Demonstration of Elevated Anti-Lewis Antibodies in Sera of Cancer Patients Using a Carcinoembryonic Antigen-Polyethylene Glycol Immunoassay

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ABSTRACT

The serum levels of carcinoembryonic antigen (CEA) and CEA-binding proteins of 68 controls and 170 cancer patients were determined. The CEA levels were determined by a double-antibody radioimmunoassay using radiolabeled CEA, and the CEA-binding proteins were determined by an immunoassay utilizing radiolabeled CEA and polyethylene glycol. The major CEA-binding proteins in serum were anti-blood group antibodies as demonstrated by differential binding of serum proteins from A, B, or O positive individuals to radiolabeled CEA's which were previously shown to carry specific blood group determinants. No statistically significant differences were observed for the binding of control versus cancer patient sera to CEA-A (carrying blood group A), except as expected, A negative (O or B positive) individuals gave high binding to CEA-A, while A positive individuals gave low binding to CEA-A. Statistically significant differences were observed for controls versus cancer patients for binding to CEA-Lewis^a^ (CEA-Le^a^). CEA-Le^a^ binding activity was higher in females and smokers in the control group, but this distinction was not found in the cancer patients. The high levels of anti-Le^a^ antibodies may be explained in females by exposure to fetal antigens during pregnancies and in smokers or cancer patients by exposure to precursors to blood group substances.

The sero 7% of the patients with colon cancer, 18% of the patients with breast cancer, and 23% of the patients with bronchogenic carcinoma bound more CEA-Le^a^ than did the serum of the highest male control. A correlation between CEA-Le^a^ binding activity and the levels of serum CEA was found for patients with colonic carcinoma but was not significant for the groups with other cancers or the control group. Serial determinations of CEA-Le^a^ binding activity for 21 patients with bronchogenic carcinoma changed congruently with the serum levels of CEA in 12 cases.

The significance of these results in terms of expression of immature blood group antigens, the subsequent production of antibodies against them, and the prognostic value of this response is discussed.

INTRODUCTION

CEA^2^ is a glycoprotein which possesses multiple antigenic determinants on the protein portion of the molecule (2, 3, 21, 25) as well as on the carbohydrate moieties (1, 11, 15, 18). In the preceding paper, the carbohydrate determinants of several CEA preparations were characterized by a lectin and antibody binding assay using PEG to precipitate CEA-protein conjugates (23). It was shown that of the CEA preparations studied some expressed the blood group determinants A and B, and all expressed the H, Le^a^, and Le^b^ determinants, which can be considered precursors to Blood Groups A and B. In this work, the binding properties of control sera and cancer patient sera to a CEA preparation with A^1^ specificity and to a CEA preparation with only the precursor blood group specificities as used in previous affinity chromatography experiments (22) were determined and compared to the serum levels of CEA as determined by binding to goat anti-CEA. The main objectives of this study were to determine the differences in CEA-binding activities between the sera of controls and cancer patients and to determine the utility of serial determinations in following the progress of various types of cancer under different treatment modalities.

MATERIALS AND METHODS

Two CEA preparations, CEA 175, prepared in this laboratory, and CEA BP160, kindly provided by Dr. H. J. Hansen, Nutley, N. J., were used for the binding assays. CEA 175 was shown to express Blood Group A, and precursor blood group specificities, and CEA BP160 was shown to express H, Le^a^, and Le^b^ but no A or B specificities (23).

Le^a^ substance was purchased from Ortho, Raritan, N. J. Anti-CEA was raised in goats as described by Egan et al. (9) and used unabsorbed. PEG 6000 was purchased from Sigma Chemical Co., St. Louis, Mo. Human control sera were obtained from 68 healthy volunteers [33 males (mean age of 35 years), 14 smokers and 19 nonsmokers; 35 females (mean age of 33 years), 18 smokers and 17 nonsmokers]. A total of 170 cancer patients provided 239 serum samples: 74 postsurgical patients with colon cancer (36 males with a mean age of 62 years, 41 females with a mean age of 63 years) of whom 57 had no sign of recurrence and 17 had a regionally spread or a metastatized carcinoma; 49 women with metastasized breast carcinoma (mean age of 57 years, 5 smokers and 44 nonsmokers); 47 patients with lung carcinoma (26 males with a mean age of 59 years, including 19 smokers and 7 nonsmokers, and 21 females with a mean age of 60 years, including 17 smokers and 4 nonsmokers), of whom 7 had localized disease, 8 had regionally spread carcinoma, and 32 had metastasized carcinoma. Of the 170 patients, CEA-binding activity was determined once for 149 patients and serially (3 to 9 times) for 21 patients with lung cancer. All sera were stored at -20° until assayed.

Absorption of Human Sera. Human sera (500-µl aliquots)
were absorbed with 300 µl of packed human erythrocytes for 12 hr at 4°. After centrifugation, the supernatants were used for the assay.

Radioiodination of CEA. Ten-µg portions of the CEA preparations were labeled with 1 mCi 125I by a modified chloramine-T method (9). Their specific activities were about 90 µCi/µg.

CEA-binding Assay. Duplicate assay tubes contained: 200 µl of serum; 100 µl of 0.2 m sodium borate buffer (pH 8.0), with sodium chloride, (4.4 mg/ml; 0.5 ng of radiolabeled CEA; and about 10,000 cmp of 57Co as a volume marker (10). The incubation was performed at 37° for 1 hr and at 4° for another 15 hr. The 125I-CEA-antibody complexes were precipitated for 16 hr at 4° in 4 mI of a 7.5% PEG solution. The samples were centrifuged at 1500 x g at 4° for 30 min. The pellets were counted in a Beckman Gamma 300 counter with an efficiency of about 50%. In each assay, the CEA-binding activities of 5 samples of a control serum from a B positive individual were measured, 4 of them containing goat anti-CEA in dilutions of 1:1000, 1:2000, 1:4000, and 1:8000, relative to the incubation volume of 300 µl. The CEA binding results of the goat anti-CEA dilutions and those of the human sera were calculated as a percentage of goat anti-CEA (1:1000). Correlations between assays using radiolabeled CEA 175 (A1, Leab, and H specificities) and CEA BP160 (Leab, H specificities) were determined by comparison of the standards containing goat anti-CEA. The AB and B control sera for CEA binding activity contained goat anti-CEA and were used to determine repeatability within an assay.

Determination of CEA. Serum levels of CEA were measured by a double-antibody radioimmunoassay as described by Egan et al. (9).

Statistical Analysis. The nonparametric H test [Kruskal-Wallis (17)] and the U test [Mann-Whitney (19)] were used for statistical analysis. For determining correlations, the product-moment correlation coefficient (Pearson) was calculated. The Kruskal-Wallis test (H test) is a nonparametric test used to compare several independent samples which are not gaussian (or normally) distributed as is the case in this work. The test checks the null hypothesis of whether or not the samples originate from one population. In the nonparametric Mann-Whitney U test, 2 independent, not normally distributed samples are compared. It is used when the conditions of the parametrical t test are not fulfilled. In this study, both the H test and the U test were used, since the samples studied were not normally distributed.

RESULTS

For the precipitation of 125I-CEA BP160 in the presence and absence of goat anti-CEA in excess (1:50), an optimal concentration of PEG 6000 was chosen as 7.5% in this assay system. The CEA-binding activity of 10 control AB sera and of 10 control B sera in the absence and presence of different dilutions of goat anti-CEA was determined in one assay with 125I-CEA 175 in order to determine the intrassay variability (Table 1). The mean percentages and standard deviations of the CEA-binding activity of control B sera in 10 consecutive assays performed with 125I-CEA BP160 were calculated to obtain the interassay variability (Table 2). When the binding to goat anti-CEA (1:1000) is normalized to 100% for each assay, the above values for binding in the presence of goat anti-CEA are recalculated as shown in Table 3.

Inhibition of the binding activity of an AB control serum containing 1:2000 goat anti-CEA and a 1:6 dilution of the serum of a healthy smoker (Blood Group A) to 125I-CEA BP160-binding activity with increasing amounts of unlabeled CEA BP160 is shown in Chart 1.

The binding activity of sera from patients with breast cancer to CEA 175 (Blood Group A1) varied according to the expected pattern of blood group antibodies: 53.3 ± 12.1% (S.D.) for patients with Blood Group B or O (n = 21) and 6.4 ± 5.2% for patients with Blood Group A or AB (n = 28). Absorption of the patients' sera with A1 RBC reduced the binding activity for CEA BP160 normalized to the binding activity of anti-CEA (1:1000) by 6.1 ± 1.2% (Chart 2).

Since CEA BP160 does not possess A or B determinants (23), the binding of human sera to it is independent of the presence of anti-A or anti-B. The binding activities for controls and cancer patients to CEA BP160 are shown in Chart 3. The result of the Kruskal-Wallis test at the 5% level indicates that there are differences between the groups. The difference in binding activity between male and female controls is statistically significant (Mann-Whitney U test, 0.001 < p < 0.01), as is the difference between smokers and nonsmokers in male controls (Mann-Whitney U test, 0.001 < p < 0.01). See "Materials and Methods" for a discussion of the statistical tests used. The difference between smokers and nonsmokers could not be shown in female controls. The reasons for this apparent discrepancy are discussed later. The majority of the sera of the cancer patients show a binding activity to CEA BP160 in the range of the controls. The binding activity does not differ for male and female colonic carcinoma patients, for male and

### Table 1

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<td>AB</td>
<td>84.1 ± 0.9</td>
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<td>B</td>
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female bronchogenic carcinoma patients, or according to whether lung cancer patients were smokers or nonsmokers (data not shown).

Five sera of the patients with colonic carcinoma \((n = 74)\) exceeded the highest binding activity value of the male controls. There was no clear relationship between binding activity and tumor burden, but a significant correlation does exist between CEA BP160-binding activity and the serum CEA level (Pearson correlation coefficient, \(p < 0.05\)). Of the breast carcinoma patients \((n = 49)\), 9 sera showed a CEA BP160-binding activity higher than the highest value of the male controls. Five of these 9 sera also had elevated serum CEA levels.

Of the lung carcinoma patients \((n = 47)\), 11 had a CEA BP160-binding activity higher than the highest value of the male controls. Ten out of these suffered from a metastasized cancer, whereas in one case only localized disease was doc-
Twenty-three of the lung carcinoma patients had elevated serum CEA levels. All 10 patients with metastasized carcinoma and elevated CEA BP160-binding activity also had elevated serum CEA levels. The patient with localized lung cancer and elevated CEA BP160-binding activity showed a normal CEA value. No statistically significant correlation was found for all lung carcinoma patients between CEA-binding activity and serum CEA levels or between binding activity and tumor burden.

Serial determinations of CEA BP160-binding activity and CEA in serum was performed in 21 patients with bronchogenic carcinoma (one to 16 months, mean of 7 months). During the followup, 12 patients showed a progressive elevation of both parameters, 5 patients showed only CEA elevation, 3 patients showed only an elevation of CEA-binding activity, and one patient had normal CEA and CEA binding activity as well.

During the course of the follow-up, 11 patients died. Six of these showed a rise of CEA-binding activity and were undergoing radiation or chemotherapy, 4 showed no alteration, and one patient showed a fall in CEA-binding activity. The serum CEA level rose in 8 of those patients, remained constant in one case, and fell in 2 other patients. Ten of the 21 patients were still alive at the end of the follow-up period. Two of those showed a rise of CEA-binding activity at the end of the follow-up, whereas 5 showed decreased and 3 others showed constant CEA-binding activities. The serum CEA levels decreased in 7 of the observed cases and stayed constant in 3 of them. Clinically, no change or partial remissions were observed.

The course of the CEA BP160-binding activity and CEA levels for 4 representative patients is shown in Chart 4. Patient A was a Caucasian female (smoker) in her seventh decade. A small-cell carcinoma of the lung, metastatic to the liver, was treated by irradiation of the primary tumor, followed by chemotherapy with cyclophosphamide. The patient died 5 months after diagnosis. CEA-binding activity rose to high levels and was followed after a short delay by high serum levels of CEA. Patient B was a Caucasian female (smoker) in her sixth decade. A small-cell carcinoma, metastatic to the skeleton, was diagnosed 1 year before the follow-up study was made. Treatment consisted of irradiation followed by Adriamycin. The patient died 6 months after the start of chemotherapy. Two months prior to death, CEA-binding activity and the level of CEA began to rise. Patient C was a Caucasian male nonsmoker in his sixth decade. A small-cell carcinoma of the lung with regional disease was treated by radiation therapy of the primary tumor. After metastases to the ribs were detected, radiation therapy followed by Adriamycin was administered. Prior to death, CEA-binding activity and CEA levels were elevated. Patient D is a Caucasian female in her seventh decade. A small-cell carcinoma, metastatic to the skeleton, was diagnosed 1 year before the follow-up study was made. Treatment consisted of irradiation followed by Adriamycin. The patient died 6 months after the start of chemotherapy. Two months prior to death, CEA-binding activity and the level of CEA began to rise. Patient E was a Caucasian male smoker in his seventh decade. A small-cell carcinoma of the lung, metastatic to the liver, was treated by irradiation of the primary tumor, followed by chemotherapy with cyclophosphamide. The patient died 5 months after diagnosis. CEA-binding activity rose to high levels and was followed after a short delay by high serum levels of CEA.

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In addition to previous experiments with CEA-agarose (22), this research was undertaken to develop a radioimmunoassay which measures the binding of serum proteins to radiolabeled CEA. As demonstrated by other investigators (5–7), at optimal conditions PEG precipitates immune complexes but not radiolabeled antigen. The formation and precipitation of labeled immune complexes are inhibited competitively by unlabeled antigen or by prior absorption of antisera. In our preceding paper (23), we have demonstrated that different CEA preparations bear various blood group determinants. The fact that human sera bind to the blood group determinants of CEA is demonstrated in this work by the inhibition of binding by absorption of human sera either with the appropriate erythrocytes or with purified blood group substances. For example, CEA 175, which bears the Blood Group A determinant, was precipitated with the sera of individuals belonging to Blood Groups O and B, but not by type A individuals. This binding was inhibited by prior absorption of the sera with type A erythrocytes. CEA BP160, which bears H, Le<sup>a</sup>, Le<sup>b</sup>, and MN determinants, was not precipitated according to the ABO system. Absorption of sera with type O erythrocytes showed no inhibition of binding. However, binding was significantly in-
hindered by purified Le\textsuperscript{a} substance, thus demonstrating that CEA BP160 specifically measures anti-Le\textsuperscript{a} activity. The finding that Le\textsuperscript{a} substance did not inhibit the binding of goat anti-CEA to CEA BP160 suggests that xenosignals primarily recognize the protein portion of the CEA molecule but that human sera primarily recognize carbohydrate portions of CEA. More specifically, the blood group determinants of CEA which correspond to the blood group type of the individual from whom the CEA was isolated are recognized by anti-blood group antibodies. Although we did attempt to demonstrate which immunoglobulin class of antibodies was precipitating CEA in this study, it is generally recognized that IgG or IgM may precipitate the blood group substances A or Le\textsuperscript{a} (13, 14, 20).

A significant finding in this work is that there are irregular or unexpected antibodies to Le\textsuperscript{a} substances in cancer patients (irregular in the sense that greater anti-Lewis activity is observed in cancer patients than in controls). Irregular anti-Le\textsuperscript{a} antibodies were also found in the female control group of nonsmokers and in the smoker control group. The exposure of females to fetal antigens during pregnancies is probably responsible for the higher levels of anti-Le\textsuperscript{a} in this group. In one study of 200,000 blood group donors and patients, a significant number of women, 75% of whom were in the fertile age group, had irregular antibodies to Le\textsuperscript{a} (16).

The higher binding activity to CEA BP160 in the male smoker control group must be due to another reason. Although it is tempting to speculate that since smokers have higher serum levels of CEA they may produce an immune response to CEA, this explanation can be ruled out. This study shows no correlation between serum levels of CEA and the binding activity of the serum to CEA BP160 in the male smoker control group. It is more likely that smokers are being exposed to immature blood group substances as a result of tissue destruction and regeneration, or altered glycoconjugate biosynthesis. Although lung cancer patients showed statistically higher levels of anti-Le\textsuperscript{a} as measured by CEA BP160-binding activity, there was no difference between smokers and nonsmokers in that group. From these results, we decided to use the level of anti-Le\textsuperscript{a} obtained from male nonsmoker controls as "normal." Based on these normal levels, up to 23% of the cancer patients studied showed irregular antibodies to Le\textsuperscript{a}.

In the group of cancer patients, a correlation between CEA BP160-binding activity and serum levels was found only for the colorectal carcinoma patients. In addition, there is no possible relationship between CEA BP160-binding activity and the tumor burden, but insufficient data were obtained to give statistically significant results. Serial determinations of the CEA BP160-binding activity revealed an increase in binding activity for patients with poor prognosis even when undergoing radiation therapy and chemotherapy. In patients with remissions or no changes, a decrease of the binding was seen more often. This was well correlated with the alterations of the serum CEA levels for the individual patient.

In this assay, the CEA-binding activity measured depends on the amount of intrinsic CEA, added labeled CEA, CEA-binding immunoglobulins, and their affinities for intrinsic and added CEA. If the added CEA behaves immunologically like the intrinsic CEA, the measured CEA-binding activity would allow an estimation of the bound intrinsic CEA. Therefore, an attempt was made to calculate a value composed of both the information from CEA serum levels and CEA-binding activity. Using these values, it is possible to differentiate patients with metastases from patients without metastases versus controls, independent of smoking habit or sex. In studies on patients with bronchogenic carcinoma, the presence of immune complexes, measured as C1q binding activity, was linked to clinically evident disease with unfavorable prognosis, if found beyond the immediate postoperative period (8).

From our results, we conclude that the irregular anti-blood group antibodies as measured by CEA BP160-binding activity are likely to come from sensitization to embryonic proteins or incompletely biosynthesized blood group substances. This may occur in healthy females during pregnancies, in smokers through a chronic bronchial inflammation, and in cancer patients by exposure to tumor tissue. It should be noted that all CEA preparations studied by us expressed Le\textsuperscript{a} determinants and that some expressed ABO determinants (23). The presence of irregular blood group determinants on CEA makes CEA an excellent antigen to screen for irregular blood group antibodies such as Le\textsuperscript{a} in this study.

It still remains to be established whether the irregular antibodies, measured by this CEA binding assay, give early information regarding the development or the recurrence of a tumor. From the follow-up data, it seems that the increase of CEA-binding activity is a poor prognostic sign for the patient. The rise of CEA-binding activity before or without an increase of CEA serum levels may indicate tumor growth at an early stage. A further question is whether or not these antibodies influence the development of a malignant disease. As soluble immunocomplexes in the case of pregnant females, they should not have a pathological function. If the antibodies are fixed on tumor cells, they may either protect the tumor by blocking T-cell killing of the tumor cells (12, 24) or cause lysis of tumor cells by the action of complement fixation (4).

The original intention of these studies was to measure the levels of anti-CEA in cancer patients; however, there is no evidence at this time that the antibodies measured are specific to CEA. On the contrary, those patients whose sera bind CEA appear to do so only through the carbohydrate determinants of CEA. This observation together with the finding that various CEA's express different blood group determinants led to the finding that female controls, smokers, and a significant number of cancer patients express irregular antibodies to blood group substances. Since it is convenient to measure the levels of these antibodies with this CEA-PEG assay, it is likely that other anti-blood group antibodies may be measured using other radiolabeled glycoproteins. Based on our results, it is likely that following the levels of irregular antibodies to blood group determinants and perhaps other carbohydrate determinants may be useful in monitoring disease progress in certain types of cancer or other diseases.

REFERENCES


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