Tumor Antigens
II. Immunological Studies of Tissue and Tumor Antigens and Serum Protein Production by Murine Interstitial Cell Tumors of the Testis and a Hepatoma[1] (34175)

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In an earlier communication (1) it was reported that the presence of a specific soluble antigen could be detected in significantly larger quantities in a transplantable mouse rhabdomyosarcoma than in the isogeneic normal skeletal muscle. In contrast, tumor antigens were not detected in a transplantable mouse mammary adenocarcinoma. The serum proteins produced by both tumors and their corresponding normal tissues were also studied.

The present communication describes the results of studies with two additional murine transplantable tumors, the interstitial cell tumor (ICT) of the testis and a hepatoma with the same objectives, to demonstrate tumor specific antigens and to study the differences in serum protein production by normal and neoplastic tissue, using the technique of radioimmunoelectrophoresis (2).

Deckers *et al.* (3), in a study of soluble antigens in tumor extracts of hepatomas using the technique of double diffusion, found a decrease in organ specific liver antigens and, in some cases, the appearance of a new antigen apparently specific for the hepatoma. More recently, tumor antigens have been detected in a diethylnitrosamine-induced transplantable hepatoma of recent origin by delayed hypersensitivity (4). Soluble antigens have been reported in bovine testes (5). However, there are no studies to date on the detection of tumor antigens in murine interstitial cell tumors.

Materials and Methods. Tumors. Four lines of interstitial cell tumors of the testis were obtained from Dr. W. U. Gardner of Yale University School of Medicine. They are designated as 36 Balb/c, 14LC, 17AA, 15 Balb/c. (i) The 36 Balb/c arose spontaneously in a Balb/c male mouse. During these experiments the tumor was in transplant generations 21 and 22. (ii) The 14LC arose in a F_1 hybrid mouse (C_{57}BlxBalb/c) that had received stilbesterol, 250 mg, and which bore a pituitary gland transplant in the testis. This tumor grows best in females and regresses after hypophysectomy. Hosts bearing tumors in transplant generation 17 were received. (iii) The 17AA arose in a stilbesterol treated Balb/c male mouse. The tumor bearing animals were females. The transplant generation was unknown. (iv) The 15 Balb/c, a pituitary-dependent, androgen-producing tumor, arose in a stilbesterol treated Balb/c male mouse. During these experiments, it was in its transplant generation 27.

BW 7756 Hepatoma. This tumor, obtained from Jackson Memorial Laboratories, Bar Harbor, Me., arose spontaneously in a C_{57}L/J mouse. It was palpable in 2 weeks and killed the host in 4–8 weeks. During these experiments the tumor was in its transplant generation 162.

All tumors were maintained in passage by subcutaneous grafts in hosts of the strain of origin by means of a 22-gauge needle.

Preparation and Absorption of Specific Antisera. Specific antisera to each of the four

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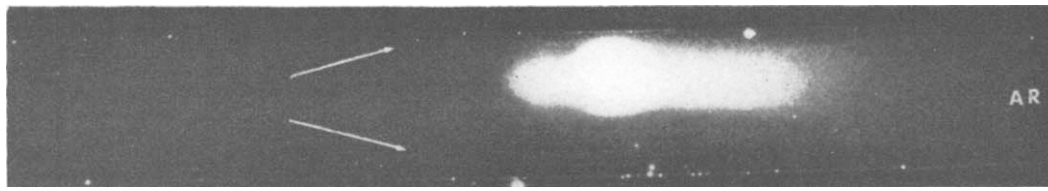

FIG. 1. Autoradiograph (AR) of immunoelectrophoretic (IE) pattern prepared with culture fluid obtained from ICT, 14LC, and specific antiserum to the tumor absorbed with normal serum. A labeled arc, in the γ region, remained after absorption.

testicular tumors, the hepatoma and the corresponding normal tissues were prepared in individual adult male New Zealand white rabbits weighing 2.0 kg. The animals were immunized by 4 weekly injections of a suspension of lyophilized tumor or normal tissue dissolved in 2.5 ml of 0.15 *M* saline and mixed with an equal volume of incomplete Freund's adjuvant. The injections were divided between the subcutaneous route and the four foot pads. Normal testis served as the control for the testicular tumors. In the text, a specific absorbed antitumor antiserum refers to one that has been absorbed with normal serum from isologous hosts.

Absorptions of rabbit antisera against mouse tumors or tissues were performed with 0.1-ml aliquots of fresh isologous mouse serum or 50-mg aliquots of a mixture of lyophilized mouse liver and spleen. The absorptions were performed in order to absorb out normal components and to identify specific components. Ten to 12 additions were usually necessary in each case.

Cultures and Analysis of Culture Fluids. Tissues or tumors to be cultured were removed under sterile conditions. After mincing, approximately 100 mg (wet wt) of each of the specimens was cultured in each of 6–10 roller tubes for 24–48 hr at 37° in 2 ml of modified minimal essential medium (Grand Island Biological Co., Grand Island, New York) to which uniformly labeled ^{14}C-L-lysine and ^{14}C-L-isoleucine (1 μc/ml each) had previously been added (2). After the culture period, the culture fluids were dialyzed against 0.015 *M* phosphate buffer, pH 7.2, for 48 hr. They were then lyophilized and redissolved with 0.1 or 0.15 ml of distilled water. For immunoelectrophoresis (IE), the culture fluids were added to the antigen well three times and then developed with the appropriately absorbed and unabsorbed antisera in order to detect specific precipitation arcs. The ^{14}C-labeled mouse proteins present in the culture fluids were thus coprecipitated in the corresponding IE lines. The labeled lines on the film strip were identified by comparing these lines with those on the IE slide. A labeled line indicates the synthesis of this protein *in vitro*. The slides were stained with amido black subsequent to autoradiography. For clarity in the text, an absorbed antiserum refers to one that has been absorbed with normal serum from isogeneic animals.

Examination of Sera. Mice bearing tumors were bled from the retro-orbital plexus 8–10 days after tumor grafting. The sera were stored at −20° until analyzed by microimmunoelectrophoresis according to the method of Scheidegger (6). The sera were added to the antigen well three times and developed with rabbit antimouse whole sera (Hyland

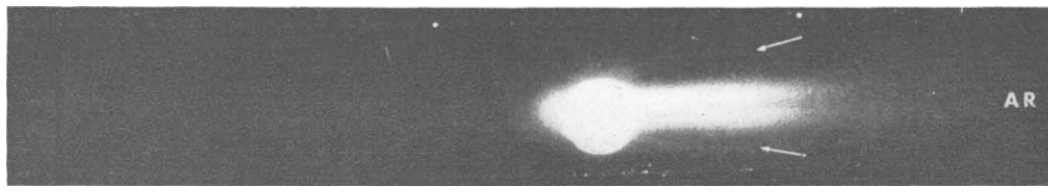

FIG. 2. Autoradiograph (AR) of immunoelectrophoretic (IE) pattern prepared with culture fluid from ICT, 15Balb/c and specific absorbed antiserum. The labeled arc is in the α region.

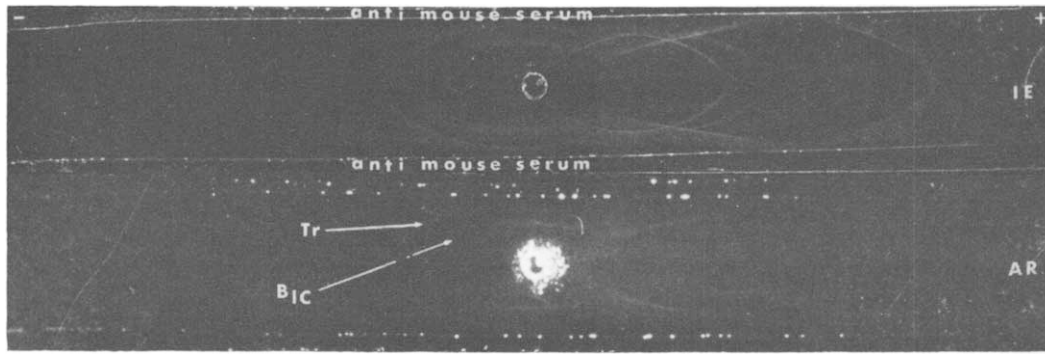

FIG. 3. Autoradiograph (AR) of immunoelectrophoretic patterns (IE) prepared with culture fluid obtained from ICT, 14LC, and developed with a mouse serum carrier and anti-whole mouse serum. Labeling of B_{1C} and Tr is present.

Laboratories, Los Angeles, California). The immunoelectrophoretic diagrams were stained with amido black 10B according to the method of Uriel and Scheidegger (7). The double diffusion method was that of Ouchterlony (8).

Results. Interstitial Cell Tumors. Three of the four ICT's examined originated in stilbesterol-treated mice. A soluble antigen could be detected in culture fluids from two of the three when developed with the specific absorbed antitumor antisera. A labeled arc, with γ-mobility, remained when the culture fluid from the 14LC tumor line was developed with its specific absorbed antitumor antiserum (Fig. 1). With the tumor line, 15 Balb/c, a labeled arc was present in the α region (Fig. 2). However, when examined by double diffusion, the specific absorbed antiserum to tumor 15 Balb/c gave a precipitation line with culture fluids from tumor 14LC, while the absorbed antitumor antisera to 14LC did not give any precipitation reaction with culture fluids obtained from the 15 Balb/c tumors. When culture fluids from both of these tumors were developed with their specific antitumor antisera absorbed with testes from isologous hosts, the labeling was removed. The culture fluids obtained from each of the four tumors or from the normal testis did not show labeling when developed with the antisera to the normal testis.

All four varieties of ICT's labeled B_{1C} and in addition, tumor lines 36 Balb/c, 14LC, 15 Balb/c labeled transferrin (Tr). In general, the labeling of Tr, when present, was weak. The 14LC tumor line showed the strongest labeling of B_{1C} (Fig. 3). The normal testis labeled only B_{1C}, the intensity of which was

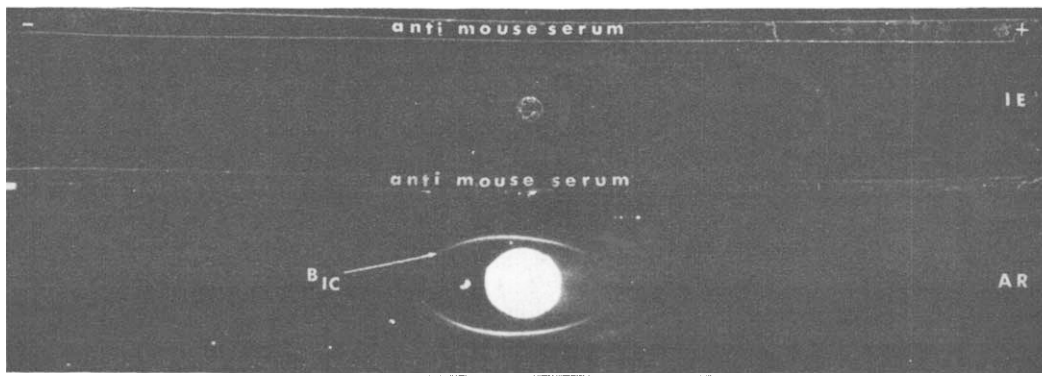

FIG. 4. Autoradiograph (AR) of immunoelectrophoretic patterns (IE) prepared with culture fluid obtained with normal testes from Balb/c mice developed with a mouse serum carrier and anti-whole mouse serum. Strong labeling of B_{1C} is present.

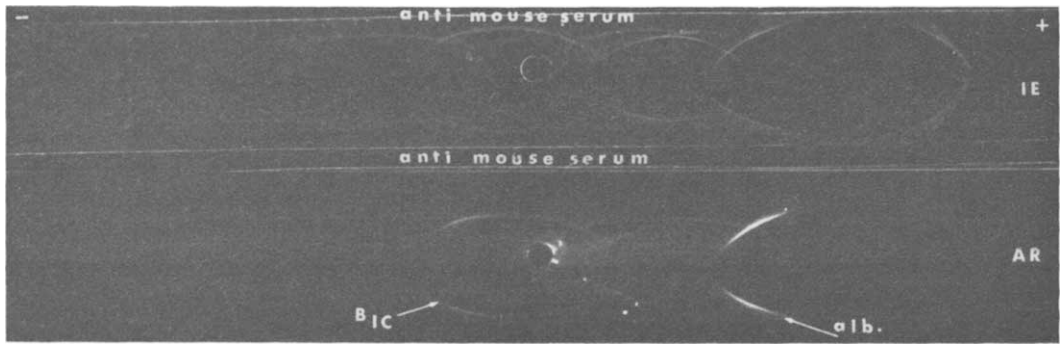

FIG. 5. Autoradiograph (AR) of immunoelectrophoretic patterns (IE) prepared with culture fluid from hepatoma BW7756. A mouse serum carrier and anti-whole mouse serum were used to develop the pattern. Labeling of albumin (alb.) and B_{1C} is present.

stronger than that seen with any of the tumor lines (Fig. 4).

Histological examination of these tumors showed a rather uniform population of densely packed epithelial cells with large vesicular nuclei containing course chromatin granules and prominent nucleoli surrounded by a moderate amount of basophilic cytoplasm with indistinct cell borders. Small collections of lymphocytes were present in each tumor line.

Hepatoma. A soluble antigen could not be detected with culture fluids from the hepatoma or normal liver.

When developed with a mouse serum carrier and anti-whole mouse serum, culture fluids from the hepatoma showed strong labeling of B_{1C} (C'3) and albumin but did not label immune globulins or Tr (Fig. 5). The results obtained with culture fluids from the tumors and normal tissues, are summarized in Table I.

On histological examination, this tumor was noted to be composed of large round or oval cells with extremely prominent hyperchromatic nuclei containing frequent mitotic figures, multiple nucleoli and clumped chromatin granules. There was an abundance of granular eosinophilic cytoplasm surrounded by distinct cell borders. The tumor cells closely resembled normal liver cord cells. A minimal diffuse chronic inflammatory infiltrate was noted.

Sera obtained 8–10 days after transplantation from several animals bearing the four varieties of ICT's and the hepatoma failed to reveal any detectable differences in the immunoelectrophoretic diagrams when compared with the patterns from nontumor bearing isologous hosts.

Discussion. Soluble antigens with γ and α mobility, respectively, were present in two of

TABLE I. Specific Antigens and Serum Protein Production by Tumors and Tissues *in Vitro.*

Tumor or tissue	Strain of origin	Method of induction	Soluble tissue or tumor antigen	Serum protein production
Interstitial cell tumors				
36Balb/c	Balb/c	Spontaneous	—	B_{1C}, Tr
14LC	$C_{57}Bl/6 \times$ Balb/c	Stilbesterol	+	B_{1C}, Tr
17AA	Balb/c	Stilbesterol	—	B_{1C}, Tr
15Balb/c	Balb/c	Stilbesterol	+	B_{1C}
Testis	Balb/c		—	B_{1C}
Hepatoma, BW7756	$C_{57}L/J$	Spontaneous	—	Albumin, B_{1C}
Liver				Albumin, Tr, B_{1C} [Ref. (10)]

the four interstitial cell tumors as showed by radioimmunoelectrophoresis. Both of these tumor lines originated in estrogen treated Balb/c or (Balb/c × A) F_1 hybrid mice. The fact that the antiserum to 15 Balb/c had antibody to 14LC suggests that the antigens found in these two tumors might possibly be the same but with different mobilities. A soluble antigen was not detected in culture fluids obtained from the normal testes when developed with antiserum to the latter. However, when the antisera raised to these two tumors were absorbed with lyophilized testes, the labeling was removed. This suggests that an antigen was present in the testes but in concentrations too low to be detected by direct precipitation.

Of interest was the fact that all varieties of ICT's examined labeled B_{1C}. Three of the four showed very weak labeling of Tr. None of the tumors labeled immune globulins. The labeling is presumably due to the presence of infiltrating host cells. Although these findings differ from those of Stecher and Thorbecke (9), who were unable to demonstrate any serum protein production *in vitro* by a Leydig cell line, it should be pointed out that Leydig cells comprise only a small proportion of the total cell population of the normally functioning adult testis.

It is known that there is very active synthesis of Tr, albumin and B_{1C} in livers from normal mice and that stimulation by infection can enhance the synthesizing activity of the liver. However, the synthesis of B_{1C} by the liver is influenced by the age of the animals, in mice as well as in other species, and shows a constant change of pattern (10).

The hepatoma, BW7756, was derived from a C'5 active strain, $C_{57}L/J$ (11–13) and histologically consisted of well differentiated parenchymal cells. Although labeling of Tr and immune globulins was not shown, labeling of albumin and B_{1C} was seen. The labeling of the latter is consistent with the minimal lymphocytic infilatrate that was observed histologically in the tumor. It was previously reported (14) that the same culture fluids developed with serum from $C_{57}Bl/6$ mice as a carrier and a specific antiserum prepared in mice against $C_{57}Bl$ serum showed labeling of the MuBl precipitation arc, known to be associated with C'5 activity. However, liver obtained from A (C'5 deficient) and $C_{57}Bl/6$ (C'5 active) strains of mice did not show labeling of the MuBl globulin.

Stecher and Thorbecke (9) found that TAPER tumor cells (a transplantable mouse hepatoma) when taken from subcutaneous nodules labeled B_{1C}, Tr, and γ-globulin, but when grown in the peritoneal cavity did not produce serum proteins. The TAPER tumor is a spontaneous mouse tumor, the cells of which do not resemble normal mouse liver, but has been classified as a hepatoma predominantly because of its localization solely within the liver. The labeling of serum proteins *in vitro* by subcutaneous nodules from the TAPER tumor is in accord with the present findings in which active serum protein production *in vitro* was demonstrated by the transplantable mouse hepatoma BW 7756. The same authors found that a cell line (H_4-II-EC_3) derived from rat hepatoma, H_{35} was extremely active in the production of various rat serum proteins, including albumin, B_{1C} and Tr (9). The absence of immune globulin production by these cells is typical.

To some extent this study differs from others (15–18) in that a specific antigen could not be detected in this particular variety of hepatoma. This could be in part due to the fact that genetic changes occurred in the tumor itself or in the mouse strain of origin, as a result of many generations in passage.

Some transplantable mouse and rat hepatomas synthesize a specific α-globulin, designated αf (17). This antigen was found to be a normal constituent of embryonic and newborn mouse sera which disappears from the same with age and reappears in the serum of adult hepatoma-bearing mice as a result of its synthesis by the tumor tissue and its secretion into the circulation. It is not present in normal liver and other organs or the serum of normal adult mice. In the present study, if such an antigen was being produced by the tumor, the antitumor antiserum did not have antibody to it. There were no detectable differences in the serum immunoelectrophoretic

or Ouchterlony patterns produced by tumor bearing and normal isologous hosts. However, no attempt was made to specifically identify this antigen.

Summary. Several murine ICT's and a murine hepatoma were studied for soluble antigens and differences in serum protein production *in vitro* by the tumors as compared to their normal counterparts. The technique used was that of radioimmunoelectrophoresis. A soluble antigen was not found in culture fluids obtained from the hepatoma but was present in two of the four ICT's. Its concentration in the normal testes was significantly less than that of the tumors. All of the ICT's synthesized B_{1C} but the labeling was not as prominent as that obtained with culture fluids obtained from the normal testes. Three of the four varieties also produced Tr. The labeling of Tr and B_{1C} by these tumors and tissues is believed to be due to the presence of infiltrating host cells. The hepatoma was found to synthesize albumin and B_{1C}.

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