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CEA-containing immune complexes in sera of patients with colorectal and breast cancer – analysis of complexed immunoglobulin classes

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Summary. A sandwich enzyme immunoassay was developed to detect circulating immune complexes containing carcinoembryonic antigen (CEA) and immunoglobulin (Ig) G, IgA, or IgM using a nitrocellulose-bound anti-CEA antibody as the solid phase reagent. Elevated levels of CEA-containing circulating immune complexes (CEA-IC) were found in 15.4% of 117 sera from patients with colorectal cancer in a postsurgery follow-up study. Also in 24.5% of 102 sera from patients with breast cancer in different states of disease CEA-IC were found. The predominant Ig determined in CEA-IC of colorectal cancer patients was IgA, followed by IgG and IgM, whereas IgG and IgM were the most frequent Igs in CEA-IC of breast cancer patients. Elevated CEA levels were found in 12.0% of the colorectal cancer patients and in 25.4% of sera from breast cancer patients. No significance for the coincidence of elevated CEA levels and CEA-IC was recorded in all patients sera tested. In sera of patients with disease recurrence, however, both parameters were shown to be elevated (CEA 80.7% and CEA-IC 42.3%). The data presented indicate the detection of CEA-IC as an additional parameter for the identification of patients at increased risk for disease recurrence.

Introduction

Carcinoembryonic antigen (CEA), a 180,000 dalton glycoprotein with a carbohydrate portion of 40%–60%, is an extensively characterized tumor-associated molecule [5, 17]. Elevated serum levels have been recorded in patients with different epithelial tumors. In contrast to patients with colorectal tumor or medullary carcinomata of the thyroid, significantly raised CEA serum levels in other epithelial carcinomata are only recorded in patients with an extended tumor burden and/or with metastatic disease [22, 23]. An increase in individual CEA serum levels has been described to indicate the presence of disease recurrence or metastasis, although only in about 70% of documented cases in colorectal cancer [11]. As possible reasons for this finding differences in production and release of CEA from the tumor surface have been discussed [14]. Furthermore the CEA molecule could be masked by antibodies which render it unaccessible for measurement in immunoassays. Although there exist controversial results concerning the antigenicity of CEA in man [1, 3, 10, 13], antibodies reacting with CEA have been demonstrated in sera from tumor patients [6, 15, 20]. CEA-containing immune complexes (CEA-IC) were first identified in kidney eluates of a colorectal cancer patient [4]. In sera from this patient group evidence for the existence of CEA-IC has been published by several authors, using extraction [9], precipitation [2], dissociation [12], and solid phase immune complex detection methods [16, 21].

In the present study a sandwich enzyme immunoassay for the measurement of CEA-IC is described. Patients after surgical treatment of colorectal carcinoma, and patients suffering from several states of breast cancer were investigated. Levels of CEA and CEA-anti-CEA-immunoglobulin (Ig) aggregates (immune complexes) of IgG, IgA, and IgM classes were determined and correlated to the state of disease.

In addition, in a number of CEA-IC positive and CEA-IC negative sera, the CEA content of the probe treated by dissociating and nondissociating conditions was compared to provide another type of evidence for the presence of CEA-IC.

Materials and methods

Patients' sera and CEA serum level analysis. Sera were obtained from 117 patients suffering from colorectal cancer taking part in a postsurgery follow-up study and 102 patients suffering from breast cancer. The CEA serum levels were determined using a radioimmunoassay (Isotopendiagnostik, Dreieich, FRG) and an enzyme immunoassay (Abbott, Wiesbaden, FRG). As controls serum was obtained from normal bloos donors (laboratory personnel and unselected volunteers from the local blood bank). Blood samples were allowed to clot for 2 h at room temperature and were then centrifuged at 1200 g for 10 min. The mean CEA serum value of the control group was 3.6 ng/ml (Abbott polyclonal CEA assay).

Sandwich enzyme immunoassay for the detection of CEA-IC. For immune complex determination sera from controls and different tumor patients were aliquoted and stored at −70° C until use. Rabbit anti-CEA antibodies (Dako, Hamburg, FRG) were coupled to nitrocellulose

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disks (Schleicher & Schüll, Einbeck, FRG) of 6 mm diameter by overnight incubation using a 1:200 dilution of the antiserum in 500 µl Tris saline (10 m*M* Tris, 152 m*M* NaCl, pH 7.5) per disk. After three washes in Tris saline, free protein binding capacities of the nitrocellulose were blocked by incubation in Tris saline containing 1% bovine serum albumin (Sigma, München, FRG) and 0.1% Tween 20 (Serva, Heidelberg, FRG) at 37° C for 1 h. The nitrocellulose disks were washed and transferred to plastic tubes for the immune complex assay.

The nitrocellulose disks were reacted for 2 h at 37° C with 500 µl of a 1:2 dilution of serum in Tris saline containing 1% bovine serum albumin and 0.1% Tween 20. After three washes, 500 µl of enzyme antiserum coupled to human IgG, IgA, or IgM was added for a second incubation step of 2 h at 37° C. All three antisera were used at a dilution of 1:1000 in Tris saline containing 1% bovine serum albumin and 0.1% Tween 20. The enzyme conjugate initially used was horseradish peroxidase-coupled goat anti-human IgG, IgA, or IgM (Tago, Medac, Hamburg, FRG). For practical reasons in the experiments using sera from breast cancer patients, the same antiserum coupled to alkaline phosphatase was used. Quantitation was achieved by a color reaction, using orthophenylenediamine (1 mg/ml, Sigma, Heidelberg, FRG) for the horseradish peroxidase-coupled antisera; the reaction was stopped after 20 min by 1 *M* HCl and photometrically quantitated at 462 nm. Alkaline phosphatase-coupled probes were reacted with para-nitrophenylphosphate (10 m*M*, Sigma, Heidelberg); the reaction was stopped by 0.5 *M* Na_2CO_3 and quantitated at 405 nm. All tests were performed in duplicate.

As no CEA-anti-CEA standard containing human Igs exists the normal range for the detection of CEA-IC was defined in every test by the mean value ($\bar{x}$) and the 3-fold SD of 10 normal sera. The SD was used to define classes of positivity of the samples: results exceeding $\bar{x}$ by less than 1 SD were given the value 0, those ecxeeding $\bar{x}$ by 1–2 SD were given the value 1 and so on. Results exceeding $\bar{x}$ + 3 SD (value and higher) were regarded as positive.

Model system. To determine the influence of the antigen-antibody ratio of CEA-IC on their detectability in the sandwich test system used experimental CEA-anti-CEA model complexes were used composed of different amounts of CEA (Abbott, Wiesbaden, FRG, 4–60 ng/ml) and rabbit anti-CEA antibody (Dako, Hamburg, FRG, $1/2 \times 10^2 - 1/2 \times 10^6$). Antigen and antibody were incubated for 1 h at 37° C, centrifuged to remove insoluble aggregates and stored at −70° C until use. For the test, plastic beads coupled with monoclonal anti-CEA antibody (Abbott) were then added as a solid phase reagent and peroxidase-labeled goat anti-rabbit IgG antibodies (Behringwerke, Marburg, FRG) served as fluid phase reagents.

Dissociation experiments. Dissociation experiments were performed with 70 of the colorectal cancer sera (17 CEA-IC positive, ⩾3 SD in the CEA-IC test, 7 revealed values between $\bar{x}$ +2 SD and $\bar{x}$ +3 SD, and 46 were CEA-IC negative, <2 SD in the CEA-IC test). In order to achieve dissociating conditions for immune complexes (pH 2.5), 20 µl of 1 *N* HCl was added to 200 µl of serum. The samples were incubated for 15 min on a shaker. Then they were neutralized by 100 µl of 0.1 *M* phosphate-buffered saline, pH 7.5 (neutralizing buffer). To avoid reassociation CEA was extracted immediately by addition of 640 µl of 0.2 *M* sodium acetate (extraction buffer) and the samples were incubated at 70° C for 15 min. Precipitated material was removed by centrifugation for 10 min at 1200 *g*. The CEA content of the supernatants was determined by the Abbott polyclonal CEA assay and compared to the CEA content of undissociated control samples of the same sera which were diluted to the same volume as the dissociated probes by addition of 120 µl of a mixture of neutralizing buffer (100 µl) and 1 *N* HCl (20 µl) before the extraction step, and which were run in parallel in the same assay.

Results

1. Experimental evaluation of the method used

To define the conditions in which CEA-IC are detectable in the sandwich assay, model immune complexes containing CEA and rabbit anti-CEA antibody were formed and tested in a system using mouse monoclonal anti-CEA antibody as a solid phase reagent and peroxidase-labeled goat anti-rabbit IgG antibodies as the fluid phase reagent. With regard to different CEA (0–60 ng/ml) and anti-CEA antibody ratios (final concentration of anti-CEA $1/2 \times 10^2 - 1/2 \times 10^6$), CEA-IC containing 10–60 ng/ml of CEA and anti-CEA over a concentration range of $1/2 \times 10^2$ to $1/2 \times 10^4$ were detected. Increasing the antibody component by more than a factor of 100 meant CEA-IC were no longer traceable, presumably because of steric hindrance of the binding of CEA to the solid phase antibody by complexing rabbit anti-CEA antibody (Fig. 1). The effect of the antigen-antibody ratio of CEA-anti-CEA immune complexes on the detectability of CEA is illustrated by an example in Fig. 2. From these data it is evident that false-negative CEA levels can be created by CEA-IC.

2. CEA-IC in patients sera and normal controls

The CEA-IC were detectable by the sandwich test in 25 of 102 sera from patients with breast cancer (24.5%), and in 18 of 117 colorectal cancer sera (15.4%). Interestingly there was a marked difference in the distribution of the Ig classes determined in the immune complexes between the

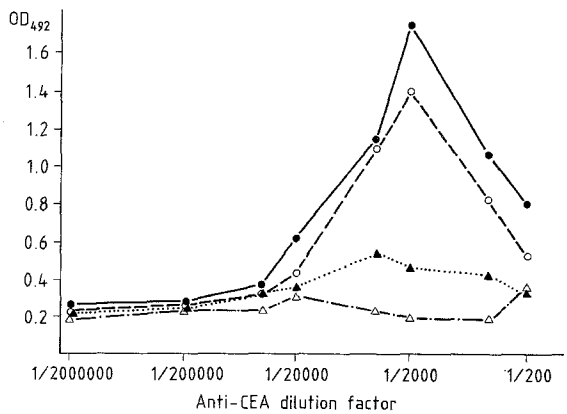

Fig. 1. Influence of antigen-antibody ratio on the detectability of CEA-IC in a solid phase enzyme immunoassay system. ● CEA = 60 ng/ml; ○ CEA = 30 ng/ml; ▲ CEA = 10 ng/ml; △ CEA = 0 ng/ml

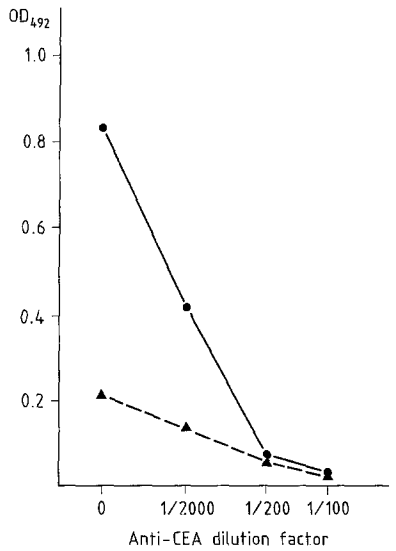

Fig. 2. Influence of CEA complexation by anti-CEA antibodies on the detectability of CEA in a CEA enzyme immunoassay system (CEA/rabbit anti-CEA model system). ● CEA serum level = 24.8 ng/ml; ▲ CEA serum level = 3.4 ng/ml

two patient groups investigated. In sera of patients with breast cancer the frequency of IgG and IgM in CEA-IC was about the same (11.7% and 13.7%), whereas IgA was very rare (1.9%). In contrast, IgA was the most common Ig present in CEA-IC of colorectal cancer patients (10.2%), complexed IgG was found in 6.0%, and CEA-IgM-IC were only detected in 1 serum (0.8%) (Table 1). Different Ig classes complexed with CEA in the same serum were detected in 3 patients with breast (2.9%) and 2 patients with colorectal cancer (1.7%) (data not shown).

In control experiments using 35 sera from normal blood donors, 10 were arbitrarily chosen to define the normal range and the results of the other 25 sera were evaluated like patients' sera. None of the results of these sera exceeded $\bar{x}$ +3 SD of the 10 samples defining the normal range for CEA-IgG, CEA-IgA or CEA-IgM-IC; applying the $\bar{x}$ +2 SD range, 1 positive serum was found for CEA-IgG-IC, none for CEA-IgA-IC, and 1 for CEA-IgM-IC.

3. Dissociation experiments

As additional evidence for the existence of CEA-IC in the sera investigated by the sandwich enzyme immunoassay, 70 of the colorectal cancer sera (17 CEA-IC positive, 7 between $\bar{x}$ +2 SD and $\bar{x}$ +3 SD of the normal controls, 46 CEA-IC negative by the sandwich test) were treated by acid conditions for 15 min in order to dissociate the putative CEA-IC; CEA was extracted immediately after reneutralization of the sample and the CEA content was compared to control probes which had not been exposed to acid conditions before CEA extraction. Figure 3 shows the results of our model CEA-IC and rabbit anti-CEA at different antigen-antibody ratios. The CEA that was no longer measurable in conventional CEA assays because it was captured in immune complexes could be recovered by the dissociation/extraction step.

The results of the colorectal cancer sera are given in Table 2. The CEA-IC positive sera revealed an average in-

Table 1. CEA-containing immune complexes in patients with breast cancer and colorectal cancer

	CEA-IgG	CEA-IgA	CEA-IgM
Breast cancer	$n = 12$ (11.7%)	$n = 2$ (1.9%)	$n = 14$ (13.7%)
$n = 102$	4.25±2.52	3±0	5.35±2.81
Colorectal cancer	$n = 7$ (6.0%)	$n = 12$ (10.2%)	$n = 1$ (0.8%)
$n = 117$	4.71±1.70	5.91±2.87	4
Total	$n = 19$ (8.7%)	$n = 14$ (6.4%)	$n = 15$ (6.8%)
$n = 219$	4.42±2.22	5.50±2.84	5.26±3.59

Incidence of carcinoembryonic antigen-containing immune complexes (CEA-IC) in patients with breast cancer and colorectal cancer. Percentage of positive results and mean values of positivity classes (= factor of SD exceeding the mean value of 10 normal sera, see *Materials and methods)*

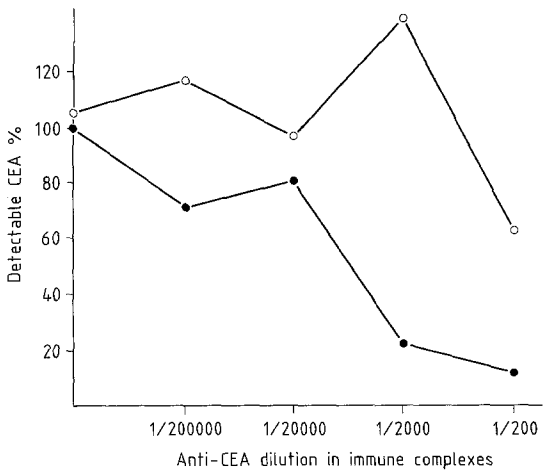

Fig. 3. Dissociation experiment of CEA-anti-CEA model immune complexes containing human CEA and rabbit anti-CEA antibodies at different antigen-antibody ratios (CEA concentration 14.8 ng/ml; anti-CEA dilution 1:200–1:200,000 in the immune complex preparation). ● CEA concentration in the untreated probe; O CEA concentration in the dissociated probe

Table 2. Dissociation experiments of CEA-IC in sera of colorectal cancer patients ($n = 70$) and healthy controls ($n = 6$)

Patients' sera	Average change in CEA level	
	In ng	In %
Positive (≥3 SD) in the CEA-IC sandwich test ($n = 17$)	+0.83 (±2.10)	+14.8 (±26.1)
2 SD ≤ $\bar{x}$ <3 SD in the CEA-IC sandwich test ($n = 7$)	+0.85 (±1.08)	+24.5 (±24.1)
Negative (<2 SD) in the CEA-IC sandwich test ($n = 46$)	+0.43 (±0.95)	+19.9 (±40.3)
Normal controls ($n = 6$)	−0.04 (±0.26)	− 6.6 (±43.5)

Results of CEA measurements in samples treated by immune complex dissociating conditions compared to results of the undissociated samples; data of 17 patients sera positive for CEA-IC (≥3 SD), 7 sera exhibiting values between $\bar{x}$ + 2 SD and $\bar{x}$ + 3 SD of the normal controls, 46 negative for CEA-IC, and 6 normal control sera (mean values and SD)

crease in their CEA level of 0.83 ± 2.10 ng (14.8% ± 26.1%); the 7 sera with increased CEA-IC levels below the limit for positivity (values between $\bar{x}$ +2 SD and $\bar{x}$ +3 SD of the normal controls) showed a similar increase of 0.85 ± 1.08 ng CEA (24.5% ± 24.1%) in the dissociation experiment, while CEA-IC negative sera gave a significantly lower increase of only 0.43 ± 0.95 ng CEA (19.9% ± 40.3%). No CEA increase in the dissociated probes was found in 6 sera from apparently healthy individuals.

Taking into account the limitations of the sandwich test in the detection of CEA-IC at a restricted antigen-antibody ratio, these results support the hypothesis that a least part of the CEA in colorectal cancer sera remains hidden in CEA assays because it is captured in immune complexes.

4. Correlation of CEA serum levels and CEA-IC

The CEA levels were found to be elevated in 21% of all sera tested (12% in sera from colorectal cancer patients and 25.4% in patients with breast cancer). Of the tested sera, 20.1% were positive for CEA-IC (15.4% in colorectal cancer patients and 24.5% in breast cancer patients). Both parameters were simultaneously elevated in 5.9% of the investigated sera (3.4% in colorectal cancer patients and 7.8% in breast cancer patients).

5. CEA-IC and disease progress

Of the 219 sera tested for CEA-IC, 26 derived from patients with documented disease recurrence or metastases (21 of 102 breast cancer sera and 5 of 117 colorectal cancer sera). Of these 26 sera 9 (34.6%) were positive both in the CEA assay and in the test for CEA-IC, 12 (46.1%) only in the CEA assay, and 2 (7.7%) only in the CEA-IC test; 3 of the 26 sera (11.5%) gave negative results in both tests. Of the further 193 sera, obtained from patients without apparent disease recurrence, 4 (2.1%) were positive for both parameters, 21 (10.9%) for CEA only, and 29 (15%) for CEA-IC, whereas 139 (72%) were negative in both assay systems (Table 3a). In total, 13 of the sera were positive in both tests. It is remarkable that 9 of them (69.2%) were from patients with recurrent disease, whereas the corresponding ratio for CEA was only 36.3%.

In summary, 80.7% of the sera obtained from patients with disease recurrence revealed increased CEA values, and 42.3% contained CEA-IC; of the patients without documented disease recurrence 13% had elevated CEA serum levels and in 17.1% of the sera CEA-IC were detected (Table 3b).

Discussion

By means of a model system using artificial CEA-anti-CEA immune complexes it was demonstrated that the sandwich technique is suitable for the detection of CEA-IC. However, as demonstrated, a limitation of this approach lies in its failure to detect CEA-IC in extreme antibody excess. In spite of this restricition CEA-IC were demonstrated in 19.6% of sera from patients suffering from two types of epithelial tumors (colorectal carcinoma and breast cancer). The data correspond to previously published results obtained with colorectal cancer sera by an assay system based on similar test principles but limited to the detection of IgG-containing immune complexes [16]. Other approaches for the detection of circulating CEA-IC include the perchloric acid precipitation of immune complexes followed by resolubilisation of the precipitated CEA in 0.01 *M* ammonium acetate. Positive results for CEA-IC were found in 24.5% of sera from colorectal cancer patients using this method [18]. However, CEA could not be demonstrated as part of the immune complexes by precipitation using polythylene glycol, followed by the detection of complexed CEA by a radioimmunoassay [2].

By the two latter methods no information concerning the complexing antibody is available, in contrast to the present study, where CEA-IC containing IgG, IgA, and IgM were demonstrated. The CEA-IgA complexes were significantly restricted to sera from colorectal cancer pa-

Table 3. (**a**) CEA-IC, CEA, and disease progress

	n	CEA+/IC+	CEA+/IC−	CEA+/IC+	CEA+/IC−
Patients with disease recurrence/metastasis	26	9 (34.6%)	12 (46.1%)	2 (7.7%)	3 (11.5%)
Patients without disease recurrence/metastasis	193	4 (2.1%)	21 (10.9%)	29 (15.0%)	139 (72.0%)
Total	219	13 (5.9%)	33 (15.1%)	31 (14.2%)	142 (64.8%)

(**b**) CEA-IC, CEA, and disease progress

	n	CEA positive	CEA-IC positive
Patients with disease recurrence/metastasis	26	21 (80.7%)	11 (42.3%)
Patients without disease recurrence/metastasis	193	25 (13.0%)	33 (17.1%)
Total	219	46 (21.0%)	44 (20.0%)

Incidence of CEA and CEA-IC in patients with and without disease recurrence: patients positive for both parameters, for one parameter alone, or negative for CEA and CEA-IC

tients, followed by IgG and – in only 1 case – IgM-containing CEA-IC. This is in contrast to results obtained by other investigators describing predominantly IgM anti-CEA antibodies in CEA-IC [7, 8]. The finding of predominantly IgA-containing immune complexes in colorectal cancer sera in the present communication corresponds with data describing the Ig pattern obtained by incubation of serum probes with colorectal tumor samples and acid elution of the adsorbed material [19]. In the study cited the author suggests that IgA may represent a major humoral antitumor immune response in patients with colorectal cancer in contrast to cancers of different origin. In sera from breast cancer patients IgG- and IgM-containing CEA-IC were found in about the same frequency; IgA-CEA-IC were very rare. Compared to the results in colorectal cancer sera, this finding may indicate differences in antibody responses to tumor-associated antigens influenced by different anatomical tumor situations.

In sera of patients with disease recurrence and/or metastasis, levels of both CEA and CEA-IC were found to be elevated (80.7% CEA, 42.3% CEA-IC) more than in sera of patients without disease relapse (13% CEA, 17.1% CEA-IC). These data are in agreement with previously published results [18] recognizing an increased incidence of CEA-IC in patients who subsequently developed metastatic disease.

The importance of CEA-IC as a factor contributing to low or even false-negative results in CEA assays as depicted in this communication has been indicated by dissociation experiments. Splitting of CEA-IC by 1.25 *M* $MgCl_2$ and removal of IgG by a protein A-containing *Staphylococcus aureus* preparation yielded up to 2800% increase in CEA concentration in sera from colorectal cancer patients [12]. In our tests 17.1% of the patients without apparent disease recurrence exhibited elevated CEA-IC levels compared to 42.3% of the patients with disease relapse whereas the corresponding data for CEA were 13% and 80.7%. These data show that the occurrence of CEA-IC may precede the presence of demonstrable CEA levels. Further studies are necessary to find out whether the detection of CEA-IC can be used to select patients with increased risk for disease recurrence.

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