ABSTRACT

A monoclonal antibody to carcinoembryonic antigen (CEA) labeled with 111In (Indacea) was used to image tumors in patients with colorectal cancer. The anti-CEA antibody has a high affinity (2.6 × 10^10 M^-1) for CEA and does not cross-react with normal cross-reacting antigen, biliary glycoprotein-1, or tumor-extracted, CEA-related antigen. During the course of these studies, it was noted that a significant number of male patients (20 of 27, 74%) showed uptake of Indacea in the testes. In order to determine if the Indacea uptake was specific, 20 testicular specimens were analyzed by immunohistological methods using five different anti-CEA monoclonal antibodies recognizing five different epitopes on CEA. In 18 cases (90%) germ cells were uniformly stained by all five antibodies. Fresh frozen testis tissue was homogenized in water and precipitated with 1.0 M perchloric acid. The supernatant contained a CEA-like material as measured by an enzyme immunoassay specific for CEA. The same supernatant was radiolabeled with 111In and immunoprecipitated with anti-CEA monoclonal antibodies. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the immunoprecipitates revealed a single species (M, 180,000) which was indistinguishable from CEA. This study documents the first description of CEA in the germ cells of normal testis. The CEA in the testis was accessible to circulating monoclonal antibodies in the majority of male patients tested.

INTRODUCTION

CEA is a high-molecular-weight (M, 180,000) glycoprotein first identified in human fetal colon and carcinomas of the adult gastrointestinal tract by Gold and Freedman in 1965 (1). It is one of the best characterized tumor markers, and comprehensive reviews have been published in this area (2, 3). Production of MABs to CEA has led to several studies examining the ability of radiolabeled anti-CEA MAB to target colorectal tumors in nude mice bearing human xenografts (4-7) and in human patients (8-12). The focus of these studies has been the identification and localization of CEA-bearing tumors. Optimizing the uptake of radiolabeled antibody by the tumor and minimizing the concomitant uptake by the surrounding normal tissues have been the main strategy. However, in most cases, the uptake of radioactivity in normal tissues has been significant.

Early human studies used 111In to label polyclonal anti-CEA antibodies, but substantial radioactivity in the blood pool and other normal tissues necessitated the use of subtraction techniques (13-15). More recently, 111In has been bound to chelate-conjugated monoclonal antibodies against CEA and other tumor-associated antigens (12, 16-19). The advantages of this radiisotope have been discussed by Bradwell et al. (20). Uptake of monoclonal antibodies labeled with 111In by normal organs such as liver, spleen, kidney, bone, bladder, colon, and blood pool has been reported in animals bearing human tumor xenografts (5, 7). To image human colon carcinoma xenografts in nude mice, we used a high affinity anti-CEA MAB (21) which did not cross-react with other normal tissue CEA-like antigens (22). From the study reported in the companion paper (12), in which Indacea was used to image colorectal cancer patients, we noted that a significant number of male patients showed uptake of Indacea in the testes. This observation was not expected and prompted further examination of the tissue for possible presence of CEA-like material. Although CEA has been found in some testicular germ cell tumors, it has not previously been detected in normal testis (23-26). In this paper, we documented the existence of CEA in the germ cells of normal human testis.

MATERIALS AND METHODS

Preparation of Indacea and Immunoscintigraphy

The anti-CEA monoclonal antibody T84.66 (22) was conjugated with DTPA as described by Paxton et al. (16). The DTPA-conjugated anti-CEA monoclonal antibody was then labeled with 111In as outlined in the companion paper (12). The resultant preparation, "Indacea," was approved for human use (BB-IND-2014). Twenty-seven male patients with colorectal carcinoma that were scheduled to have surgery for diagnosis and/or treatment of their disease as previously described (12) received Indacea prior to operation.

Immunohistopatology

Tissue Preparation. Normal testicular tissue from 20 consecutive adult autopsy cases was selected. Portions of testes were fixed in 10% formalin or B5 fixative, embedded in Tissue Prep 2, and stained with hematoxylin: eosin for routine histological examination. The testicular tissue samples were free of hematological and nonhematological neoplastic processes and had sufficient germ cells for evaluation.

Preparation of the paraffin-embedded, fixed tissue for immunohistochemical studies has been previously described in detail (27). Our procedure can be summarized as follows. The sections were cut at 6 µm, and two sections were placed on one glass slide. After deparaffinization and rehydration, sections were studied with well-established immunohistochemical techniques.

Reagents. The primary antibodies in this study include five anti-CEA monoclonal antibodies which recognize five different epitopes on the CEA antigen (21, 22). The five monoclonal antibodies are designated as CEA.66, T84.1, CEA.41C, CEA.281, and T84.66. Biotinylated, affinity-purified, anti-mouse antibody and preformed complex of avidin and biotinylated horseradish peroxidase were obtained from Vector Laboratories, Burlingame, CA. 3-Amino-9-ethylcarbazole used as chromogen was obtained from Polysciences, Inc., Warrington, PA (28).

Immunohistochemical Techniques. A modification of the highly sensitive avidin:biotin complex technique (29) was used for evaluation of CEA reactivity with germ cells in testicular tissue samples. Our procedures are briefly summarized in the companion paper. As a positive control, ten histologically documented cases of colon adenocarcinoma were selected and studied in a fashion similar to the testis. A section was
considered to be CEA positive when germ cells showed distinctly positive staining of cytoplasm or membrane and cytoplasm, which made them easily distinguishable from the adjacent unstained cells or surrounding stroma. The intensity of immunostaining was graded as markedly positive (+++), moderately positive (++), mildly positive (+), or negative (−). The pattern of immunostaining of individual cells was classified as surface membrane or cytoplasmic, depending on the predominant localization of the stain.

**Tissue Extraction**

Testicular tissue was obtained from a postmortem examination of a patient who had previously shown a weakly positive testicular scan (Patient 21; Table 1). The tissue was flash-frozen and stored at −70°C until analyzed. The tissue was extracted using the method developed for the isolation of CEA (30). A portion of the tissue (3.9 g) was cut into small pieces and homogenized in 13.6 ml of ice-cold deionized water for 10 s using a Brinkmann Polytron PT 10/35 homogenizer equipped with a PTA 10 generator. An equal volume of ice-cold 2 M perchloric acid was added dropwise with stirring of the homogenate. Stirring was continued for 20 min after the addition of perchloric acid was completed. The precipitate was removed by centrifugation at 13,000 × g for 30 min in a Beckman centrifuge equipped with a JA20.1 rotor. The supernatant was dialyzed against frequent changes of deionized water at 4°C for 72 h and then lyophilized. The lyophilized testicular extract was dissolved in deionized water at 50 mg/ml, and insoluble material was removed by centrifugation for 2 min using a Beckman microfuge. The supernatant, which will be referred to as the testicular extract, was carefully removed and submitted for further analysis.

**Amino Acid Analysis**

The protein concentration and the glucosamine content of the testicular extract were determined by amino acid analysis on a Beckman 121MB analyzer by the method of Del Vallee and Shively (31). The sample was hydrolyzed in an evacuated tube for 48 h at 110°C with 6 N HCl containing 0.02% 2-mercaptoethanol.

**CEA Determination**

The CEA concentration of the testicular extraction was determined using the Roche Diagnostics CEA enzyme immunoassay kit (a gift from Roche Diagnostic Systems, Nutley, NJ). One of the two monoclonal antibodies in this kit is T84.66, the same MAB used in the immunohistology and one of the two MABs used in the immunoprecipitation.

**Radioiodination**

An aliquot of the testicular extract (5 μl) was mixed with an equal volume of deionized water and 10 μl of 1 M PB, pH 7.5. One mCi of Na125I (ICN Biomedicals) and 4 μg of chloramine T in 10 μl of 75 mM PB, pH 7.5, were added; the reaction was stopped after 90 s by the addition of 3 μg of Na2S2O5 in 10 μl of 75 mM PB, pH 7.5. Bovine serum albumin (200 μg) was added as carrier, and the solution was chromatographed on a 0.7- x 25-cm Sephadex G25 column equilibrated with phosphate-buffered saline. Fractions of 1 ml were collected, and the peak fractions (Nos. 5 to 7) of radioiodinated testicular extract were saved for immunoprecipitation. Highly purified CEA (10 μg) was radioiodinated using the same procedure.

**Immunoprecipitation**

Aliquots of iodinated CEA and iodinated testicular extract were added to 0.5 ml of bovine serum albumin (10 mg/ml) in 50 mM Tris:150 mM NaCl:1 mM EDTA:0.1% Nonidet P-40, pH 8.0. These solutions were agitated gently with 10 μl of mouse ascites fluid containing anti-CEA MAB T84.1 or anti-CEA MAB T84.66, or with 10 μl of normal mouse serum for 6 h at 4°C. Immune complexes were adsorbed with 100 μl of a 50% suspension of Protein A:Sepharose CL-4B beads with gentle agitation for 16 h at 4°C. The beads were washed twice with 0.8 ml of Buffer A (10 mM Tris:150 mM NaCl:0.5% Nonidet P-40, pH 8) containing 1 M LiCl and twice with 0.8 ml of Buffer A. Immune complexes were eluted from the beads by incubating with 100 μl of SDS:PAGE sample buffer (62 mM Tris:20% glycerol:2% SDS:0.005% bromophenol blue:5% 2-mercaptoethanol, pH 6.8) for 30 min at 25°C. The distribution of radioactivity for each immunoprecipitation reaction was determined by counting the nonbound fraction, the washes, the Protein A:Sepharose beads (before and after elution), and the eluted supernatant.

**SDS Electrophoresis and Autoradiography**

The eluted samples were heated for 5 min in boiling water and electrophoresed on a 10% polyacrylamide gel as described by Laemmli (32). The gel was stained with Coomassie Blue R250, destained, and dried on Whatman No. 1 filter paper. The dried gel was exposed to Kodak X-Omat film with two Cronex intensifying screens (DuPont) at −70°C for 48 h.

**RESULTS**

**Immunoscintigraphy.** Positive testicular scans were obtained in 20 of the 27 (74%) male patients studied. The intensity of the scan, which reflects the degree of Indacea uptake by the tissue, was variable (Fig. 1). Six patients showed a strongly positive scan (Fig. 1A), nine a moderately positive scan (Fig. 1B), and five a weakly positive scan (Fig. 1C). Intensity of testicular uptake did not correlate with that of other normal tissues such as blood, bone, liver, spleen, or kidney. Testicular uptake did decrease with time postinjection, with the 48-h scan showing a drop in radioactivity relative to the 24-h scan (Fig. 2). In all cases, it was found that the anterior image was stronger than the posterior, indicating that the activity was testicular uptake and not an artifact of the scan.

The age of the patients ranged from 38 to 86 yr, but no correlation was noted between age and intensity of testicular Indacea uptake. Similarly, there was no apparent relationship between the level of CEA in the serum and the degree of testicular imaging (Table 1).

The possibility that the negative testicular scans were related to previous chemotherapy was also examined (Table 1). Of the seven patients showing no testicular uptake of Indacea, three (43%) had been given chemotherapy prior to the scan. Two of these (Patients 7 and 26) received 5-fluorouracil i.v., and one (Patient 11) received FdUrd via a liver pump infusion. Four of the 20 patients (20%) showing a positive testis image had received previous chemotherapy. Three of these patients (Patients 5, 10, and 27) had their chemotherapy administered i.v., and one (Patient 8) received FdUrd via a liver pump. Two patients (Patients 26 and 27) had been given chemotherapy as recently as 3 to 4 wk prior to the Indacea injection. The other five had not received any treatment for 3 mo or longer before their scan. No statistically significant differences (Fisher's exact test) were obtained between the positive image group and the negative image group with respect to previous chemotherapy treatments. It was noted that all six patients showing a strongly positive scan had received no chemotherapy prior to the scan. Of the four positive image patients who had been given chemotherapy, two showed a weakly positive and two a moderately positive scan. The difference between these two groups was again not statistically significant (Fisher's exact test).

**Immunohistopathology.** Hematoxylin:eosin-stained sections from all 20 tests studied showed morphological features of essentially normal adult testis (Fig. 3A). Eighteen of the 20 testes studied showed variable immunoreactivity with all five monoclonal anti-CEA antibodies (90%). The immunostaining pattern in all the sections studied was
Carcinoembryonic Antigen in Normal Testis

Fig. 1. Patients received 0.2 mg of Indacea labeled with 2 mCi of \(^{111}\text{In}\) and were imaged at 48 h. Weak (A), moderate (B), and strong (C) uptake of Indacea in the testes is shown. G, gonad; B, bladder; T, tumor; L, liver; S, spleen; and O, osseous space.

Table 1. Age, serum CEA, and previous chemotherapy: effect on testicular imaging

<table>
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<tr>
<th>Patient</th>
<th>Age (yr)</th>
<th>Serum CEA (ng/ml)</th>
<th>Previous chemotherapy</th>
<th>Intensity of testicular image at 48 h</th>
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<td>++</td>
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* $++$, strong; ++, moderate; +, weak; –, no uptake.
* Chemotherapy i.v.
* Chemotherapy via hepatic pump infusion.

Immunoprecipitation and SDS Electrophoresis. \(^{125}\text{I}\)-labeled CEA and testicular extract were immunoprecipitated with two anti-CEA MABs and two normal mouse sera. Anti-CEA MAB T84.1 precipitated 42% and anti-CEA MAB T84.66 precipitated 3.4% of the \(^{125}\text{I}\)-CEA in their respective immunoprecipitation reactions. The decreased binding by T84.66 was due in part to radioiodination damage of the antigenic site on CEA (21). In addition, the stringent binding and washing conditions used in the experiment may be less favorable for T84.66 compared to T84.1. The two normal mouse sera bound less than 0.1% of the \(^{125}\text{I}\)-CEA. SDS:PAGE of \(^{125}\text{I}\)-CEA, both before and after immunoprecipitation with the anti-CEA MABs, revealed...
Fig. 4. SDS-polyacrylamide gel electrophoresis and autoradiography of radiolabeled CEA and testicular extract. ¹²⁵I-labeled CEA or testicular extract was immunoprecipitated with different antibodies and electrophoresed as described in "Materials and Methods." The samples were ¹²⁵I-labeled CEA before immunoprecipitation (Lane A) or after immunoprecipitation with anti-CEA T84.1 (Lane B), anti-CEA T84.66 (Lane C), and normal mouse serum (Lanes D and E). ¹²⁵I-labeled testicular extract is shown before immunoprecipitation (Lane F) or after immunoprecipitation with anti-CEA T84.1 (Lane G), anti-CEA T84.66 (Lane H), and normal mouse serum (Lanes I and J). Values along the ordinate, molecular weight (x 10⁻³).

a broad band with a molecular weight of 180,000 (Fig. 4, Lanes A to C). ¹²⁵I-CEA was not visible in the normal mouse sera immunoprecipitates (Fig. 4, Lanes D and E).

The amount of ¹²⁵I-testicular extract precipitated by anti-CEA MABs T84.1 and T84.66 was less than 0.1% of the total radioactivity in each immunoprecipitation reaction. The result is not surprising, considering that CEA represented 0.022% of the total protein of the testicular extract.

Despite the small amount of radioactivity bound by the anti-CEA MABs, the immunoprecipitates were analyzed by SDS:PAGE. Fig. 4, Lane F, shows the ¹²⁵I-testicular extract before immunoprecipitation. The majority of the radioactivity is associated with two proteins with molecular weights of 43,000 and 30,000. In addition, a large amount of radioactivity traveled with the dye front. Anti-CEA MAB T84.1 (Fig. 4, Lane G) precipitated a M, 180,000 protein that migrates exactly like CEA (Lanes A to C). No specific proteins were precipitated by anti-CEA MAB T84.66 (Fig. 4, Lane H) or the normal mouse sera (Lanes I and J).

DISCUSSION

CEA was originally thought to be associated only with colonic tumors. However, elevated plasma levels of CEA have subsequently been found in numerous malignancies of epithelial origin such as breast, lung, ovary, pancreas, and stomach. In addition, several benign disorders, mainly inflammatory in nature, can also give rise to elevated levels of CEA in the plasma. As more sensitive immunoassays have developed, it has been shown that certain normal tissues and fluids associated with the digestive tract such as colon, saliva, gastric juice, and feces also contain CEA (2).

CEA is still regarded as a good marker for colorectal carcinoma, and radioimaging of colorectal tumors utilizing antibodies to CEA labeled with ¹²⁵I, ¹²⁳I, or ¹¹¹In has stimulated much interest. Using radioactive iodine, subtraction techniques were found necessary to improve tumor images due to substantial
uptake of label in normal tissues (13–15). Uptake of radiolabeled anti-CEA antibodies by normal tissues has also been reported by groups using $^{111}$In (10, 12, 17–19). Strategies such as transaxial tomoscopy (8, 9), antibody fragments (6), and antibody cocktails (11) have been tried in order to diminish the level of the antigen obtained by the perchloric acid method of purification was 46 ng/g of testicular tissue, compared to levels of 10 to 200 µg/g obtained from liver metastases (2). Immuno-precipitation of this antigen using T84.1 followed by SDS/PAGE revealed a single protein with a molecular weight of 180,000. This protein was indistinguishable from CEA by SDS/PAGE, which further supports the claim that the antigen found in normal testis is CEA. Failure of immuno-precipitation of testicular CEA using T84.66 is presumably due to the modification of the specific epitope on CEA by the radioiodination of the antigen as noted with CEA extracted from tumors.

Since CEA was identified in the germ cells by histochemical and immunochemical methods, the possibility that the 7 patients who didn’t show testicular uptake may have had their germ cells compromised by previous chemotherapy was examined. Chemotherapy can result in cytotoxic damage and loss of germinal epithelium in the testis (34). Our results indicated, however, that in this study there was no significant correlation between prior exposure to chemotherapy and the ability of Indacea to localize in the testis. The intensity of the positive images was variable from patient to patient, suggesting that the amount of accessible CEA in the germ cells was variable. The patient (Patient 21; Table 1) whose testicular tissue was used for the CEA extraction showed a weak (+) testis image and weak positive immunoperoxidase staining, and the level of testicular CEA was 46 ng/g of tissue. It appears that the negative images, therefore, may reflect CEA levels too low to be detected by immunoscintigraphy. Other factors known to adversely affect testicular function, such as chronic disease, malnutrition, and metabolic abnormalities may also have contributed to the negative images.

The fact that the testes could be demonstrated on the CEA scan in a significant proportion of the male patients challenges a current concept of the accessibility of testicular germ cells to circulating drugs. It has been suggested that the testes may be “sanctuary organs” with a “blood-gonad” barrier similar to that of the blood-brain barrier (35, 36). In this paper we have shown that the testis contains CEA which is accessible to circulating antibodies and can be imaged by Indacea immunoscintigraphy.

REFERENCES

Carcinoembryonic Antigen in Normal Testis


Testis Imaging with $^{111}$In-labeled Anticarcinoembryonic Antigen Monoclonal Antibody: Identification of Carcinoembryonic Antigen in Normal Germ Cells
