	76.1	77.6
Baboon	76.2	73.3	75.3
Mouse	64.1	64.0	65.1

^{125}I-CEA overnight capture at 37°C

indicating that the CEA once bound to the bead surface is quite firmly held on the surface by the antibody.

Determination of the Maximal Capture Capacity of Antibody Coated Beads for CEA

The four types of anti-CEA antibody coated beads (goat, baboon, guinea pig, and mouse monoclonal) were tested to determine their maximal capacity to bind CEA (see methods). The goat anti-CEA antibody coated beads could bind approximately 160 ng of CEA per bead; the mouse monoclonal antibody coated beads bound approximately 60 ng CEA per bead; and the guinea pig antibody coated beads bound approximately 18 ng CEA per bead (Table 3). In order to test whether the concentration of antibody utilized to coat the bead surface was important in the resultant capture capacity of the beads for CEA three different baboon anti-CEA antibody coated bead preparations were tested (see methods). The baboon anti-CEA antibody coated beads which were prepared with a baboon antibody coating solution concentration of 100 mg/L had a

TABLE 3

Bead Capture Capacity for CEA

	Guinea Pig Beads		Mouse Monoclonal Beads		Goat Beads	
Cold CEA Added	% ^{125}I-CEA Bound	Cold CEA Bound	% ^{125}I-CEA Bound	Cold CEA Bound	% ^{125}I-CEA Bound	Cold CEA Bound
0	38.7	-	62.7	-	78.9	-
25 ng	19.2	4.8 ng	63	15.8 ng	82.6	20.7 ng
50	15.3	7.7	57.5	28.8	76.7	38.4
100	12.4	12.4	39.1	39.1	74.7	74.7
200	8.6	17.2	24.1	48.2	57.7	115.4
300	6.2	18.5	17.6	52.8	45.9	137.7
400	4.5	17.8	14.3	57.2	34.6	138.4
500	3.3	16.7	11.5	57.5	31.0	155
1000	2.0	19.6	5.1	51.0	15.9	159
1500			4.3	64.5	11.5	172

CEA capture capacity of 230 ng per bead; in contrast, the baboon anti-CEA antibody coated beads coated at a concentration of 25 mg/L had a CEA capture capacity of 90 ng per bead (Table 4).

The binding capacity for CEA by the different antibody coated bead preparations (Tables 3 and 4) was performed as a competitive inhibition of cold versus hot CEA (see Methods). Thus a Scatchard analysis of each type of antibody coated bead's binding avidity and capacity for CEA could be derived (see Figure 3). Evident from this analysis is that the guinea pig antibody coated beads had the least avidity and the lowest binding capacity. The mouse monoclonal and the goat antibody coated beads showed similar avidity (slopes) but different capacities to bind CEA (X-axis intercepts). Of interest, the baboon antibody coated beads showed

TABLE 4

Baboon Antibody Coated Bead CEA Capture Capacity

Cold CEA Added	Prep A - 100 mg/L % ^{125}I-CEA Bound	Prep A - 100 mg/L Cold CEA Bound	Prep B - 50 mg/L % ^{125}I-CEA Bound	Prep B - 50 mg/L Cold CEA Bound	Prep C - 25 mg/L % ^{125}I-CEA Bound	Prep C - 25 mg/L Cold CEA Bound
0	73.7	-	78.6	-	74.7	
25 ng	77.9	19.5 ng	81.3	20.3 ng	80.3	20.1 ng
50	78.3	40	81.9	41	76.1	38.1
100	77.7	78	78.2	78	63.3	63
200	73.9	148	73.5	147	37.5	75
300	67.6	203	64.6	194	35.0	105
400	57.3	229	55.7	222	22.0	88
500	50.1	250	51.6	258	16.6	83
1000	22.1	221	18.8	188	9.2	92
1500	16.2	243	15.3	30	5.6	92

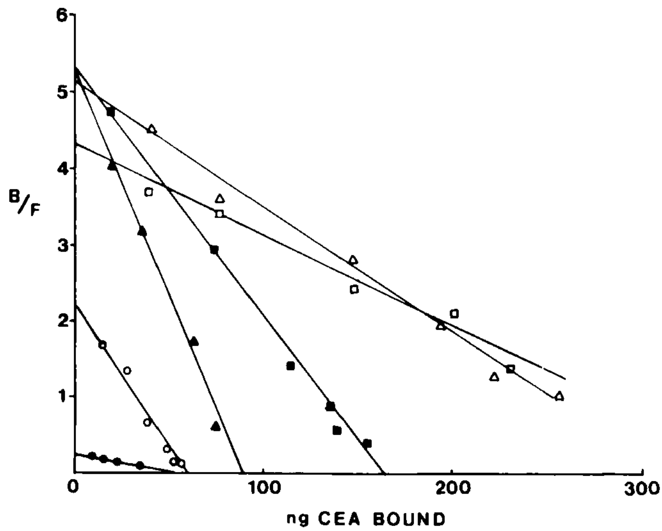

Figure 3 Scatchard analysis for guinea pig (●), mouse (○), goat (■), and baboon (▲ 25mg/L), (△ 50mg/L) (□ 100mg/L) antibody coated beads. B/F versus ng CEA bound was calculated from the data presented in Tables 3 and 4.

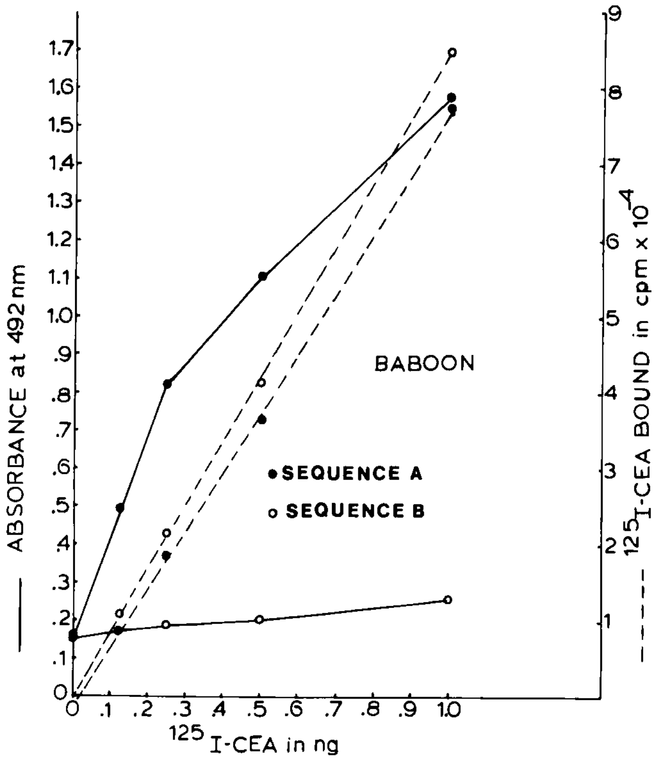

Figure 4. Effect of the sequence of reagent addition on the number of counts captured of ^{125}I-CEA by baboon anti-CEA coated beads and determination of the adsorbance generated at 492 nm from OPD development due to the binding of goat anti-CEA-HPO conjugate to the ^{125}I-CEA on the bead surface (see methods for description of the sequence A and B reagent addition conditions). Total counts added for 1 ng of ^{125}I-CEA for both reagent sequences was 117,500 cpm.

increased capacity to bind CEA as the concentration of antibody to coat the beads was increased, however, the apparent avidity of the antibody for CEA on the bead surface was decreased by the increasing concentration of antibody.

Effect of the Sequence of CEA and Goat-Anti-CEA-HPO Conjugate Addition on the Percentage Capture and the Degree of Enzymatic Detection of the CEA Bound to Antibody Coated Beads

Two different sequences of reagent addition were tested for their effects on the capability of antibody coated beads to bind ^{125}I-CEA and for the bound CEA to be enzymatically detected (see methods). In reagent addition sequence A the goat anti-CEA-HPO conjugate was complexed with ^{125}I-CEA prior to exposure of the ^{125}I-CEA to the antibody coated bead. In sequence B the ^{125}I-CEA was bound to the antibody coated bead prior to exposure to the goat anti-CEA-HPO conjugate. Each of the four types of antibody coated beads were tested in both reaction sequences (Figures 4-7). For each type of antibody coated bead the exposure of ^{125}I-CEA to the bead prior to the complexing of the CEA with anti-CEA-HPO conjugate (sequence B) resulted in a greater degree of capture of the ^{125}I-CEA on the bead surface. The degree of difference in capture of ^{125}I-CEA on the bead surface was greater between sequence A and B for the mouse monoclonal and guinea pig antibody coated beads than for the goat and baboon antibody coated beads.

Enzymatic detection of captured CEA on the bead surface was superior for sequence A of reagent addition compared to sequence B for all four antibody bead types. With baboon and goat antibody coated beads the enzymatic detection of the bound ^{125}I-CEA was at background level for sequence B of reagent addition, even though in sequence B slightly more ^{125}I-CEA had been bound to the

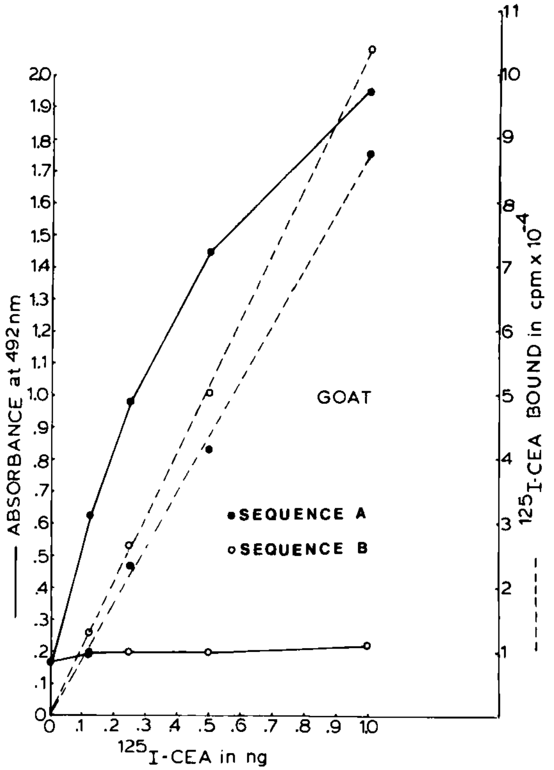

Figure 5. Effect of the sequence of reagent addition on the number of counts captured of ^{125}I-CEA by goat anti-CEA coated beads and determination of the adsorbance generated at 492 nm from OPD development due to the binding of goat anti-CEA-HPO conjugate to the ^{125}I-CEA on the bead surface (see methods for description on the sequence A and B reagent addition conditions). Total counts added for 1 ng of ^{125}I-CEA for both reagent addition sequences was 124,000 cpm.

antibody coated beads at each assay point, compared to sequence A (Figures 4 and 5). The mouse monoclonal antibody coated beads showed the least effect of enzymatic detection of CEA relative to the sequence of reagent addition (Figure 6).

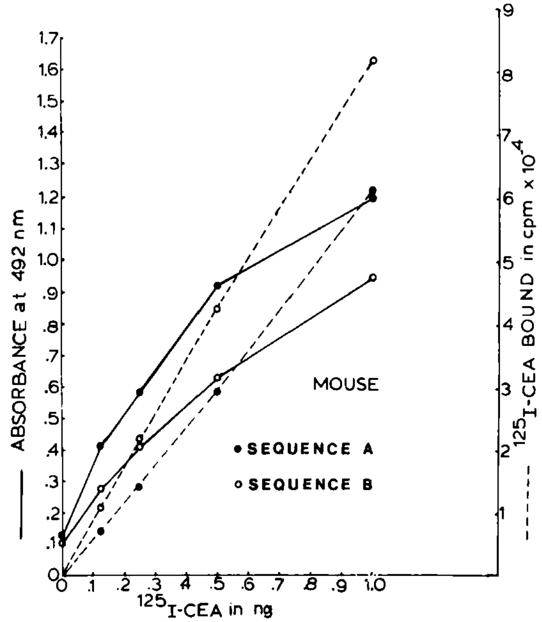

Figure 6. Effect of the sequence of reagent addition on the number of counts captured of ^{125}I-CEA by mouse monoclonal anti-CEA coated beads and determination of the adsorbance generated at 492 nm from OPD development due to the binding of goat anti-CEA-HPO conjugate to the ^{125}I-CEA on the bead surface (see methods for description of the sequence A and B reagent addition conditions). Total counts added for 1 ng of ^{125}I-CEA for both reagent addition sequences was 124,000 cpm.

Sequence A of reagent addition for goat, baboon and mouse monoclonal antibody coated beads was also compared to simultaneous addition of all reagents (see methods). As shown in Figure 8 the simultaneous reagent addition resulted in less detection of CEA than sequence A for all three types of antibody coated beads.

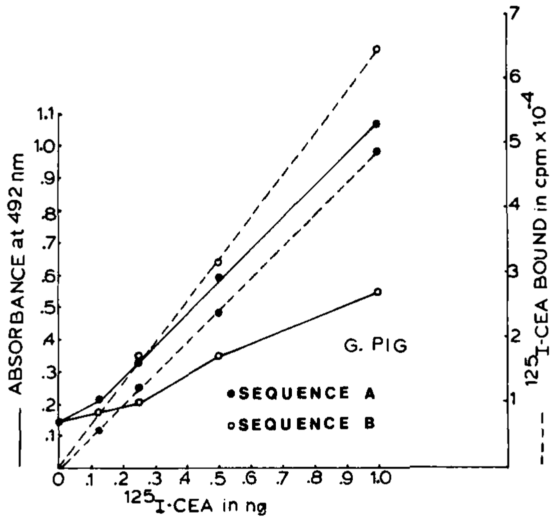

Figure 7. Effect of the sequence of reagent addition on the number of counts captured of ^{125}I-CEA by guinea pig anti-CEA coated beads and determination of the adsorbance generated at 492 nm from OPD development due to the binding of goat anti-CEA-HPO conjugate to the ^{125}I-CEA on the bead surface (see methods for description of the sequence A and B reagent addition conditions). Total counts added for 1 ng of ^{125}I-CEA for both reagent addition sequences was 120,500 cpm.

DISCUSSION

An optimal enzyme immunoassay would require 100% capture of sample CEA on the antibody coated bead surface and stiochiometric binding of the anti-CEA-HPO conjugate to the captured CEA. The ensuing enzymatic reaction would then quantitatively reflect the sample CEA concentration at optimal sensitivity. In this paper we have assessed several of the parameters which influence CEA capture and detection in a model enzyme immunoassay system.

The binding of ^{125}I-CEA to antibody coated beads was found to occur optimally in an ionic strength buffer of 0.1 mol/L or above

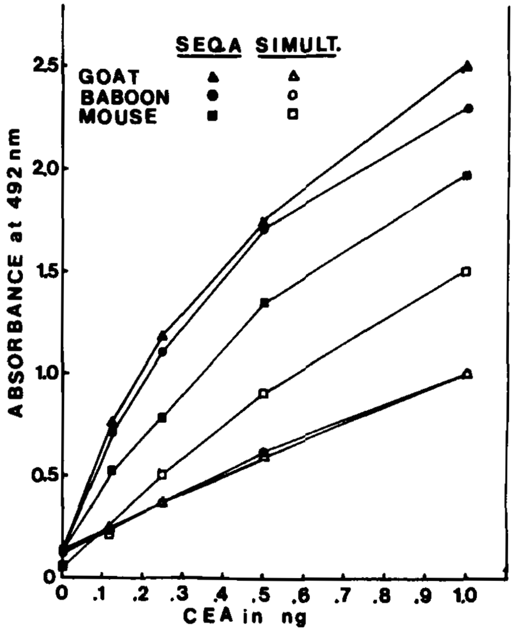

Figure 8 Effect of the sequence of reagent addition on the enzymatic detection of CEA by mouse versus goat versus baboon antibody coated beads (see methods for description of sequence A versus simultaneous reagent addition conditions).

(Figure 1). This finding is in contrast to the observation of optimal reaction kinetics of CEA with antibody in solution where low ionic strength buffers are more favorable (21-22). This difference in ionic strength effect on the two types of assay systems (antibody coated beads versus antibody in solution) is not due to the use of different antibodies since both the goat and baboon anti-CEA antibodies utilized in the present study have also been tested in soluble phase RIA for ionic strength sensitivity effects (21). We hypothesize that the optimal reaction of CEA with antibody coated beads in high ionic strength buffer is due to a neutralization of static charges around the bead surface by the high ionic strength buffer thus allowing the CEA to come into optimal contact with the antibody bound on the bead surface.

Reaction of CEA with antibody coated beads was also optimized at elevated temperatures (45°C). This is probably due to the increased Brownian motion imparted to the solution by an increased temperature which results in faster contact time between the soluble CEA molecules and the stationary antibody coated beads.

Experimentally produced goat and baboon anti-CEA antibody coated beads were found to have significantly higher CEA capture capacities (approximately 160 and 230 ng of CEA per bead respectively) than the mouse monoclonal antibody coated beads obtained from Hoffman-La Roche (capture capacity approximately 60 ng per bead) or the guinea pig antibody coated beads obtained from Abbott Laboratories (capture capacity approximately 18 ng per bead). The capture capacity of baboon antibody coated beads was in part dependent on the concentration of antibody utilized in the bead coating procedure (Table 4).

Scatchard analysis (Figure 3) of each of the antibody coated beads revealed that the guinea pig antibody coated beads had the least avidity for CEA. The mouse and goat antibody coated beads had similar avidity for CEA which was markedly better than the guinea pig. The avidity for CEA by baboon antibody coated beads appeared dependent on the initial concentration of antibody used to coat the beads with increasing concentration decreasing avidity. This probably reflects conformational interference at the bead surface for antigen binding due to crowding of antibody molecules. For each of the four types of antibody coated beads the working range for CEA analysis in the EIA was from 0 to 1 ng of CEA and each assay bead showed stiochiometric binding properties for CEA in this assay range (Figures 4-7).

Our most interesting observation on the EIA for CEA was the effect of the sequence of reagent addition on the capture and detection of CEA. In sequence B of reagent addition the actual amount of ^{125}I-CEA which was bound to the antibody coated beads was higher than for sequence A of reagent addition for all types of antibody coated beads (Figures 4-7). However, the binding of CEA to either the goat or baboon antibody coated beads prior to the addition of the anti-CEA-HPO conjugate (reagent addition sequence B) resulted in no enzymatic detection of the bound CEA. An explanation of this finding may relate to the type of CEA determinant recognition occurring with the bead coated antibody. Both the goat and the baboon affinity purified antibodies against CEA recognize predominately specific site determinants on CEA (21-23). The goat-anti-CEA-HPO conjugate antibody is also predominately directed against specific site determinants (unpublished observations, H. Hansen). In contrast, reagent addition sequence B only mildly decreased the enzymatic detection of CEA compared to sequence A of reagent addition for the mouse monoclonal antibody coated beads (Figure 6). The mouse monoclonal antibody used in these experiments recognizes a common site determinant on CEA (unpublished observations, H. Hansen). The guinea pig antibody coated beads reacted in EIA in a similar fashion to the mouse monoclonal antibody coated beads (Figures 6 and 7). We interpret the above findings to indicate that the specific site determinants on CEA are in a steric arrangement such that once CEA is bound to an antibody coated bead by specific site antibody these determinants are no longer exposed for binding of goat-anti-CEA-HPO conjugate. In contrast, the binding of CEA to antibody coated beads by common site determinant anti-CEA antibody still allowed exposure of specific site determinant recognition by goat-anti-CEA-HPO conjugate antibody.

The uniqueness of CEA is defined immunologically by its specific site determinant recognition (2, 22-23). In our model EIA analysis the beads coated with specific site determinant antibody (goat and baboon affinity purified antibody) showed a higher percentage capture capacity for ^{125}I-CEA than the mouse monoclonal antibody coated beads against a common site determinant or the guinea pig antibody coated beads (Figure 1). Also the goat and baboon antibody coated beads showed in sequence A of reagent addition the most sensitive detection of CEA by EIA. They were approximately twice as sensitive as the mouse monoclonal or guinea pig antibody coated beads for CEA detection (Figures 4 and 5 versus Figures 6 and 7). When antibody against common site determinants on CEA is utilized in the capture of CEA on antibody coated beads this resulted in a lower percentage of the CEA molecules present being bound by the antibody coated beads and a lower degree of detection of the bound CEA occurred versus the use of a CEA specific site antibody coated bead system. The clinical relevance of these laboratory observations is under investigation.

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