[CANCER RESEARCH 36, 3260-3264, September 1976]

Identification and Characterization of a Circulating Tumor-associated Oncofetal Protein from a Radiation-induced Adenocarcinoma of the Rat Small Bowel[1]

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SUMMARY

A tumor-associated protein from the cellular membranes of a radiation-induced rat small bowel adenocarcinoma was identified, found to be serologically unaltered in the circulatory system, and was observed to be susceptible to acid hydrolysis. The immunochemical reactivity was unchanged by heat, alkali, or neuraminidase digestion. The protein appeared to be a single immunologically active species, but it was structurally composed of a heterogeneous group of proteins.

INTRODUCTION

CEA[3] was isolated in 1965 by Gold and Freedman (7) from spontaneously occurring human adenocarcinoma of the colon. The antigen has since been characterized as an oncofetal glycoprotein localized at the glycocalyx of the neoplastic cells (8). It has also been detected in the circulatory system of individuals with colon carcinoma; thus, it is believed that the antigen arises from the turnover of the membrane carbohydrate layer (4). The biological importance of such antigen "shedding" is clear if it is accepted that the circulating antigens would compete for the immune reactions that otherwise could lead to the destruction of the tumor *in situ* (1). Cells of colon adenocarcinoma are not unique in their ability to release antigens, since both chemically induced hepatomas (2) and sarcomatas (15) have also been observed to release soluble antigens.

In a previous communication, it was demonstrated that a tumor-associated protein existed in tissue derived from X-irradiation-induced adenocarcinoma in the small bowel of rats (13). The protein was observed to be immunologically identical in every lesion studied and to exist at the tumor membranes. It was also tested for fetal characteristics and found to share common antigenic determinants with a protein existing in 17- to 19-day-old rat embryos. Since the protein was not detected in either normal tissues or the lungs, spleen, liver, kidneys, large bowel, urine, and feces of animals possessing the tumors, it was considered to be an oncofetal protein. Additional studies, reported here, establish the existence of the tumor-associated protein in the circulatory system of animals possessing the carcinoma and describe some characteristics of the unique protein. Evidence is offered that such a protein occurs in detectable quantities and represents a phenomenon that occurs during the development of radiation-induced intestinal adenocarcinoma in 3 strains of rats.

[1] This work was supported by the National Cancer Institute Research Contract NOI-CB-43891.

[2] Supported by NIH Training Grant IT32CA09125-01.

[3] The abbreviations used are: CEA, carcinoembryonic antigen; LBN, Lewis Brown Norway rats.

Received July 17, 1975; accepted May 25, 1976.

MATERIALS AND METHODS

Animals and Tumors. Intestinal adenocarcinomas were induced in 3 strains of rats: (*a*) Holtzman, obtained from the Holtzman Co., Madison, Wis.; (*b*) LBN, from Microbiological Associates, Inc., Walkersville, Md.; and (*c*) Buffalo, from the Simonsen Co., Gilroy, Calif.

Tumor induction in the rat small bowel was effected by X-irradiation of the hypoxic, temporarily exteriorized ileum and jejunum as previously described (3).

Tumor-associated Protein Isolation. The neoplastic cellular membranes were isolated by discontinuous centrifugation (13). Membrane proteins were solubilized in 0.1% sodium dodecyl sulfate in 0.05 M Tris-HCl (pH 7.4) buffer for 18 hr at 30°. The detergent solution was applied to a 90- x 1.5-cm column packed with preequilibrated Sephadex G-200. Elution was carried out with 0.05 M Tris-HCl (pH 7.4) buffer and 2-ml fractions were collected utilizing a flow rate of 8.0 ml/hr. Antigenic activity was assayed by the Mancini (11) single radial diffusion technique, and the protein concentration was determined by absorption at 280 nm.

Membrane proteins were iodinated by the technique of Hunter and Greenwood (10). Approximately 5 μg protein of the cellular membranes in 10 μl of distilled water were added to 2 mCi $Na^{125}I$ in 50 μl of 0.05 M phosphate buffer at pH 7.5. Then 50 μl of chloramine T (3.5 mg/ml in phosphate buffer) were added and the iodination was allowed to continue for 2 min. The ^{125}I-radiolabeled membranes were applied to the Sephadex G-200 column both in the presence and in the absence of the 0.1% sodium dodecyl sulfate and eluted as previously described. The iodinated proteins were also mixed with 50% sucrose in 0.1 M Tris-borate (pH 9.2) buffer and subjected to electrophoresis on 0.5- x 100-mm polyacrylamide gel columns (total acrylamide T, 5.2%; cross-linking with *N*,*N*'-methylenebisacrylamide C, 3.8%) cast in 0.025 M Tris-borate (pH 9.2) buffer. Polymerization was catalyzed by 0.025% ammonium persulfate supported by 0.5% tetramethylenediamine. Electrophoresis was conducted, utilizing 2 ma/gel in a 0.025 M Tris-borate (pH 9.2)

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buffer at 20° until 0.05% bromophenol blue tracking dye migrated to the end of the gel. The gels were sliced in 2-mm sections, and the iodinated proteins were eluted into 0.05 M Tris-HCl (pH 7.4) buffer. The immunologically active protein was identified by its ability to bind to tumor antiserum absorbed with normal components as previously described (13). The assay consisted of 40 to 50 x 10^3 cpm of ^{125}I-labeled protein in 125 µl of 0.05 M Tris-HCl (pH 7.4) buffer containing 25 µl of antiserum. The reaction was allowed to continue for 18 hr at 4°, and then 20 µl of human IgG (10 mg/ml) Cohen II fraction and 180 µl of saturated ammonium sulfate were added. Precipitation of the protein-antibody complex by this Farr technique (5) was allowed to proceed for 1 hr at 4°, after which separation of the unbound antigen from the antibody-bound fraction was achieved by centrifugation at 500 x *g* for 5 min.

Heat extraction of the tumor-associated protein was accomplished through heating either tumor tissue homogenates, the cellular membranes, or 20 ml of sera from tumor-bearing animals diluted (1:1) with distilled water at a temperature of 100° for 20 min. The insoluble components were precipitated by centrifugation at 800 x *g* for 10 min. The supernatant was lyophilized to dryness, and the resulting material was dissolved in 200 µl distilled water for assay.

Immunoassays. The antisera utilized in these studies were prepared in male New Zealand White rabbits and were made specific to the oncofetal protein by our previously described methods (13). Microdouble diffusions were measured in 1% Noble agar (12), immunoelectrophoresis in 0.05 M Veronal (pH 8.6; Γ/2, 0.1)-buffered 1% Noble agar (9), and Mancini single radial diffusions in 1% Noble agar containing absorbed antiserum (11).

RESULTS

During a 6- to 8-month postirradiation observation period, some interstrain differences in size of tumor and frequency of occurrence were noted. In general, over 50% of the Holtzman rats had large lesions (6 to 10 g) by 8 months, 10% of the LBN animals had tumors varying from 2 to 10 g, and 50% of the Buffalo strain developed small neoplasms (1 to 2 g) during the same time period.

To date, tumor-associated protein isolated from individual tumors taken from 50 Holtzman, 7 LBN, and 7 Buffalo rats has been studied by Ouchterlony analysis and immunoelectrophoresis (Fig. 1). Interanimal and interstrain immunological identity has been demonstrated.

An acid-soluble protein sharing some common antigenic determinants with those possessed by the tumor-associated protein was isolated by the acid extraction (13) of pooled rat serum (Fig. 2). The isolation of a serum protein possessing identical serological determinants was accomplished by heating a pool from tumor-bearing animals to 100° for 15 min followed by precipitation of the insoluble proteins and concentration of the supernatant (Fig. 2). Heat stability of the tumor-associated protein was also established through comparisons of the immunological activity of the protein existing both in the tumor homogenate and at the cellular membranes after heating to 100° for 20 min with that of the activity possessed by the protein not subjected to these conditions (Fig. 2).

The immunological activity of the tumor-associated protein was observed to be acid labile with all detectable activity by Ouchterlony analysis (12) lost through incubation of the protein in 0.1 N HCl for 20 min at 30°. However, in contrast, an incubation in 0.1 N NaOH for this period did not appear to alter the serological activity. Both endo- and exopeptidases eliminated discernible immunological activity of the protein, while neither nucleotidases nor neuraminidase affected the activity (Table 1).

Solubilization of the cellular membranes of the tumor tissue was accomplished with sodium dodecyl sulfate. When the components were separated according to molecular weight on a molecular exclusion column, there was evidence for antigenic activity in at least 6 different peaks as indicated by Mancini single radial diffusion analysis (Chart 1). An analogous broad range of activity was observed when the ^{125}I-radiolabeled membranes were solubilized, eluted under identical conditions, and assayed by the binding of the radioactive components to a specific antiserum generated to the tumor oncofetal protein (Chart 2). However,

Table 1

Enzyme effects on the serological integrity of the tumor-associated protein

Serologically active Holtzman rat small bowel adenocarcinoma homogenate fractions (500 µg) were incubated at 37° for 3 hr with (Sigma Chemical Co., St. Louis, Mo.) enzymes having the following units: peptidase, 6; protease (VI), 800; trypsin (XI), 10,000; catalase, 3600; papain, 150; deoxyribonuclease (I), 2000; RNase (X-A), 1000; hyaluronidase (VI), 3000; and neuraminidase (VI), 4.

Enzyme	Serological reactivity
Peptidase	–
Protease	–
Trypsin	–
Catalase	–
Papain	+
DNase	+
RNase	+
Hyaluronidase	–
Neuraminidase	+

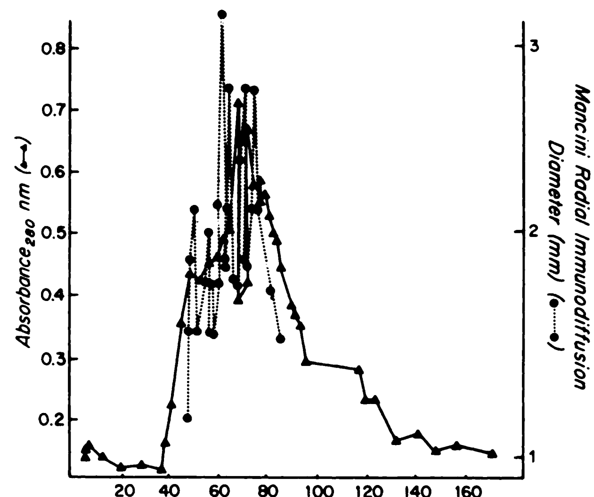

Chart 1. Molecular exclusion chromatograph with Sephadex G-200 of sodium dodecyl sulfate-solubilized cellular membranes from Holtzman rat small bowel adenocarcinoma. *X-axis*, fraction number of 2-ml aliquots collected.

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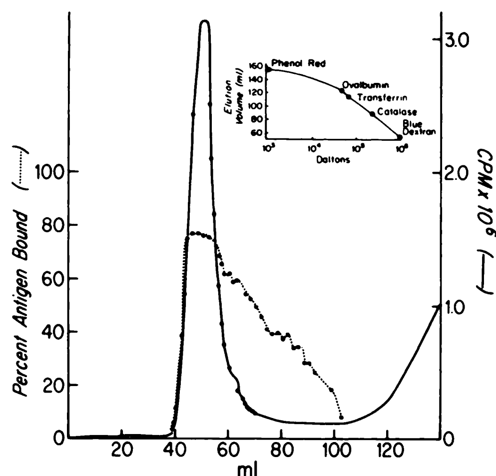

Chart 2. Molecular exclusion chromatograph with Sephadex G-200 of sodium dodecyl sulfate-solubilized ^{125}I-radiolabeled cellular membranes from Holtzman rat small bowel adenocarcinoma. Column prestandardization was accomplished with the following compounds: phenol red, 354; ovalbumin, 43,000; transferrin, 74,000; catalase, 232,000; blue dextran, 2,000,000 daltons.

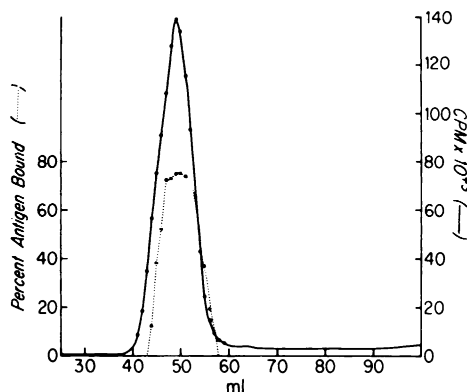

Chart 3. Molecular exclusion chromatograph with Sephadex G-200 of nonsolubilized ^{125}I-radiolabeled cellular membranes from Holtzman rat small bowel adenocarcinoma.

when the ^{125}I-tagged membranes were not initially solubilized with the detergent, a single immunologically active peak was observed (Chart 3).

The cellular membrane proteins were tagged with covalently bound ^{125}I and were separated by polyacrylamide gel electrophoresis. After electrophoresis, the gels were sliced into 2-mm sections and the protein eluted into buffer. Total radioactivity of the slices indicated that there were at least 9 regions where various proteins were tagged. Immunological measurements indicated a single broad region of activity with relative mobility of 0.12 to 0.18 (Chart 4). The activity of this region was observed to be susceptible to radiation damage, because antibody-binding capacity was severely reduced with the storage of the radiolabeled protein in buffer at 4°. Complete loss of activity was observed after 3 to 4 weeks. Accompanying the loss of antibody binding capacity was the formation of a high-molecular-weight species that has not yet been identified. Separation of the antibody-bound radiolabeled proteins from the unbound ones was possible utilizing 50% saturation ammonium sulfate (Farr technique), because the immunologically active proteins

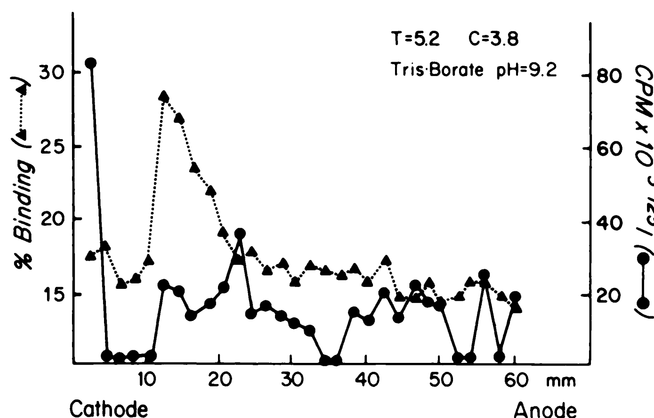

Chart 4. The polyacrylamide gel electrophoresis of ^{125}I-labeled proteins occurring in the cellular membrane fraction of Holtzman rat smal bowel adenocarcinoma.

were found to be soluble in 100% saturated ammonium sulfate.

DISCUSSION

The induction of small bowel adenocarcinoma in the rat by local X-irradiation apparently triggers a synthetic mechanism for the formation of a membrane-localized protein for the neoplastic cells that develop. We reported in an earlier communication that this protein was immunologically identical to a protein existing in 17- to 19-day-old rat embryos. The induction of an immunologically identical protein occurred in individual tumors of all 3 strains of rats studied. Such proteins were not detected in irradiated animals that failed to develop the tumor or in any other tissue or excretable products of the animals possessing tumors. However, it must be recognized that failure to detect the protein may be simply the lack of a sufficiently sensitive assay, but certainly it is clear from the results that a quantitative increase in the protein concentration parallels the progressive development of the neoplasm. The acid lability of the tumor-associated protein was unexpected, since characteristics of the protein reported both in this report and an earlier communication (13) suggested it was much like CEA. A quite common isolation technique for CEA involves a dilute perchloric acid extraction (14); however, in utilizing this method, partial hydrolysis of some of the antigenic determinants of the radiation-induced tumor-associated protein occurred. Complete hydrolysis and loss of all immunological activity were observed when 0.1 N HCl was substituted for the perchloric acid. In contrast, incubation of the protein in 0.1 N NaOH had no observable effect on the immunological activity.

The susceptibility of the immunological activity of the protein to both endo- and exopeptidase emphasizes the importance of the intact protein moiety for binding to the rabbit antibody generated against it. The failure of neuraminidase to affect the serological activity indicated that sialic acid residues had no detectable influence on the serological activity.

The heat stability of the tumor-associated protein offered a characteristic that could be utilized for its extraction. The

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method presented a quick and convenient means to isolate in an unaltered form the protein from the other tumor cellular or serum components. This heat stability suggested that the antigenically active form of the protein was the most stable of all the possible molecular conformations.

Serologically, the protein from the adenocarcinoma appeared to exist as a single species (13). The chromatograph of the non-detergent-solubilized ^{125}I-radiolabeled membrane components tended to support this conclusion with the presence of a single high-molecular-weight fraction having immunological activity (Chart 3). However, the molecular exclusion chromatograph of the detergent-solubilized components (Chart 2) and the polyacrylamide electrophoresis (Chart 4) suggest that what appears to be a single, immunologically active protein may actually be a heterogeneous population of proteins, all of which contain the common antigenic determinants. Existence of such a population would explain the broad β-globulin precipitin bands previously observed in the immunoelectrophoresis of the protein (13). Whether this protein population is heterogeneous structurally or represents a different degree of dissociation of monomers remains to be determined.

These studies indicate that the tumor-associated protein in the X-irradiation-induced adenocarcinoma cell is released and exists in an unaltered immunologically active form in the serum of tumor-bearing animals. The investigations suggest that the single, serologically active protein may actually be composed of a heterogeneous population of structurally different proteins that possess the common antigenic determinants. The biological role of the protein(s) remains to be elucidated. Studies are presently underway to determine whether they have a function in the animal's immune response to the neoplastic tissue.

ACKNOWLEDGMENTS

The authors are indebted to Dr. K. L. Coop, Los Alamos Scientific Laboratory, and Dr. J. G. Sharp, University of Nebraska, for their many helpful suggestions, and to N. Hug, L. Wightman, F. Kirchner, and J. Peiffer for expert technical assistance.

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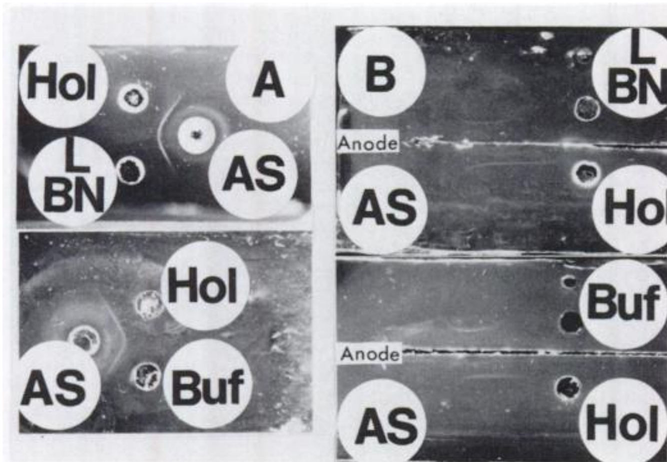

Fig. 1. Immunological analysis of the tumor-associated protein occurring in the rat small bowel adenocarcinoma. *A*, Ouchterlony analysis; *B*, immunoelectrophoretic analysis; *Hol*, Holtzman (38 μg); *LBN*, Lewis Brown Norway (5.3 μg); *Buf*, Buffalo (1.4 μg); *AS*, absorbed rabbit antiserum generated against the tumor-associated protein occurring in the Holtzman rat small bowel adenocarcinoma.

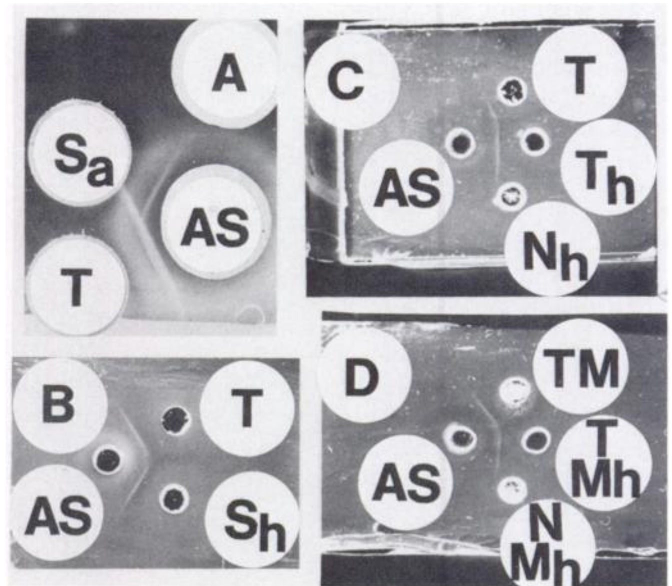

Fig. 2. Ouchterlony analysis of the Holtzman rat tumor-associated protein. Comparisons between the cellular protein with the serum protein extracted by the perchloric acid method (*A*) and heating technique (*B*). Heat stability of the tumor-associated protein was demonstrated in the total cellular homogenate (*C*) and cellular membrane fractions (*D*). *AS*, absorbed rabbit antisera generated against the Holtzman rat small bowel adenocarcinoma; *N*, normal cellular homogenate (38 μg); N_h, heated normal cellular homogenate (38 μg); NM_h, heated normal cellular membrane homogenate (38 μg); S_a, perchloric acid serum extract (5 μg); S_h, heated tumor serum extract (10 μg); *T*, tumor cellular homogenate (23 μg); T_h, heated tumor cellular homogenate (23 μg); *TM*, tumor cellular membranes (30 μg); TM_h, heated tumor cellular membranes (30 μg).

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