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Differential Association of Fetal Antigen with Hepatoma Tissue Grown *In Vivo* and *In Vitro**

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Abstract—*Production of two distinct fetal antigens, alpha-fetoprotein (AFP) and γ-fetal antigen (γ-FA) was associated with growth of the BW7756 mouse hepatoma* in vivo. *Synthesis of AFP, but not γ-FA, continued during* in vitro *propagation of the tumor cells. After re-inoculation of these cultured hepatoma cells into inbred mice, both AFP and γ-FA could be detected again in the growing tumor tissue and in the sera of tumor-bearing mice. It is evident that different growth conditions effect synthesis of these two tumor-associated antigens.*

INTRODUCTION

MALIGNANT transformation of mammalian cells is frequently accompanied by various cellular alterations that include the re-expression of embryonal antigens [1–3]. Hepatomas are particularly interesting in this regard since certain cell lines derived therefrom synthesize alpha-fetoprotein (AFP) while retaining particular differentiated hepatocyte functions [4–5]. Indeed, it has been postulated that hepatocellular carcinoma may arise from proliferating de-differentiated parenchymal cells in which the maturation pathway has been blocked [6]. Neoplasia may be viewed, therefore, as a disease of cellular differentiation [7].

As part of a study of the antigenic composition of tumor cells, we examined the extent of de-differentiation in a transplantable murine hepatoma as measured by fetal antigen production. Growth of this neoplasm *in vivo* was found to be associated with the synthesis of both AFP and a late-gestational neo-natal phase antigen (γ-fetal antigen; γ-FA). Unlike AFP which was synthesized by hepatoma cells *in vivo* as well as *in vitro*, γ-FA could not be detected in tumor cells grown *in vitro*, but it was re-expressed in the resulting tumor tissue and serum of mice inoculated with cultured hepatoma cells.

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MATERIALS AND METHODS

1. *Experimental animals and tumor line*

The BW7756 hepatoma (Jackson Laboratories, Bar Harbor, Maine) was maintained in a subcutaneous transplantation passage in C57L/J male mice. Fetuses, obtained from primiparous C57L mice, were dissected free of placentae and fetal membranes and washed in phosphate-buffered saline (PBS) to remove amniotic fluid proteins. Time of conception was determined by vaginal plug observations [8]. Homogenates of whole fetal and neonatal mice were prepared in PBS and the 5000 *g* supernatant obtained [9]. Extracts of tumor were similarly derived from homogenates of fresh tissue.

2. *Preparation of anti-mouse alpha-fetoprotein*

New Zealand white rabbits were immunized with 0.5 ml of day 18 fetal mouse serum

emulsified with 0.5 ml of complete Freund's adjuvant according to a previously described schedule [9]. Anti-fetal mouse serum was absorbed by addition of an equal volume of adult mouse serum, the mixture incubated at 24°C (2 hr), 4°C (1 hr), and the supernatant collected after centrifugation at 5000 ***g*** for 20 min at 4°C. Specificity of the absorbed antiserum (anti-AFP) was determined by agar double-diffusion and immunoelectrophoresis. Electrophoresis of 10 μl samples of day 18 fetal and adult mouse sera was carried out at 20 mA/200 V for 45 min on 7.5 × 3.8 cm glass slides coated with 5 ml of 1% agarose (w/v), pH 8.6, using Beckman B-2 barbiturate buffer. Antisera were then added and the precipitin pattern allowed to develop at 37°C for 24 hr. Mouse alpha-fetoprotein was isolated from crude fetal homogenates by alternating molecular sieve chromatography on Sephadex G-100 and G-200 followed by ion-exchange chromatography on DEAE-Sephadex A-25 [10, 11]. The 5000 ***g*** homogenate supernatant of day 16.5–19.0 fetal mice [9] was dialyzed against 10^{-3}M Tris–HCl, pH 7.2, clarified by centrifugation at 5000 ***g*** and chromatographed on Sephadex G-100. The approximately 70,000 dalton region was pooled, lyophilized, and reconstituted in 10^{-3}M Tris–HCl for chromatography on Sephadex G-200. Those fractions corresponding to a mol. wt of approximately 70,000 daltons were again pooled and aliquots subjected to ion-exchange chromatography on DEAE-Sephadex A-25 using a 0.01–0.3 M NaCl gradient [10]. Several peaks obtained were found to contain only AFP as determined by immunochemical tests (manuscript in preparation). Antiserum to one AFP-containing ion-exchange fraction, isolate 5, was prepared in New Zealand white rabbits using the immunization protocol described previously [9]. To assure specificity for AFP, this antiserum was absorbed with adult mouse serum as described above.

3. *Anti-γ-fetal antigen*

Preparation of a rabbit antiserum to a late-gestation neo-natal phase mouse antigen (γ-fetal antigen; γ-FA), and its identification as a tumor-associated fetal antigen, have been described [12].

4. *Single radial immunodiffusion*

Radial immunodiffusion plates [13] contained 50 μl of antiserum per 2.5 ml of 1% agarose (w/v) in Beckman B-2 buffer; sodium azide was added as a preservative to a final concentration of 0.1%. Antigen wells, 3.7 mm in diameter, were filled with 10 μl of whole-body saline extract adjusted to 1 mg protein/ml of PBS [14]. Immunodiffusion plates were developed for 72 hr at 37°C before precipitin ring diameters were measured. The radial immunodiffusion unit used for antigen quantitation is defined as the $(\text{dia})^2$ of the resultant precipitin disc minus the $(\text{dia})^2$ of the antigen well per microgram of protein assayed [12].

5. *Tissue culture*

A transplanted BW7756 tumor, approximately 1 cm in diameter, was removed from a 10-week old C57L/J male mouse, rinsed in sterile PBS, and the necrotic area dissected away. The fresh, healthy tumor tissue was minced and fragments placed into 60 mm Petri dishes containing 5 ml of Ham's F-12 medium supplemented with 20% fetal bovine serum for incubation at 37°C in a humid 95% air: 5% CO_2 atmosphere. Tissue fragments and media were removed by aspiration 3 days after initiation of culture and the remaining adhering cells were washed with PBS and 5 ml of F-12 medium containing 15% fetal bovine serum, 10^{-6}M dexamethasone, and 2.5 γ/ml fungizone was added to each culture; a second medium change was made 4 days later. After 7 additional days of growth, the cells were dispersed with 0.1% trypsin-0.02% versene and thenceforth maintained in Petri dish culture with passage every 7 days.

6. *Indirect immunofluorescence*

Coverslips from 75% confluent cell culture were rinsed in PBS, the cells fixed in 100% methanol for 30 min at 24°C, and stored at −20°C. Cells were re-hydrated in PBS prior to use in the indirect immunofluorescence test [15]. Primary rabbit antisera and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Miles Laboratories, Lot no. DS291), were absorbed with fetal bovine serum as follows: 0.8 ml of PBS and 0.1 ml of fetal bovine serum were added to 0.1 ml of primary antisera or FITC-conjugated IgG fraction of goat anti-rabbit IgG, the mixture incubated at 37°C for 1 hr then at 4°C for 1 hr, centrifuged at 3000 ***g*** for 15 min and the supernatant diluted to 3 ml in PBS (final antisera dilution = 1 : 30). After incubation, the coverslips were mounted in 50% glycerol/PBS, pH 7.5, and examined by ultraviolet light microscopy.

7. *Membrane fluorescence*

In vitro passage 9 BW7756 cells, from a 75% confluent culture, were brought into suspension upon treatment with 0.02% versene in PBS and washed by three consecutive cycles of centrifugation and re-suspension in PBS. The final cell pellet (approximately 1×10^6 cells) was resuspended in 0.3 ml of serum-free growth medium. Antiserum and control rabbit serum for membrane fluorescence were prepared as follows: 0.3 ml aliquots of 15% fetal bovine serum-supplemented F-12 medium were added to each 0.2 ml aliquot of anti-AFP, or normal rabbit serum, incubated at 56°C for 1 hr, then centrifuged at 3000 ***g*** for 15 min and the supernatant used without further dilution. The FITC-conjugated IgG fraction of goat anti-rabbit IgG was similarly absorbed. For immunofluorescence tests, 0.1 ml aliquots of cell suspension were added to 0.1 ml of primary serum, incubated for 60 min at 24°C and the cells washed by three successive cycles of centrifugation and re-suspension in serum-free growth medium. The final cell pellets were brought up in the FITC-conjugated secondary antibody and incubated once again for 60 min. After the final series of washes, the cells were mounted in 50% glycerol/PBS for examination in ultraviolet light.

8. *Double-diffusion tests*

Agar double-diffusion tests of various sera and tissue extracts were done on pattern "D" Hyland Immuno-Plates (Hyland Laboratories, Costa Mesa, California). Antigen concentrations and antisera dilutions were as indicated in the text. Precipitation patterns were allowed to develop for 72 hr at 37°C before staining with Amidoschwarz 10B [16].

9. *Assay of culture fluids for γ-fetal antigen*

Culture fluid was collected from semi-confluent and confluent BW7756 cultures and replaced with serum-free growth medium. After 24 hr, the medium was removed, clarified by centrifugation at 10,000 ***g*** for 15 min, and both the serum-supplemented and serum-free culture supernatants concentrated by lyophilization for antigen determination.

RESULTS

The late gestational neo-natal phase antigen (γ-FA), which we previously identified in fetal and neoplastic mouse tissue [12], and AFP proved indeed to be two distinct antigens. Both showed unique concentration profiles, relative to total body saline-extractable protein, during pre- and post-natal life (Fig. 1). Moreover, the electro-

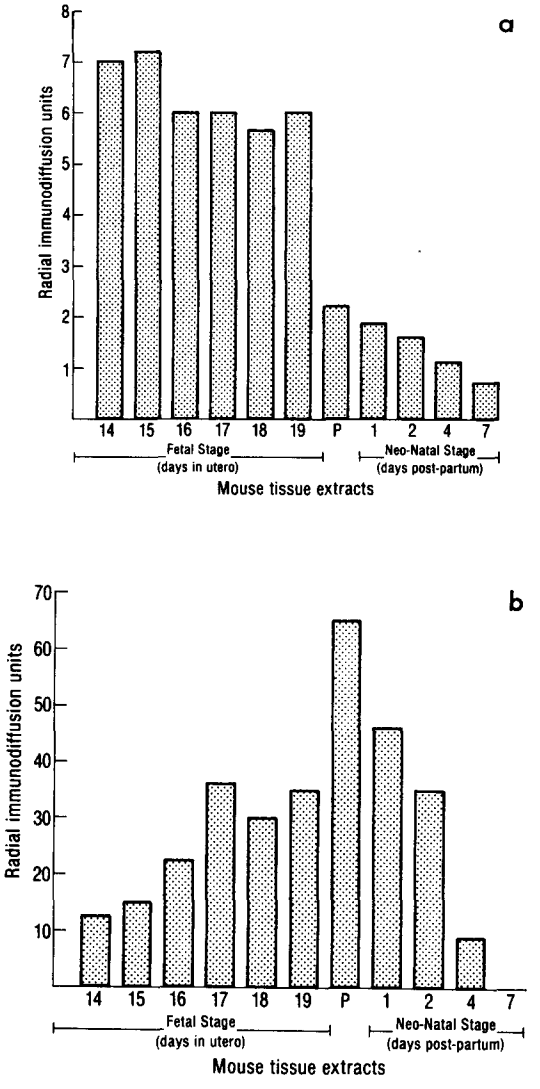

Fig. 1. Concentration profiles of alpha-fetoprotein (a) and γ-fetal antigen (b) as a function of total body saline-extractable protein in the late-gestational and early post-natal mouse as determined by single radial immunodiffusion; P indicates parturition.

phoretic mobilities of AFP and γ-FA were different; AFP possessed α migration (Fig. 2) while the latter antigen was clearly γ in mobility [12].

Both AFP and γ-FA could be detected in extracts of BW7756 tumor tissue, as well as in the sera of tumor-bearing mice (Fig. 3). The serum concentrations of both antigens increased concomitantly with increased tumor size (data not shown). Liver extracts and sera of normal C57L/J mice were negative in parallel assays even when tested at con-

centrations 6–8-fold greater than those which yielded positive tests for tissue derived from tumor-bearing animals.

Preliminary experiments showed γ-FA to be soluble in 10^{-3}M Tris–HCl, pH 7.5, and that the ability of this antigen to react with specific antiserum was retained upon heating of the tumor extract to 56°C for 30 min.

Growth of hepatoma cells in culture resulted in the appearance of monolayer aggregates of epithelioid cells interdispersed by occasional fibroblasts. AFP, but not γ-FA, could be identified in the cytoplasm of 60–80% of the epithelioid cells by indirect immunofluorescence test of *in vitro* grown hepatoma-derived tumor cells (Fig. 4). Fibroblasts in these same cultures were consistently negative when tested for both antigens. AFP could also be found on the surface of 60% of cultured hepatoma cells brought into suspension with versene (Fig. 4). Immunoprecipitable AFP could be identified in concentrates of BW7756 cell culture fluids (20X and 50X for serum-containing and serum-free media, respectively) by double-diffusion test; parallel assays on these same concentrates failed to disclose the presence of γ-FA.

Although γ-fetal antigen was not demonstrable in cultured hepatoma cells by immunofluorescence test or in concentrates of culture supernatant fluids, subcutaneous inoculation of 2×10^6 cultured hepatoma cells into C57L/J mice resulted in tumors from which both AFP and γ-FA could again be extracted (Fig. 5).

DISCUSSION

The BW7756 hepatoma and cell lines derived therefrom display a considerable spectrum of differentiated hepatic functions [4, 5]. This tumor, however, has been largely neglected in immunological studies relating to the neoplastic process. Several reasons which may account for this include that it is derived from a spontaneous liver tumor of uncertain etiology and that it induces only a weak humoral immune response upon transplantation in syngeneic animals (unpublished observations).

The production of alpha-fetoprotein (AFP) and a late gestational neonatal phase antigen (γ-FA) was associated with growth of BW7756 hepatoma cells *in vivo*. The latter antigen had been originally observed in the tumor tissue of a transplantable 3-methylcholanthrene-induced mouse fibrosarcoma [12]. This is of particular interest since rat fibrosarcoma and hepatic tumors, induced with 3-methylcholanthrene and 4-dimethylaminoazobenzene respectively, have also been found to share common embryonic antigens [17]. The mechanisms underlying "spontaneous" and chemical oncogenesis in fibroblastic and epithelial cell types may therefore be related. AFP was also detected on the surface of *in vitro* propagated hepatoma cells in confirmation of previous reports concerning the occasional cell surface localization of this protein [18–19]. AFP has been implicated to be immunosuppressive [20–21] under the appropriate conditions, although this has recently been disputed [22–23]. Should AFP be immunosuppressive, the presence of this protein on the surface of tumor cells which secrete it may facilitate their growth.

The production of γ-FA by mouse hepatoma cells ceased upon *in vitro* propagation but could be detected again in extracts of tumors which resulted from inoculation of cultured hepatoma cells and in the sera of tumor-bearing animals. This suggests that γ-FA is either produced by the tumor itself or reflects a host response to the growth of this particular neoplasm. Both AFP and γ-fetal antigen are consistently associated with *in vivo* proliferation of the BW7756 tumor. It remains to be ascertained whether they function, either synergistically or independently, in the initiation and/or maintenance of the malignant phenotype.

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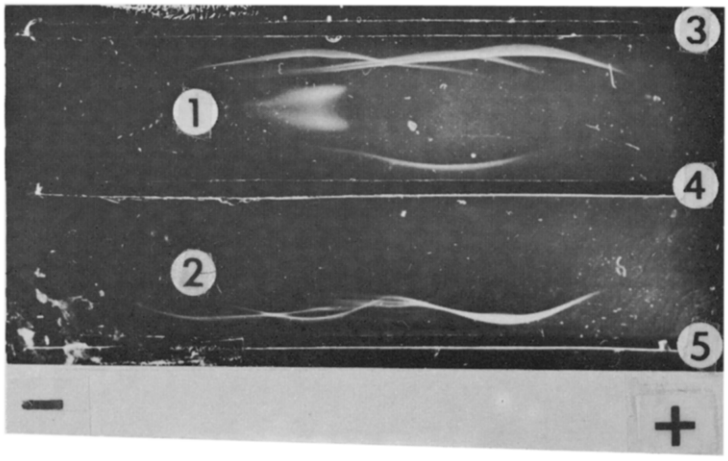

Fig. 2. *Determination of the specificity of anti-AFP serum by immunoelectrophoresis. Designations are as follows:* 1. day 18 *fetal mouse serum* (10 μl, 50 mg *protein*/ml): 2. *adult mouse serum* (10 μl, 50 mg *protein*/ml); 3. *antiserum to* day 18 *fetal mouse whole serum* (10 μl, *undiluted*); 4. *anti-AFP* (10 μl, *undiluted*); 5. *anti-adult mouse whole serum* (10 μl, *undiluted*).

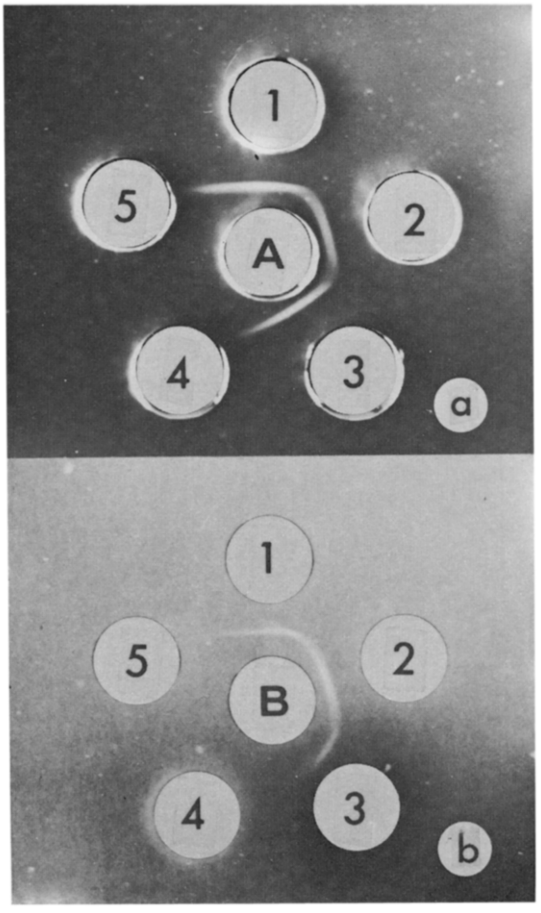

Fig. 3. *Detection of AFP* (*a*) *and γ-fetal antigen* (*b*) *in extracts of BW*7756 *mouse hepatoma and fetal mouse tissue and in the sera of tumor-bearing mice by agar double-diffusion. Peripheral wells contained* 10 μl *of a* 10 mg *protein*/ml *solution of:* 1. *extract of* day 18 *fetal mouse tissue;* 2. *extract of BW*7756 *hepatoma;* 3. *serum of hepatoma-bearing C*57*L*/*J mouse;* 4. *serum of normal C*57*L*/*J mouse; and* 5. *extract of normal C*57*L*/*J liver tissue. Central wells contained* 10 μl *of A. anti-AFP* (*undiluted*) *or B. anti-γ* FA (*diluted* 1:10).

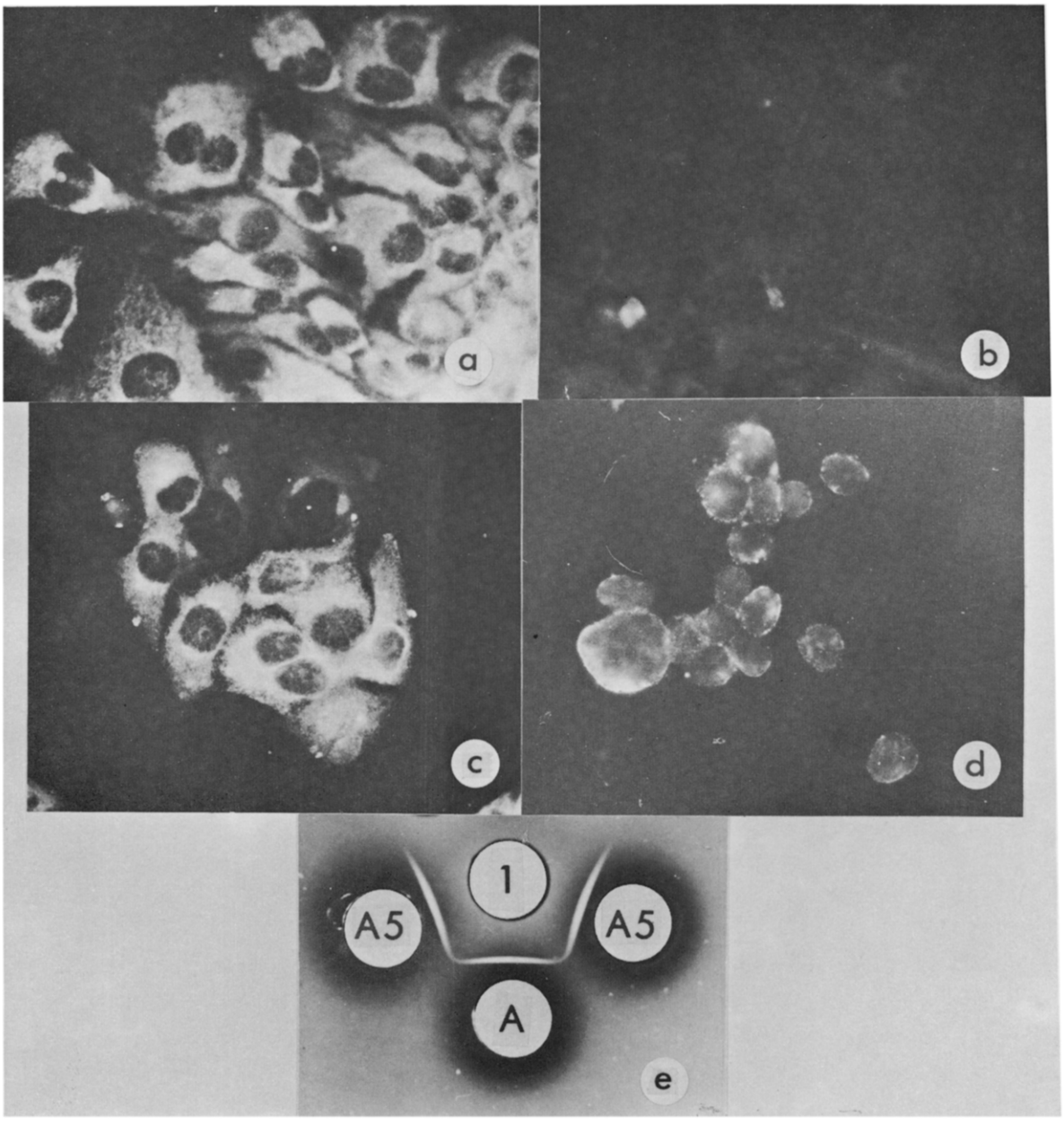

Fig. 4. *Positive indirect immunofluorescence test for cytoplasmic AFP in methanol-fixed cultured mouse hepatoma cells as detected with anti-AFP* (*a*). *The cells grown on a second coverslip from this same culture were negative for γ-fetal antigen* (*b*). *Specificity of the staining reaction for AFP is demonstrated in* (*c*) *using antisera prepared to chromatographically-purified AFP* (*isolate* 5). *AFP was also identified on the surface of* in vitro *passage* 9 *hepatoma cells brought into suspension with versene* (*d*); *a–d*, *X*330, *u.v. optics. Control experiments in which pre-immunization rabbit serum was used as the primary antibody source were negative in each case. The precipitin line of identity which formed between anti-AFP* (*A*) *and anti-isolate* 5 (*A*5) *sera against* day 18 *fetal mouse serum* (1) *attests to the specificity of anti-isolate* 5 *serum for AFP* (*e*). *Spurs were not in evidence even after staining of the precipitin pattern with Amidoschwarz* 10*B*.

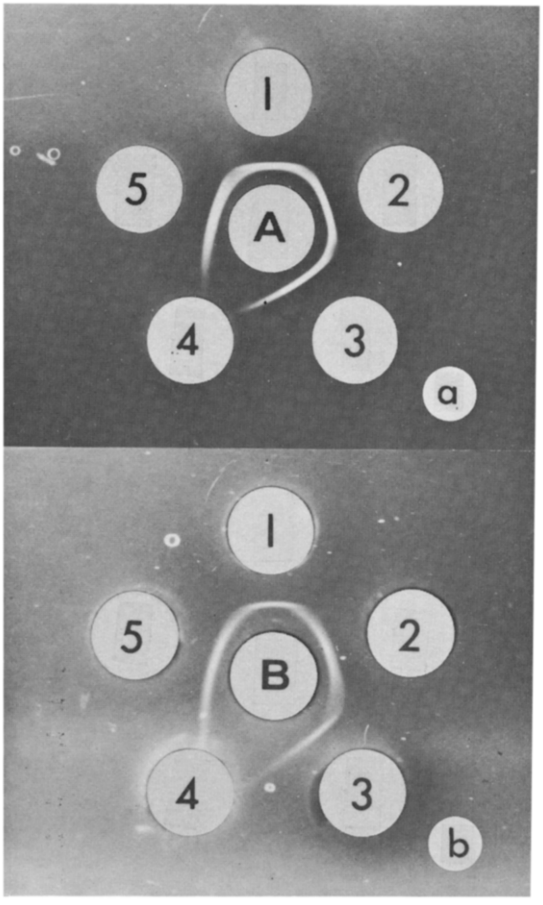

Fig. 5. Detection of AFP (a) and γ-fetal antigen (b) in tumor tissue and serum of a mouse inoculated with BW7756 hepatoma-derived cells which had been maintained in vitro *for 9 continuous passages. Peripheral wells contained 10 μl of a 10* mg *protein/*ml *solution of the following: 1. extract of* day *18 fetal mouse tissue; 2. extract of BW 7756 hepatoma tissue derived from tumors maintained on* in vivo *passage; 3. serum of C57L/J mouse carrying tumor produced upon inoculation of hepatoma-derived tissue-cultured cells; 4. normal C57L/J mouse serum; 5. extract of tumor produced upon inoculation of* in vitro *passage 9 hepatoma cells into a C57L/J mouse. A. anti-AFP (undiluted) or B. anti-γ FA (1:10) were placed in the central wells.*

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