Immunoradiometric assay for carcinoembryonic antigen using avidin-biotin separation technique

SONG Shiping, TANG Guozhong, LI Bin
(*Shanghai Institute of Nuclear Research, the Chinese Academy of Science, Shanghai 201800*)

Abstract A sensitive, specific, noncompetitive, sandwich-type radioimmunoassay for carcinoembryonic antigen (CEA) has been developed in our laboratory, which can be performed conveniently. The assay involves two monoclonal antibodies, selected for high affinity and specificity and also for reaction against antigenic sites on CEA that are distal from each other. One of these antibodies was labeled with ^{125}I and the other was conjugated covalently to biotin. Polystyrene tubes were conjugated covalently to avidin. These tubes represent a rapid, simple method for separating the CEA-bound antibody from the free antibody. The biotin-antibody-CEA-^{125}I-labeled antibody complexes bind to the tubes and CEA concentration is directly related to counts per minute. This assay can detect the CEA at a concentration of 0.22 μg/L in serum.

Keywords Carcinoembryonic antigen(CEA), Immunoradiometric assay(IRMA), Biotin-avidin system(BAS)

1 Introduction

Several assays have been published for the determination of carcinoembryonic antigen.[1~3] However, because the CEA is heterogeneous — the CEA from various tumors displaying different carbohydrate content — different values have been observed in different assays. The principal carbohydrate in the CEA is N-acetylglucosamine, but the galactose, mannose fucose, and neuraminic acid content may also differ in CEA from various tumors.

The CEA is a family of isoantigens with multiple antigen determinants. Another difficulty of CEA assays is that some CEA-like substances in serum may cross-react with the antibodies to various extents, e.g, nonspecific cross-reacting antigen, normal glycoprotein, CEA-associated protein, colonic carcinoembryonic antigen 2, colon carcinoma antigen III, beta external protein and tumor-associated antigen.

Comparison of CEA assays in patients with different tumor has often yielded conflicting results. Sometime enzyme-linked immunoassays for CEA yielded, for some healthy blood donors, CEA concentrations that exceed the upper limit of the generally accepted normal reference interval. Suspecting some cross-reaction of two polyclonal antibodies with CEA-like substances, we replaced them with two monoclonal antibodies, one of which was biotinylated and the other of which was labeled with ^{125}I.

The resulting immunoradiometric assay has the advantages: improved precision, absence of a high-dose "hook" effect. The biotinylated antibody could be quantified precisely when it was immobilized to the avidin coated tube. All materials involved are commercially available and the solid-phase avidin can be used for different assays.

2 Materials and methods

2.1 Biotinylation of monoclonal anti-

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body

Biotinylated monoclonal antibody to the CEA was performed according to Meir Wilckek *et al.*[4] and William D. Odell *et al.*[5] The monoclonal antibody was dissolved at a concentration of about 2 g/L in a 0.05 mol/L phosphate-buffer saline (PBS)(pH 7.4). The amount of N-hydroxy succinimidobiotin (BNHS; Sigma Chemical Co.) added to the antibody solution is 15% of the weight of the protein. The BNHS, dissolved in a volume of N,N-dimethylformamide (DMF; Sigma Chemical Co.) that did not exceed 5% of the antibody volume, was added to the dissolved protein. The mixture was vortexmixed for 2 min, then covered with Parafilm and incubated at the room temperature for 16~24 h. The contents were transferred to dialysis tubing with Mr-2000 cutoff and dialyzed against salined solution (NaCl, 9g/L) twice in 12 h and then against PBS. The incubations above were allowed to perform at the room temperature. The contents of the tubing were transferred to an appropriate container and stored at -20°C in 500μL aliquots or diluted with an antibody buffer to an intermediate working dilution.

2.2 Preparation of avidin-coated tubes[6]

The conjugate of avidin and bovine serum albumin was dissolved at a concentration of 4 mg/L in a coating buffer of 40 mmol/L sodium hydrogen phosphate (pH 7.4) and 1 g/L sodium azide and stirred for 30 min at the room temperature. Test tubes of polystyrene were each filled with 1 mL of the solution and loaded at 20°C for 22 h. After sucking out the test tubes, there took place an after-treatment with 2 mL of a 2% sucrose solution which contains 0.9% sodium chloride and 0.05% Tween 20 (V/V), the after-treatment being carried for 30 min at 20°C. Subsequently, the test tubes were ready for use of carrying out tests.

2.3 Iodination of monoclonal antibody

Monoclonal antibody was radioiodinated by the Iodogen method. Two microliters (33.76μg) of monoclonal antibody was pipetted into a vial coated with Iodogen (4μg) and then mixed gently with 20 μL of 0.25 mol/L phosphate buffer (pH 7.4), 18.5 MBq of carrier-free ^{125}I. After shaking for 5 min, the reaction was stopped by aspirating the contents from the Iodogen vial. The combined mixture was transferred to a Suphadex G-25 column for purification. Fractions were collected in tubes and tested for binding activity.

2.4 Assay procedure

We used a CEA two-step monoclonal radioimmunoassay (RIA). Biotinylated monoclonal antibody was diluted with the antibody buffer to a concentration of 1 mg/L. 500 μL of the solution of biotinylated monoclonal antibody was added to each tube, and incubated at 37°C in a water bath for 4 h. After aspirating the contents of the tubes, the assay was performed by combining 100μL of standard or serum sample, 400μL of assay buffer. The tubes were incubated at 37°C for 4 h, and then the contents of the tubes were aspirated again. After the tubes were washed with the PBS, 500 μL of ^{125}I-labeled monoclonal antibody was added and incubated at 37°C for 20 h. Then the contents were aspirated again. The radioactivity bound to the tube wall was counted with a gamma counter.

To determine the optimal working concentration of the biotinylated monoclonal anti-CEA antibody, we tested various dilutions of stock solution, from 100~6400 fold (0.15~10 mg/L).

3 Results

3.1 Optimal dilution ratio of biotinylated antibody

As Fig.1 shows, binding of ^{125}I-labeled

monoclonal antibody is optimal when the ratio of dilution of the stock solution of biotinylated monoclonal antibody is about 1/1000. Fig.1 also shows that excess of the biotinylated antibody may result in decrease of the binding between the solid-phase antibody and the CEA or between the solid-phase CEA and the ^{125}I-labeled antibody.

3.2 Kinetics of solid-phase anti-CEA /CEA binding

The CEA is a macromolecular antigen. Its molecular weight is higher than that of antibody. In incubating, enough time often was needed for complete binding between the CEA and the anti-CEA. Fig.2 shows that solid phase assays required at least 4 h to give efficient results for the first step of immune reaction.

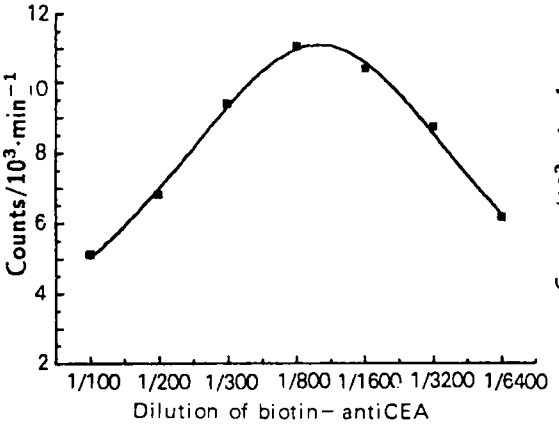

Fig.1 Working concentration of the biotin-anti CEA

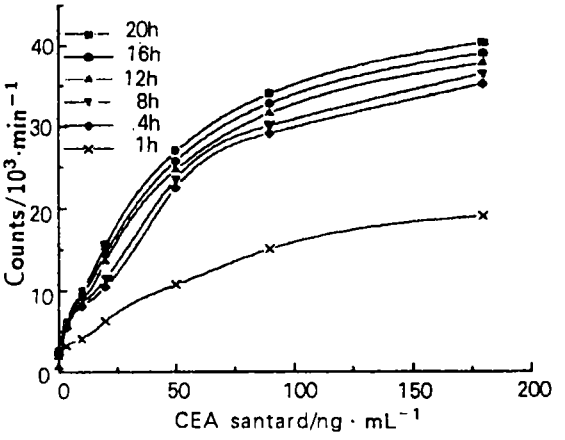

Fig.2 Kinetics of the CEA/anti-CEA of the binding: standard curves after various incubation times(1~20 h)

3.3 High-dose "hook" effect

A high-dose hook effect — falsely low CEA concentration calculated when concentration are very high — and a less-steep standard curve are obtained if standards or samples and the ^{125}I-labeled monoclonal antibody are incubated together instead of sequentially. As Fig.3 shows, no hook effect appeared at the CEA concentrations up to 10mg/L if the assay was performed under sequential conditions (4 h + 20 h).

3.4 Primary quality control

Standard Curve. The final set-up of the assay gave a typical standard curve as shown in Fig.4. The signal range was 1060 to 17677 counts/min for the CEA concentrations between 3.4 and 90 μg/L. The maximal binding (17677 counts/min) was 51% of total activity (34660 counts/min).

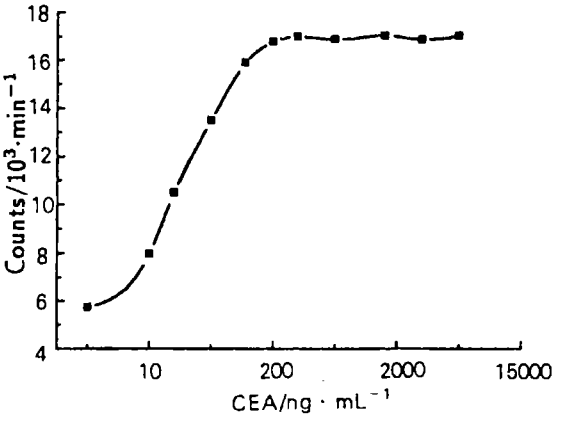

Fig.3 High-dose hook effect of the CEA-BAS-IRMA

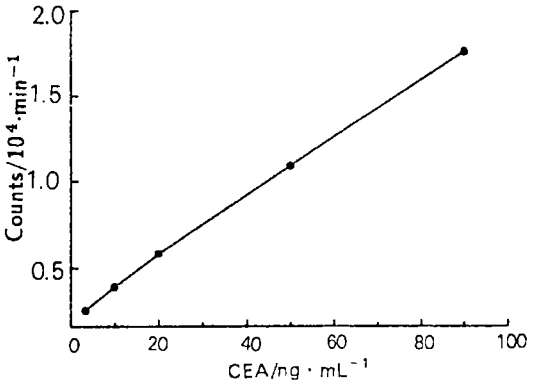

Fig.4 Standard curve of the CEA-BAS-IRMA

Sensitivity. The sensitivity was calculated as the concentration of CEA corresponding to the value of the zero standard minus two standard deviations(mean±2SD, n=10). The sensitivity is 0.22 μg/L

Precision. We measured samples with different CEA concentrations to determine the coefficient (CV) ranged between 2.56% and 4.89% in the intra-assay, and ranged between 6.03% and 7.75% in the inter-assays. The results are listed in Table 1.

4 Discussion

Although the radioimmunoassay was initial an technique used for determining CEA[2,3], antibody-excess methods such as immunoradiometric assays (IRMA) have been established in recent years because of their shorter incubation times and higher precision.

In IRMA, the use of two polyclonal antibodies does not obviously offer the specificity required for CEA determination because several cross-reacting substances are present. To increase specificity, we used two commercially available monoclonal antibodies, which direct against different epitopes of CEA.

In immobilizing biotinylated monoclonal antibody through solid-phase avidin, something unexpected had happened. Theoretically, excess of biotinylated antibody would yield a steeper curve. But, in fact, a peak curve was yielded. We think that excess of biotinylated antibody might result in steric hindrance which might affect the binding of CEA and anti-CEA.

Table 1 Precision of the CEA-BAS-IRMA

CEA control	Intra-assay variation Mean(SD) n=10		Inter-assays variation Mean(SD) n=5	
	/μg·L^{-1}	CV/%	/μg·L^{-1}	CV/%
L	1.75	4.89	1.74	7.75
M	15.2	3.71	15.0	6.58
H	43.3	2.56	42.7	6.03

The time course of binding in this solid-phase system is slower than in a liquid-phase system. We used fairly long incubation intervals to reach an endpoint of binding. Sequential incubation obviated a high-dose hook effect and yielded a steeper standard curve. Prolonging the first incubation period of immune reaction from 4 h to 20 h did not improve the standard curve significantly. Commercial CEA-ELISA ordinarily involve relatively short incubation time; however, they may not reach steady-state conditions. In our opinion, determinations of CEA should be as accurate as possible to detect small, early increases in CEA concentrations in serum. Because patients are monitored monthly or less often and because follow-up treatment is sometime based solely on a time-dependent increase of CEA concentration, the quality of the assays should not be sacrificed for speed.

In order to set up a solid-phase system used for different assays, we prepared a kind of solid-phase avidin which can immobilize any biotinylated antibody or antigen. Thus the variety of assays performed in one laboratory may be increased, or a great number of antibodies and antigens can be tested for different purposes in a very short time. The solid-phase tubes and the biotinylated antibody both can be stored for long time.

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