Europ. J. Cancer Vol. 15, pp. 475–480.

0014–2964/79/0401–0475 $02.00/0

Analysis of Human Sera for Carcinoembryonic Antigen (CEA) Binding Proteins by Affinity Chromatography with CEA-Agarose

R. POMPECKI

II. *Medizinische Klinik und Poliklinik des Universitätskrankenhauses Hamburg-Eppendorf und Physiologisch-Chemisches Institut (Molekulare Biologie) der Universität Hamburg Martinistraße* 52, 2000 *Hamburg* 20, *Federal Republic of Germany*

Abstract—*Sera from* 11 *healthy non-smokers and* 12 *healthy smokers as well as sera from* 23 *patients suffering from adenocarcinoma of the bowel were analysed for carcinoembryonic antigen (CEA) binding proteins. The methods used were affinity chromatography on CEA-agarose columns, followed by an* ^{125}I-*CEA binding assay with rabbit antihuman immune globulin (Ig) G as the precipitating antibody.*

The serum preparations of healthy non-smokers (N = 11) *bind* ^{125}I-*CEA up to* 1%. *Seven of the sera from smokers* (N = 12) *and* 7 *of the sera from patients with resectable colorectal cancers* (N = 12) *show binding up to* 10%. *On the basis of the binding assay used, it is concluded that human IgG binds the* ^{125}I-*CEA. The binding did not correlate with antiblood group antibodies. Although some findings may indicate a possible immunogenicity of CEA in man, cross-reactions of antibodies against antigens similar to CEA seem to be more likely to account for the weakness of the* ^{125}I-*CEA binding.*

INTRODUCTION

FOR SEVERAL years the question has been asked whether the carcinoembryonic antigen (CEA), first described by Gold and Freedman [1], is antigenically effective in tumor patients. Circulating antibodies against semipurified and purified CEA preparations have been found in sera of patients suffering from carcinomas by a hemagglutination method [2] and by radioimmuno-electrophoresis [3, 4]. Other authors could not confirm these findings [5, 6]. Further investigations indicated an immunological relationship between CEA and human blood group substances A and H [7–10]. However, CEA and isoantigen A were later detected by immune reactions to be separate molecules on the same tumor cell [11].

In the present study, CEA binding proteins in human sera have been sought by affinity chromatography with CEA bound to agarose. This method has been employed in order to purify heterologous hyperimmune sera against CEA and CEA cross-reactive proteins [12, 13].

Accepted 23 August 1978.

MATERIALS AND METHODS

Chemicals

Purified CEA in 0.1 molar (M) sodium borate buffer pH 8.0 (generously provided by Dr. H. J. Hansen, Nutley, N.J., USA, batch BP 160 CEA) was used for coupling to cyanogen–bromide (CNBr) activated Sepharose 4B (Pharmacia, Uppsala, Sweden) and for labelling with 125-iodine (^{125}I) supplied by Amersham, England. Human immune globulin (Ig) G (Intraglobin) was supplied by Biotest, Frankfurt, Germany, human serum albumin and monospecific rabbit anti-human IgG (y-chain) antiserum by Behring, Marburg, Germany.

The reagents to measure the plasma CEA levels, including zirconyl phosphate gel (Z-gel), were purchased from Hoffmann-La Roche, Grenzach, Germany.

Sera

Rabbit anti-CEA antiserum was used to check the function of the CEA-agarose columns. It was prepared by repeated intracutaneous injections of perchloric acid (PCA) extracts of metastases of a colon carcinoma emulsified in complete Freund's adjuvant.

Forty-six human sera were examined: sera from 11 healthy non-smokers, 12 healthy smokers and from 23 patients suffering from adenocarcinoma of the colon and rectum.

Procedures

Coupling of CEA to CNBr-activated Sepharose 4B. One milligram of CEA was coupled to 2.5 g of CNBr-activated Sepharose 4B according to the method of Axen *et al.* [14]. The determination of CEA in the eluate after coupling was carried out in the same way as in plasma [15]. The CEA-agarose suspended in 0.1 M Tris–HCl 0.5 M NaCl pH 8.0, was distributed into two columns each with a 3 ml gel bed.

Affinity chromatography

One millilitre of crude rabbit hyperimmune serum or 10 ml of human serum were dialysed overnight at 4°C against 0.1 M Tris–HCl buffer pH 8.0, 0.5 M NaCl. The sera were allowed to penetrate the column for at least 8 hr at 4°C. The column was washed with the Tris–HCl buffer until no absorption at 280 nm could be detected in the eluate. Thereafter the column was eluted extensively with 0.1 M glycine-HCl buffer pH 3.0, 0.5 M NaCl, and finally adjusted again with the 0.1 M Tris–HCl buffer pH 8.0. Acid fractions were neutralized by the addition of 1 M Na_2CO_3.

Assay for CEA binding proteins

After chromatography on CEA-agarose the rabbit serum fractions were assayed for CEA binding proteins separately or as in the case of the human sera samples were collected and concentrated. The last two alkaline fractions were combined with the first ten acid fractions and concentrated by Amicon Ultrafiltration (Model MMCA 435) to a final volume of 500 µl, resulting in a 20-fold concentration. The native fractions or concentrated samples were stored at −20°C.

Ten micrograms of CEA were labelled with 1 mCi ^{125}I by a modification of the Chloramin-T method according to Das *et al.* [16]. The ^{125}I-CEA binding of the rabbit anti-CEA antiserum was carried out in duplicate in 5 ml of 0.01 M ammonium acetate buffer pH 6.25 at a final dilution of 1 : 2500 at 45°C for 30 min. The free ^{125}I-CEA was separated from bound by addition of 2.5 ml of zirconyl phosphate gel and centrifugation at 1000 ***g*** for 5 min. The supernatant was decanted, the gel washed with 0.1 M ammonium acetate buffer pH 6.25 and counted in a Packard Auto-Gamma Scintillation Spectrometer 5260-0 after another centrifugation step. The ^{125}I-CEA binding of the human serum peak fractions was determined, after Amicon concentration in duplicate and in two series: with and without unlabelled CEA. Phosphate buffer 0.05 M pH 7.4 with 1 g/l human serum albumin was used throughout. Each incubation step was carried out for 24 hr at 20°C. Aliquots of 2.5, 5, 10 and 20 µl of each preparation were diluted with the phosphate buffer to give a volume of 100 µl. Unlabelled (500 ng) CEA was added to each assay in another 100 µl of phosphate buffer. After the first incubation period ^{125}I-CEA (0.8 ng CEA, 35×10^3 counts/min) was added to each assay and allowed to incubate. Finally, 100 µl of phosphate buffer with 2.5 ng of human IgG were added as a carrier followed by addition of 100 µl of rabbit anti-human IgG antiserum at a 1 : 10 dilution.

After the last incubation period the tubes were centrifuged for 30 min at 2500 ***g*** at 4°C, decanted, gently washed with ice cold distilled water, centrifuged again without stirring, decanted and counted.

The human plasma CEA levels were determined according to the method of Hansen *et al.* [15].

RESULTS

After binding of 1 mg of CEA to 2.5 g of CNBr activated agarose 2.7 µg CEA was found in 100 ml of the washing solution. Apparently, the binding of CEA to agarose was almost quantitative.

In order to investigate the binding capacity of the CEA-agarose columns 0.1 ml and 1 ml of the rabbit anti-CEA antiserum were applied. While the bulk of the proteins appeared in the first peak (Fig. 1), only a small proportion of binding affinity was detected when 1 ml of rabbit anti-CEA was applied to the column and no binding affinity was detected when 0.1 ml was applied. Depending upon the amount of rabbit antiserum applied to the column most or all binding capacity as measured with the Z-gel

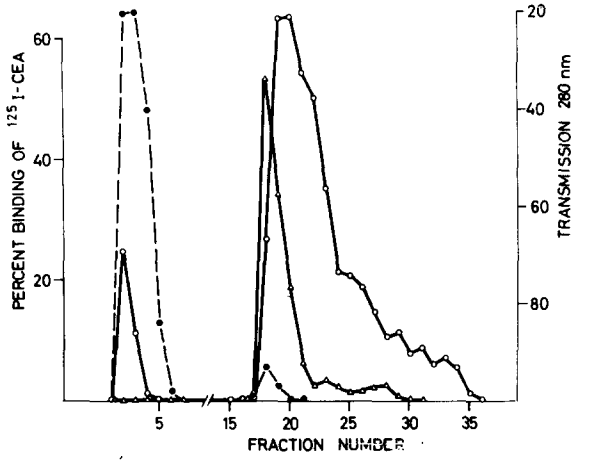

Fig. 1. Elution profile of 1 ml *of rabbit anti-CEA antiserum* ●---●, *O.D. at* 280 *nm.* pH *changed at fraction* 17. ^{125}I-*CEA binding profile of* 1 ml ○—○ *and of* 0.2 ml △—△ *of rabbit anti-CEA antiserum.*

technique appeared in the second protein peak obtained after changing the buffer from pH 8 to 3 (Fig. 1). The columns were re-equilibrated with the pH 8.0 buffer, then eluted again at pH 3.0 serum. Though small protein peaks appeared after re-equilibration and elution (data not shown) no CEA binding activity was detected when the peaks were concentrated and measured by the Z-gel technique. A column packed with mock-coupled agarose as applied to CEA coupling showed no retention of ^{125}I-CEA binding proteins (Table 1).

10 and 20 µl of the concentrated second protein peak of each run were checked for ^{125}I-CEA binding affinity using the rabbit anti-human IgG method. The specific binding curves (Fig. 2), expressed as a per cent, were calculated as the difference between ^{125}I-CEA binding without unlabelled CEA minus the binding in the presence of unlabelled CEA. This complex bundle of curves is more meaningfully arranged when the smoking habits of the control individuals and the smoking habits and clinical data of the colon cancer patients are taken into consideration. The binding capacity of 20 µl of the concentrated samples is shown in Fig. 3. Seven out of the twelve smokers have serum ^{125}I-CEA binding affinity greater than the 11 non-smokers. The distribution of serum ^{125}I-CEA binding in 12 patients (including 2 smokers) with respectable colon carcinoma resembles that of the healthy smokers; 3 out of 4 patients with metastazised colon carcinoma have low ^{125}I-CEA binding affinity in the serum. However, testing these independent groups of controls and patients by the non-parametrical test of Kruskal-Wallis (*H*-Test) with a significance level of a $=0.05$ for the type I error, the Null hypothesis could not be rejected; i.e., the differences fail to be statistically significant.

Plasma CEA level and CEA binding capacity do not correlate well for the patients examined. Healthy smokers and non-smokers,

Table 1. ^{125}I-*CEA binding of concentrated protein peaks of* 1 ml *of rabbit anti-CEA antiserum, chromatographed by CEA-agarose and mock-coupled agarose*

Protein peak at 280 nm	pH	Per cent binding of CEA-agarose	^{125}I-CEA mock-coupled agarose
1	8	20.2	100
2	3	100	0
3	8	2.6	0
4	3	0	0
5	8	0	0

During the course of these experiments, the properties of the CEA-agarose columns remained unchanged as shown by control experiments with rabbit antiserum at the end of the investigation.

Chromatography of 10 ml of the human sera was carried out under the same conditions as the rabbit serum. The transmission pattern at 280 nm showed very small peaks upon changing the buffer from pH 8 to pH 3 and back to pH 8 again. Aliquots of 2.5, 5,

however, show low ^{125}I-CEA binding with low plasma CEA levels and higher ^{125}I-CEA binding with higher CEA levels (Fig. 4). This is statistically evident by a Spearman's correlation coefficient of $P<0.025$ in one-sided test.

Of the 19 individuals examined with blood group O and B having anti-A antibodies, 8 show ^{125}I-CEA binding in an amount of more than 1%. Of the 24 individuals with blood group A or AB, 11 have raised binding

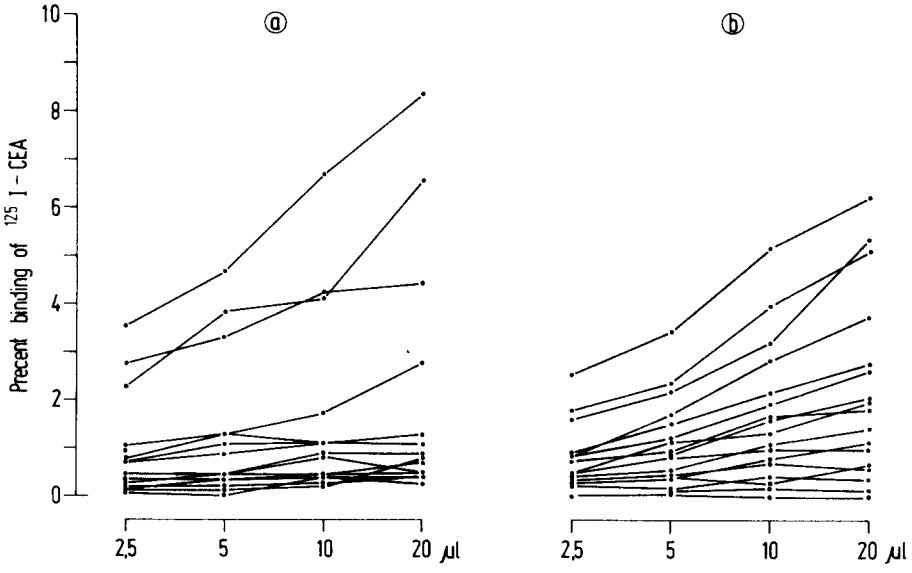

Fig. 2. ^{125}I-*CEA binding of increasing amounts of human serum proteins in some controls* (*a*) *and colon cancer patients* (*b*).

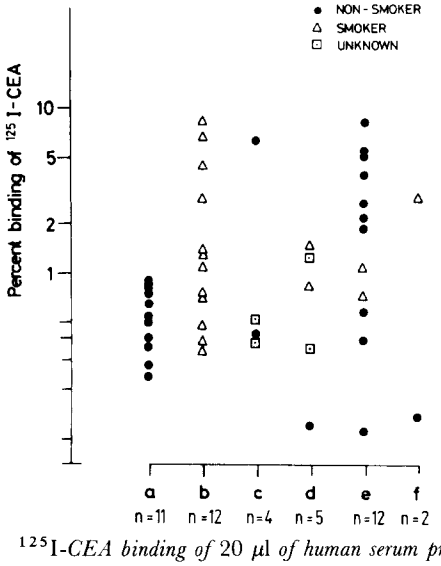

Fig. 3. ^{125}I-*CEA binding of* 20 µl *of human serum proteins:*
a = *healthy non-smokers;*
b = *healthy smokers;*
c = *patients with metastazised colon cancer;*
d = *patients with locally advanced colon cancer;*
e = *patients with resectable colon cancer;*
f = *one patient* (*smoker*) *with an early colonic cancer and one patient* (*non-smoker*) 6 yr *after resection without recurrence.*

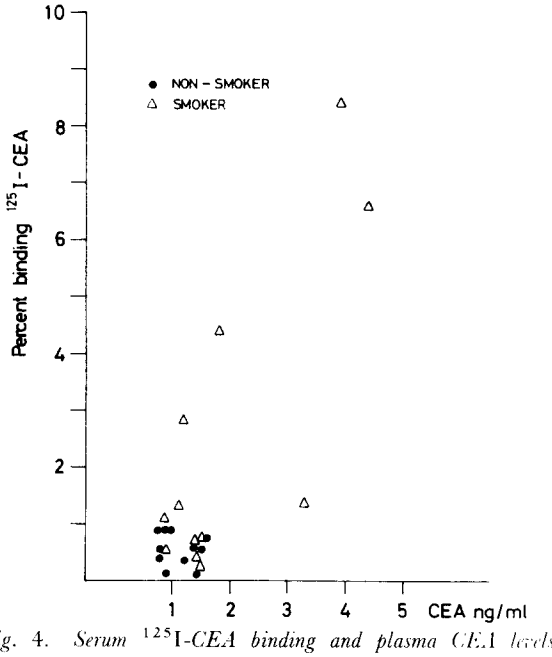

Fig. 4. Serum ^{125}I-*CEA binding and plasma CEA levels from controls* (N = 23).

proteins. For the colonic cancer patients alone these ratios are 3 out of 8 and 9 out of 14 (Table 2).

DISCUSSION

The aim of this study was to determine whether CEA binding proteins can be detected in human sera by affinity chromatography and whether these proteins are randomized among the individuals examined or not.

In order to characterize the function of the CEA-agarose columns affinity chromatography was carried out with rabbit anti-CEA antiserum. When 1 ml of rabbit anti-CEA serum was applied to a CEA-agarose column both the first protein peak (flow through) and the second protein peak (pH 3.0 elution) demonstrated CEA binding proteins. This is due to overloading of the column since runs with 0.1 ml of rabbit anti-serum show binding proteins only in the second protein peak. No anti-CEA activity was found in the further protein peaks when the buffer was changed

Table 2. ^{125}I-*CEA binding and blood group in patients and controls*

Blood group	No. studied	No. with raised binding
Blood type O, B		
Colon cancer patients	8	3
Controls	11	5
Totals	19	8
Blood type A, AB		
Colon cancer patients	14	9
Controls	10	2
Totals	24	11

once more. That means that the columns were stripped of CEA binding proteins after only one run. Mock-coupled CNBr-agarose does not bind rabbit anti-CEA activity.

The method used to measure CEA binding affinity of human protein solutions includes the precipitation of the CEA–protein complex by monospecific rabbit anti-human IgG (γ-chain) anti-serum. ^{125}I-CEA alone did not give a precipitate with the rabbit anti-IgG antiserum. Cold CEA in excess displaced the ^{125}I-CEA almost totally. Therefore it is concluded that the human proteins responsible for ^{125}I-CEA binding in this assay are IgG.

MacSween [4] tested sera from tumor patients and controls by radioimmunoelectrophoresis and found ^{125}I-CEA binding predominantly involved serum IgM. Gold *et al.* re-interpreted similar results [3] after having demonstrated that IgM anti-A antibodies are capable of binding to a site on CEA [7]. Collatz *et al.* [5] and Lo Gerfo *et al.* [6] did not observe antibodies against CEA by hemagglutination and by radioimmunoassay. The differences between the reported findings and those of the present study may be due to the concentration of reactive globulins produced by affinity chromatography of 10 ml of serum. On the other hand variations of the methods used to determine the CEA binding or immunological variations of the CEA preparations may cause the discrepancy of results.

Serum IgG from 12 to 23 patients with colonic cancer and 7 of 23 healthy individuals showed specific ^{125}I-CEA binding of more than 1%. The binding is quite low, reaching a maximum at 10%, however binding is concentration dependent (Fig. 2), i.e. binding is directly proportional to the amount of human binding proteins present in the reaction.

^{125}I-CEA binding proteins tested in this study appear not to be connected with the ABO blood groups as described by Gold *et al.* [3, 7]. About half the individuals examined, with or without anti-A antibodies showed ^{125}I-CEA binding. Only 3 out of 8 colon cancer patients having anti-A antibodies showed ^{125}I-CEA binding whereas the sera of 9 out of 14 patients without anti-A antibodies bound ^{125}I-CEA (Table 2).

The correlation of high plasma CEA level and high ^{125}I-CEA binding for healthy individuals ($P<0.025$) may indicate that CEA is immunogenic. But this impression is based on the data of only 7 persons. In contrast, 3 out of 4 patients with metastasized carcinoma and very high plasma CEA levels have CEA binding IgG in low amounts. This correlates well with the probability of a weak immune system in advanced tumor stages. Seven out of twelve patients with resectable colon carcinoma show CEA binding IgG in higher amounts, comparable with those of healthy smokers. None of these patients was a smoker. In this stage of tumor development humoral immunoreactions against CEA may occur. The data presented here indicates that CEA in man may be immunogenic. On the other hand, the low amount of CEA binding detected may be caused by antibodies generated by antigens similar to CEA. Therefore, the specifity of the ^{125}I-CEA binding IgG remains open to question.

Acknowledgements—The author is indebted to Dr. H. J. Hansen for his donation of CEA. Prof. H. Frahm, Prof. G. Koch, Priv. Doz. Dr D. Grässlin and Dr. Fehr are thanked for suggestions and much support.

REFERENCES

1. P. Gold and S. O. Freedman, Demonstration of tumour specific antigens in human colonic carcinomata by immunological tolerance and absorption techniques. *J. exp. Med.* **121,** 439 (1965).
2. P. Gold, Circulating antibodies against carcinoembryonic antigens of the human digestive system. *Cancer (Philad.)* **20,** 1663 (1967).
3. J. M. Gold, S. O. Freedman and P. Gold, Human anti-CEA antibodies detected by radioimmunoelectrophoresis. *Nature New Biol.* **239,** 60 (1972).
4. J. M. MacSween, The antigenicity of carcinoembryonic antigen in man. *Int. J. Cancer* **15,** 246 (1975).
5. E. Collatz, S. von Kleist and P. Burtin, Further investigations of circulating antibodies in colon cancer patients: on the autoantigenicity of the carcinoembryonic antigen. *Int. J. Cancer* **8,** 298 (1971).
6. P. lo Gerfo, F. P. Herter and S. J. Bennett, Absence of circulating antibodies to carcinoembryonic antigen in patients with gastrointestinal malignancies. *Int. J. Cancer* **9,** 344 (1972).
7. J. M. Gold and P. Gold, The blood group A-like site on the carcinoembryonic antigen. *Cancer Res.* **33,** 2821 (1973).
8. G. Andreu, G. Chavanel and P. Burtin, H blood group activity on CEA molecules. *Immunochemistry* **12,** 921 (1975).
9. J.-P. Mach, K. Singh, J. Perrinjaquet, S. Carrel, C. Merenda, D. MacDonald and A. Holburn, Relationship between carcinoembryonic antigen and blood group antigens A, B and Lewis. *Ann. N.Y. Acad. Sci.* **259,** 389 (1975).
10. J. P. Bali, R. Magous, C. Lecou and M. Mousseron-Canet, Presence of blood group H antigen on a carcinoembryonic antigen, and its enzymatic modification into blood group A and B specificities. *Cancer Res.* **36,** 2124 (1976).
11. K. L. Rosenthal, J. L. Palmer, J. A. Harris, W. E. Rawls and W. A. F. Tompkins, Antibody-induced redistribution of CEA on the cell surface: utilization in separation of CEA and isoantigen A. *J. Immunol.* **115,** 1049 (1975).
12. F. J. Primus, E. S. Newman and H. J. Hansen, Affinity in radioimmunoassay of antibody cross-reactive with carcinoembryonic antigen (CEA) and colon carcinoma antigen-III (CCA-III). *J. Immunol.* **118,** 55 (1977).
13. Y. D. Kim and A. A. Hirata, Carcinoembryonic antigen-like substances in human cavity fluids. *Immunol. Commun.* **5,** 619 (1976).
14. R. Axén, J. Porath and S. Ernback, Chemical coupling of peptides and proteins to polysaccharides by means of cyanogen halides. *Nature (Lond.)* **214,** 1303 (1967).
15. H. J. Hansen, K. P. Lance and J. Krupey, Demonstration of an ion sensitive antigenic site on carcinoembryonic antigen using zirconyl phosphate gel. *Clin. Res.* **19,** 142 (1971).
16. S. Das, B. R. Das and W. D. Terry, Modifications and evaluation of double antibody radioimmunoassay of human carcinoembryonic antigen. *Cancer Res.* **36,** 1954 (1976).