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Identification of a Tumor-Specific Antigen in the Insoluble Fraction of Human Nephroblastoma. (Preliminary Communication)

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Abstract—*The insoluble fraction of nephroblastomas has been solubilized by the aid of desoxycholate and the soluble product, after partial purification, used to raise antisera in guinea pigs. After suitable absorption, the antisera were reactive in gel diffusion only with nephroblastoma fractions and sera from three patients with nephroblastoma. No reactivity was observed with the fractions of normal adult and fetal organs, as well as a variety of tumors of the other histologic types tested, or to sera from 30 patients with non-malignant diseases. The antigen was shown distinct from previously described antigens. It was therefore concluded that at least one tumor-specific antigen was present in the insoluble fraction of nephroblastomas.*

INTRODUCTION

A NUMBER of reports have been documented in recent years to show the existence of tumor-associated antigens in variety of human tumors. With respect to nephroblastoma (Wilms' tumor), the immune responses directed against specific antigens by the tumor-bearing individual have been demonstrated by a variety of immunological techniques [1–7]. Other evidences for the presence of tumor-associated antigen(s) in nephroblastomas were provided by Allerton *et al.* [8], Burtin and Gendron [9], Beierle *et al.* [10], Wise *et al.* [11] and Waghe and Kumar [12]. However, much still remains unclear about the specific antigens associated with this tumor.

Using methods of extraction and solubilization employed for the organ-specific antigens [13, 14] we have undertaken the characterization of antigens in the insoluble fraction of nephroblastomas. The soluble product obtained from the insoluble fraction, after partial purification by gel filtration, was used for the production of antisera by immunization of guinea pigs. After suitable absorption, the antisera were shown reactive with the fractions of nephroblastomas and also against the sera of certain patients with nephroblastoma, in contrast with an absence of reactivity with the fractions of normal adult and fetal organs, as well as a variety of other tumors and against sera from patients with non-cancerous diseases. Therefore it was concluded that at least one tumor-specific antigen (nephroblastoma-specific antigen, or NPSA) was present in the insoluble fraction of nephroblastomas. We report here also some physicochemical characteristics of this antigen.

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MATERIALS AND METHODS

Tissues

Three nephroblastomas (W_1, W_2, W_3) were obtained after surgery. The histological appearance of W_1–W_3 was type 3b, 1 and 4. [15], respectively. W_3 was small and obtained at a time when the immunodiffusion studies had been completed and was thus used only in immunofluorescence experiments. Samples of tumors of the other histologic types were obtained from autopsy. After washing with saline, necrotic tissue and area of hemorrhage were removed by gross dissection. Normal adult organs were obtained at autopsy from several patients with non-malignant diseases. Fetal organs were obtained from normal fetuses at the second trimester of gestation. All tissues were stored at $-20°C$ until used.

Extraction procedure

The cellular insoluble lipoprotein fraction

of each tissue was prepared by the method of Smith *et al.* [16], with modifications, as follows. The freeze–thawed tissue was cut in small pieces and homogenized in a Waring blendor with three volumes of "Solution I" (0.16 M potassium chloride, containing 186 mg of iodoacetic acid per liter and 5 g of sodium citrate per liter) for 10 min at 4°C. The homogenate was strained successively through 1, 2, 4 and 8 layers of gauze and centrifuged at 10,000 ***g*** for 30 min. All centrifugations were carried out at 4°C with a refrigerated centrifuge. The supernatant (S) was saved while the residue was homogenized for 5 min in a teflon glass homogenizer with about 10 vol of Solution I and the homogenate was centrifuged at 10,000 ***g*** for 30 min. The supernatant was decanted, and the residue was homogenized in about 10 vol of "Solution II" (1 M potassium chloride, containing 186 mg of iodoacetic acid per liter, adjusted to pH 4.7 by adding concentrated hydrochloric acid) and centrifuged at 10,000 ***g*** for 30 min. Subsequently the homogenization of the residue with Solution II and the centrifugation at 10,000 ***g*** for 30 min were repeated 7 times. When the residue was extremely viscous, the homogenization was carried out by a Waring blendor. The pellet thus obtained, called LP, was stored in glass-stoppered bottles at −20°C until studied. Approximately 20 g (wet weight) of LP were obtained from a segment of W_1 (*ca.* 150 g, wet weight).

A portion of each LP preparation was homogenized in a teflon glass homogenizer with 3–5 vol of 0.2% sodium desoxycholate until a creamy homogenate was obtained. The homogenate was then stirred rapidly without bubbling for 48 hr at 4–8°C and centrifuged at 10,000 ***g*** for 30 min. To the supernatant (S_1) about 10 vol of cold acetone were added and the mixture was left overnight at −20°C while the residue (R_1) was submitted to re-extraction with desoxycholate in the same manner as described above. The resulting precipitate was isolated by low-speed centrifugation, exposed to air stream until there is little odor of acetone, and immediately dissolved in a small amount of distilled water to produce a clear or faintly opalescent solution. An aliquot of the solution was fractionated by column chromatography on Sepharose 4B at 4–8°C using distilled water as eluent. Figure 1(A) shows an example of the elution profiles. The included fractions, called LPfr, were pooled and concentrated by pervaporation in air stream at 4°C and used as antigen.

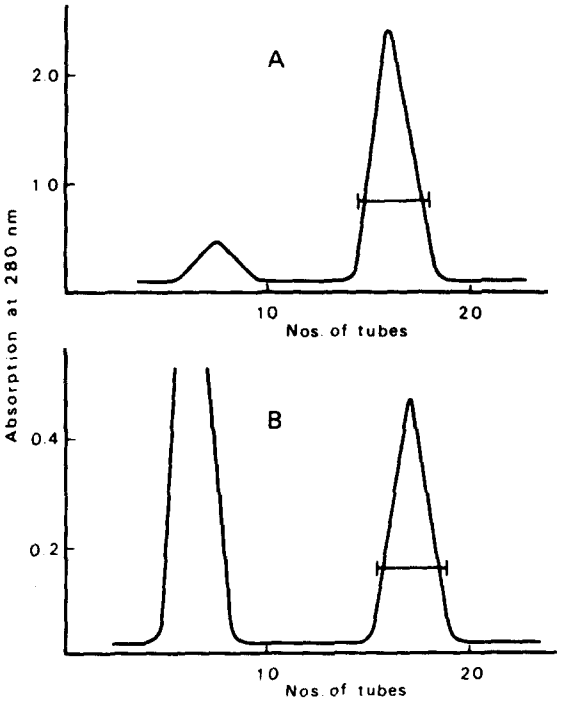

Fig. 1. Sepharose 4B (A) and Sephadex G-100 (B) elution patterns of the W_1-LPfr. The pool of the Sepharose 4B-included fractions was treated with the immunoglobulins of antisera against common antigens and subsequently passed on Sephadex G-100.

Approximately 10 mg protein of LPfr were obtained from 1 g (wet weight) of W_1-LP. Though the preparation appeared to contain somewhat more contaminants, LP was obtained from the resulting precipitate from the S which was adjusted at pH 4.3 by adding glacial acetic acid and left overnight at 4°C and LPfr, also from the R_1.

In some experiments the W_1-LPfr from the S_1 was depleted of the non-specific antigens by reverse immunoabsorption with an immunoglobulin fraction of the xenoantiserum described below. A mixture of 0.4 ml (5 mg protein) of the immunoglobulin fraction and 4 ml (5 mg protein) of the LPfr were incubated for 6 hr at 30°C and centrifuged at 2000 ***g*** for 5 min. The supernatant was submitted to Sephadex G-100 column chromatography with 0.05 M Tris–HCl buffer of pH 8.0 containing 0.15 M sodium chloride as eluent. Figure 1(B) shows a typical elution pattern. The pool of the second peak, called G-100 fraction, was concentrated by placing in a cellophane bag and embedding in solid carboxymethyl cellulose at 4–8°C.

The immunoglobulin fraction of the xenoantiserum was prepared as follows. A mixture of LP preparations of the normal spleen, liver, lung and kidney (each 100 mg, wet weight) was used to raise antisera in rabbits by 4 injections of antigens incorpo-

rated in complete Freund's adjuvant at intervals of 2 weeks. The antisera were collected 7 days after the 4th injection. The globulin fraction was obtained by bringing the antiserum to 33% saturation with a saturated ammonium sulfate solution at 4°C. This was followed by centrifugation and the pellet was dissolved in, and extensively dialyzed against saline. The solution was submitted to chromatography on Sephadex G-100 with 0.05 M Tris–HCl buffer of pH 8.0 containing 0.15 M sodium chloride as eluent. The pool of the excluded fractions was adjusted to 12.5 mg protein/ml.

The globulin fraction of the antiserum against LP preparations of the normal spleen, liver, lung, and pancreas was also prepared for the G-100 fraction of the normal kidney in a similar manner.

Protein concentration was determined by the method of Lowry *et al.* [17]. Carbohydrate contents were determined by the orcinol–sulfuric acid procedure. The method of Fings and Dunn [18] for total lipid contents was also used.

Antiserum production

Three guinea pigs weighing about 400 g were each injected into the subscapular region with 1 ml of W_1-LPfr containing 10 mg protein emulsified with 1 ml of complete Freund's adjuvant. Injections of the same composition and volume were given on days 7 and 21 after the first injection. On days 28–31 after the first injection the animals were bled and sera stored at −20°C. Antisera against the normal kidney LPfr for immunoelectrophoresis were also prepared in a similar manner.

The anti-W_1-LPfr sera were absorbed if necessary either with each 100 mg (wet weight) of LP preparations from normal organs or 100 mg of lyophilized normal human serum per ml antiserum. The homogenized mixture of antiserum and absorption material was left at room temperature for 2 hr and then at 4°C for an additional 10 hr period. Two or three absorptions were usually performed.

Patients' sera

Serum samples were obtained from 4 patients with pathologically proven nephroblastoma, inclusive and exclusive of donors for tissues. The fourth patient who received nephrectomy 8 yr ago is now clinically free of disease. Control sera were obtained from 30 patients with non-malignant diseases.

Immunological tests

Double immunodiffusion and immunoelectrophoresis in agar were performed. Antigenic fractions at 8–10 mg protein/ml and undiluted antisera were used unless otherwise stated. The precipitin patterns were allowed to develop in a moist chamber at 4°C for 7 days. Plates were sometimes dried and stained by *p*-phenylenediamine oxidation reaction [19] or Sudan black B after sufficient washing.

The indirect immunofluorescence technique was also employed. Cryostat sections of W_2 and W_3 and of their associated normal kidneys were air-dried and without fixing were incubated at 37°C for 30 min with the anti-W_1-LPfr serum absorbed triply with LP preparations of the spleen, liver, lung and kidney, diluted 1/16–1/64, treated with sodium nitrite as described [20] and incubated with FITC-conjugated anti-guinea pig IgG rabbit serum (Med. and Biol. Laboratories, Tokyo, Japan) at room temperature for 30 min. Observations were made with a BH-RFL Olympus Microscope, equipped with FITC-interference filters.

Antisera to A and B human erythrocytes were obtained from Orth Pharmaceutical Corp., Raritan, N.J., U.S.A.; anti-Forssman serum (hemolysin), from Difco Laboratories, Detroit, Mich., U.S.A.; anti-α_1-fetoprotein rabbit serum, from Behring Institute, Hoechst Pharmaceuticals Inc., Germany; and fetuin, from Gibco, Santa Clara, Calif., U.S.A. (lyophilized, "99.9%", Spiro method). A partially purified preparation of the carcinoembryonic antigen (CEA) was prepared from a fresh colonic adenocarcinoma according to the method of Krupey *et al.* [21].

Physicochemical studies

The enzymatic degradation of the antigen was carried out as previously described [14] with trypsin (Boehringer Mannheim GmbH, Mannheim, Germany, from pancreas), neuraminidase (Sigma Chemicals Co., St Louis, Mo., U.S.A., type V), and phospholipase A (Sigma, from muscle).

Disc electrophoresis was carried out with the 15% polyacrylamide gel separating at pH 8.9, the stacking gel at pH 6.7, in Tris–glycine buffer. During the run, a current of 4 mA per tube was used. Gels were stained either by Amido black 10B or sometimes by the periodic acid–Schiff reagent [22] and Sudan black B.

Molecular weights determination was carried out by column chromatography on Sepharose 6B in the presence of 6 M guanidine hydrochloride according to the method of Davison [23]. The protein in the eluate

samples was estimated turbidimetrically with trichloroacetic acid. A kit of non-enzymatic molecular weight markers (Schwarz/Mann, NY, U.S.A.) was used as a calibration standard.

RESULTS

The double diffusion experiments suggested that the anti-W_1-LPfr serum, after suitable absorption, was monospecific. It gave a single distinct line with the W_1-LPfr or W_1G-100 fraction and sometimes faint lines with LPfr of the liver and heart but failed to give visible precipitin line with LPfr of the kidney, spleen, and lung (Fig. 2). Increasing the protein concentration of liver or heart LPfr from 8 mg protein/ml to 30 mg protein/ml did not strengthen the faint line but removed it. Absorption of the antiserum with liver or heart LP also removed the faint line but did not apparently weaken the distinct line. The lines were not positively stained for carbohydrate and lipid. When the W_1-LPfr was placed in an immunodiffusion well adjacent to one containing the W_2-LPfr, and allowed to react against the anti-W_1-LPfr serum, a line of complete fusion resulted (Fig. 3), indicating antigens of immunological identity in the W_1-LPfr and the W_2-LPfr. Other experiments showed lack of precipitation line between the unabsorbed antisera and LPfr of the brain, thyroid gland, stomach, testis and placenta. No precipitin line was also observed with the LPfr preparations of fetal organs, including the spleen, lung, muscle, liver and kidney (Fig. 4). Tests with the individual LPfr of various tumors revealed that the reactive antigen appeared to be restricted to the nephroblastoma LPfr (Table 1, Fig. 5). The anti-W_1-LPfr serum failed to react with CEA (0.01–10 mg protein/ml) (Fig. 5) and with fetuin (0.1–10 mg/ml) as well. The antisera to Forssman antigen, and to A and B group human erythrocytes, as well as the anti-α_1-fetoprotein serum failed to produce any precipitin line with the W_1-LPfr even when concentrated one tenth the original volume. Furthermore, the anti-W_1-LPfr serum failed to agglutinate sheep erythrocytes and human erythrocytes of A and B group.

Table 1. Reactivity of LPfr preparations from various tumors with anti-W_1-LPfr serum by immunodiffusion

Tumor histologic type	No. positive/ No. tested
Nephroblastoma	2/2
Renal cell carcinoma	0/4
Transitional cell carcinoma	0/3
Adenocarcinoma of stomach	0/5
Adenocarcinoma of colon	0/1
Epidermoid carcinoma of cervix	0/1
Hepatoma	0/2
Seminoma	0/1
Lymphosarcoma	0/1
Leiomyoma	0/1
Leiomyosarcoma	0/1
Rhabdomyosarcoma	0/1

In Fig. 6, the immunoelectrophoretic analysis is presented in which the electrophoretically separated W_1-LPfr and the normal kidney G-100 fraction are tested against their respective antisera. The W_1-LPfr revealed a single precipitin arc with migration characteristic slightly faster than IgG while the kidney G-100 fraction showed an arc of α_1-mobility.

With immunofluorescence, the triply absorbed anti-W_1-LPfr serum, diluted 1/16–1/64, appeared to stain the tumor-cell plasma membrane of the W_2 and W_3 (Fig. 7A, B). This staining was not felt to be due to antibodies to human histocompatibility antigens because the normal kidney cells associated with the tumor were negative (Fig. 7C).

The anti-W_1-LPfr serum produced a definite though weak precipitation line with each of three patients' sera which fused with the line formed by W_1-LPfr in an identity reaction. In these tests, it was found necessary to absorb preliminarily the antiserum by whole human serum because traces of serum components contaminated the W_1-LPfr and the antibodies to them occasionally produced unrelated lines. The serum from the fourth patient and the 30 control sera failed to precipitate the absorbed antiserum.

Gross chemical analysis revealed that the W_1G-100 fraction contained protein, carbohydrate (3.6% of protein) and virtually no lipid.

The effects of enzymatic treatments on the precipitating ability of the present antigen were studied. Trypsin caused inactivation of the antigen while neuraminidase as well as phospholipase A showed no marked effects. The protein part is, therefore, of major importance to the integrity of this antigen.

Disc electrophoresis on polyacrylamide gel of the W_1-LPfr gave rise to one main band and several faint bands when stained by Amido black 10B (Fig. 8). These bands were negative for staining of carbohydrate and lipid.

By Sepharose 6B column fractionation in the presence of 6 M guanidine hydrochloride,

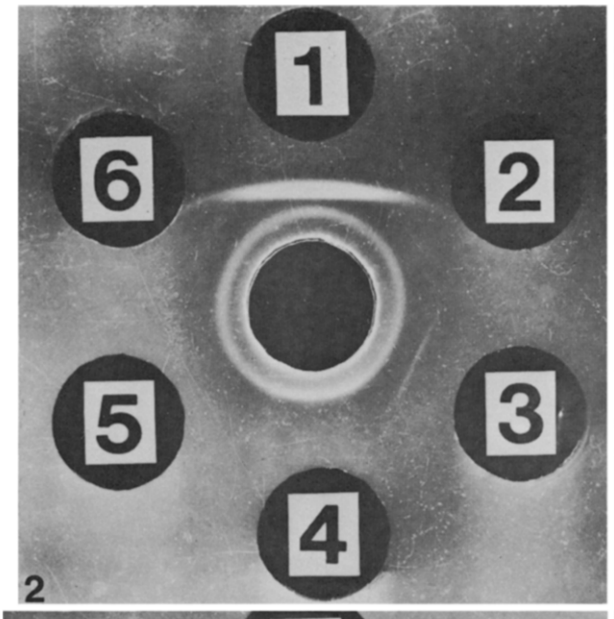

Fig. 2. Immunodiffusion patterns of anti-W_1-LPfr serum in center well against W_1-LPfr and LPfr of normal organs in pheripheral wells. (1) W_1-LPfr; (2) kidney LPfr; (3) liver LPfr; (4) spleen LPfr; (5) lung LPfr; (6) heart LPfr.

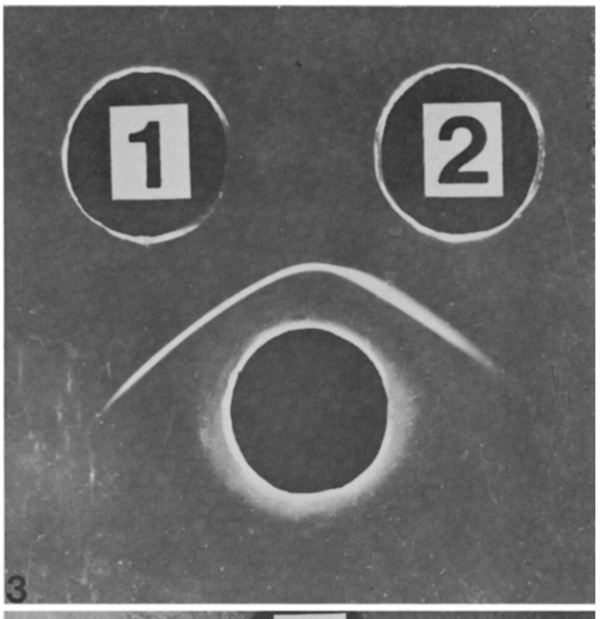

Fig. 3. Immunodiffusion patterns of anti-W_1-LPfr serum in lower well against W_1-LPfr (1) and W_2-LPfr (2) in upper wells. A reaction of complete identity can be seen between the two lines formed.

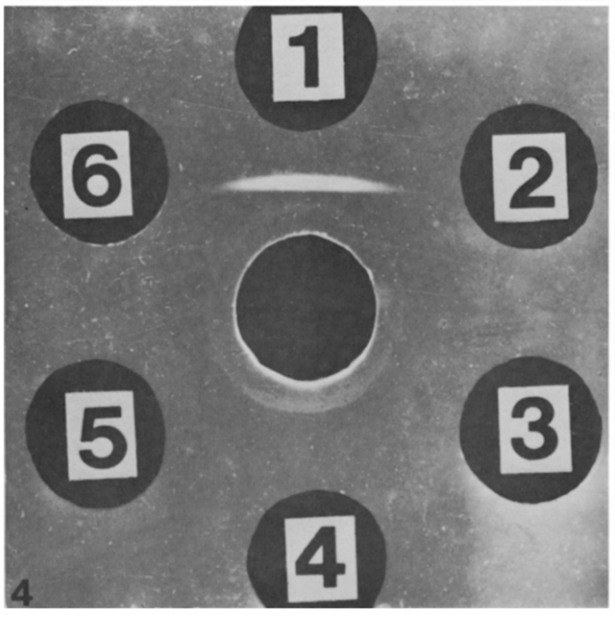

Fig. 4. Immunodiffusion patterns of anti-W_1-LPfr serum against W_1-LPfr and LPfr of fetal organs. (1) W_1-LPfr; (2) fetal spleen LPfr; (3) fetal lung LPfr; (4) fetal muscle LPfr; (5) fetal liver LPfr; (6) fetal kidney LPfr.

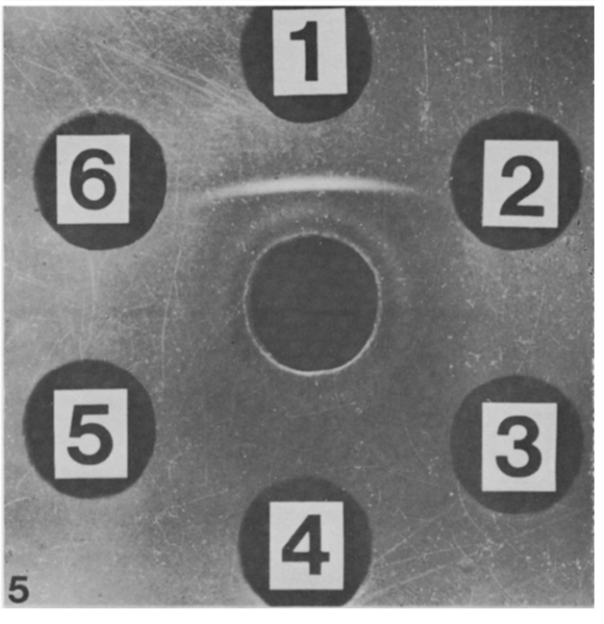

Fig. 5. Immunodiffusion patterns of anti-W_1-LPfr serum against W_1-LPfr, different tumor LPfr, and CEA. (1) W_1-LPfr; (2) renal cell carcinoma LPfr; (3) transitional cell carcinoma LPfr; (4) CEA (10 mg/ml); (5) colonic adenocarcinoma LPfr; (6) gastric adenocarcinoma LPfr.

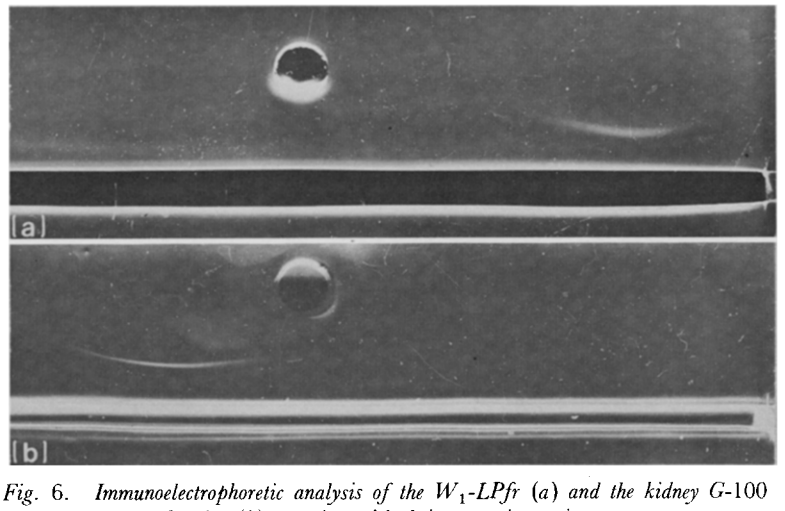

Fig. 6. Immunoelectrophoretic analysis of the W_1-LPfr (a) and the kidney G-100 fraction (b), reacting with their respective antisera.

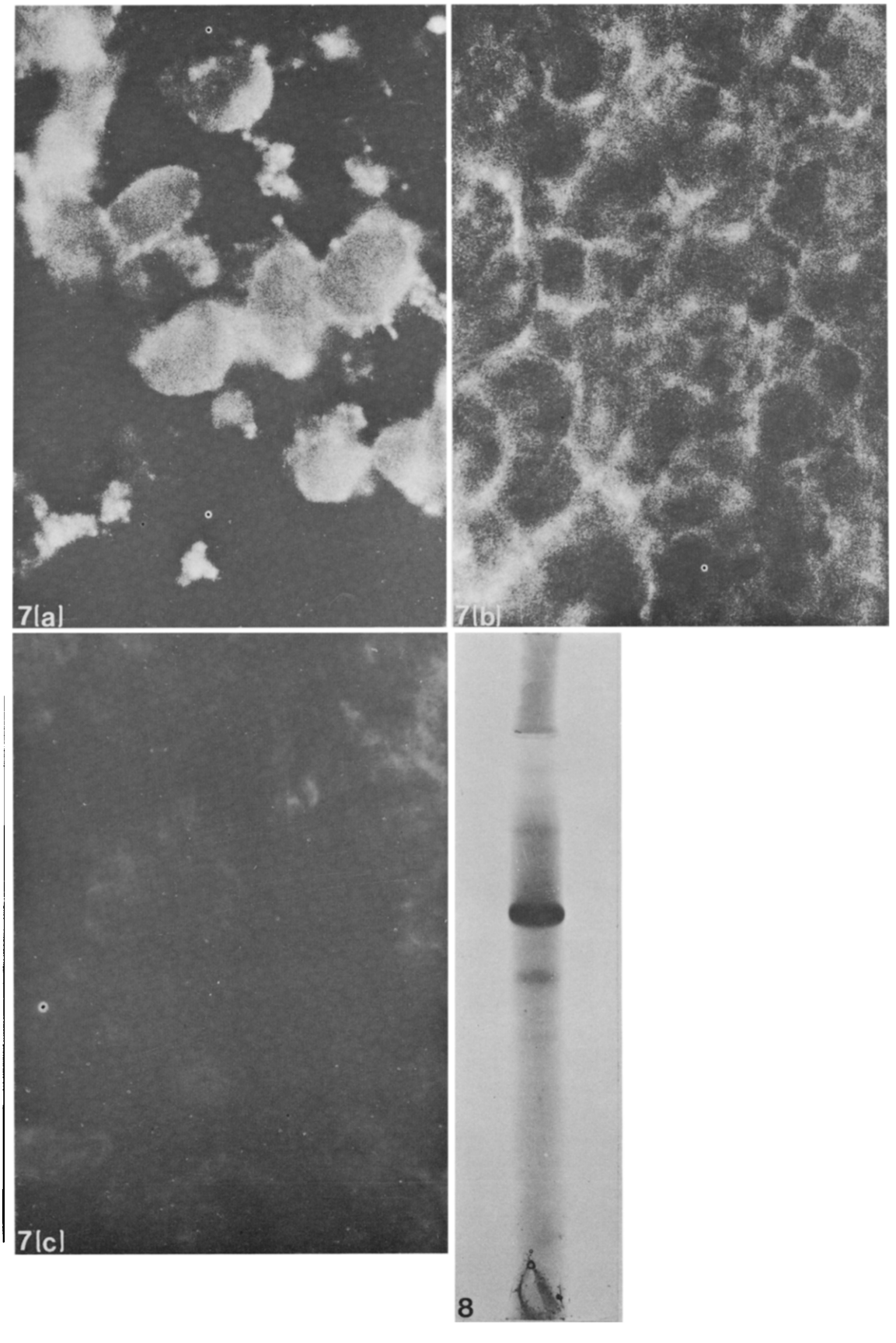

Fig. 7. Immunofluorescene of anti-W_1-LPfr serum on sections of nephroblastomas. (a) W_2. (b) W_3. (c) normal kidney cells associated with $W_3 \times 450$. The antigen appears to be located at the plasma membranes of tumor cells.

Fig. 8. Disc electrophoresis of the W_1-LPfr in 15% polyacrylamide gel, at 4mA/*tube for* 45 min. *The cathode is uppermost.*

the W_1-LPfr separated into at least 4 components of apparent mol. wt about 100,000, 72,000, 60,000 and 47,000 while the W_1G-100 fraction showed one distinct peak of mol. wt 60,000 and three small peaks of mol. wt 100,000, 72,000 and 47,000 (Fig. 9A, B). Control experiments indicated that the normal kidney LPfr and G-100 fraction contained only one component of mol. wt about 47,000 (Fig. 9C).

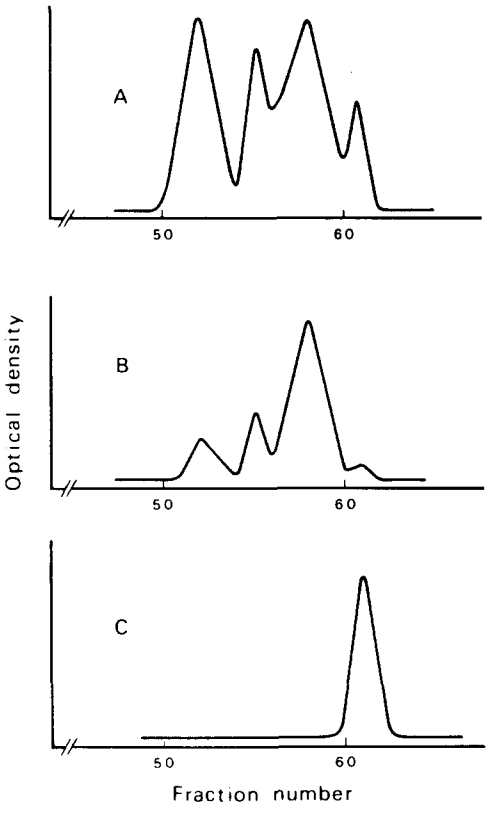

Fig. 9. Elution diagrams of the W_1-LPfr (A), the W G-100 fraction (B) and the kidney G-100 fraction (C) chromatographed on Sepharose 6B in 6 M guanidine hydrochloride. Each solvent also contained β-mercaptoethanol, EDTA and sodium chloride. The turbidity with trichloroacetic acid was measured at 420 nm.

DISCUSSION

The experimental findings suggested that the nephroblastoma LPfr preparation prevents a heavy contamination with irrelevant cell antigens, contains at least, one tumor-associated antigen which appears to be absent in normal adult and fetal organs, as well as various tumors of other origins, and is capable of stimulating tumor-specific antibody formation in heterologous animals. This antigen differs from the carcinoembryonic antigen [24] and α_1-fetoprotein [25], and appears to be also different from the previously described antigens in nephroblastomas, such as abnormal extracellular components [8], W-antigen [9], fetuin-like antigen [11], and Wilms' tumor associated antigen [12]. Abnormal extracellular components appear to be polyanionic mucoproteins while the present antigen is a cationic component. The W-antigen is trypsin-insensitive and anionic but this antigen is not. In contrast to the fetuin-like antigen, this lacks the reactivity with fetuin. The Wilms' tumor associated antigen has the α-mobility and the cross-reactivity with hypernephroma but our antigen has not. The error due to individual-specific antigenic differences was overcome in the present investigations because the antigenic identity of W_1-LPfr and W_2-LPfr and the absence of the present antigen in the normal kidney associated with the tumor were observed. The extensive absorption of the anti-W_1-LPfr serum on large quantities of LP preparations from normal organs failed to inhibit the development of the tumor-specific line formation between the antiserum and the nephroblastoma LPfr. These observations suggest that the antigen under consideration is qualitatively tumor-specific and not merely present in higher concentrations in tumor tissue than in normal tissue. In this respect, however, further studies by more sensitive techniques will be required.

In order to gain insight on the nature of the present antigen, some preliminary physicochemical studies were undertaken. The nephroblastoma LPfr appeared to be nearly homogeneous as judged by immunodiffusion, but it showed several additional faint bands except one prominent band on disc electrophoregram and was separated into at least four components by gel filtration in the presence of guanidine hydrochloride. Experiments of the W_1G-100 fraction suggested that the specific antigen is the component of mol. wt about 60,000. Due to restricted yield of the G-100 fraction, it was not decided whether the other components were resulted from incomplete absorption, or had not desired degree of tumor-specificity. It should be noted here that the normal kidney LPfr and G-100 fraction showed only one component of about 47,000. The use of solid immunoabsorbent or affinity chromatography for the reverse immunoabsorption of the antigen is now in progress.

The antigenic molecules are not truely solubilized from the insoluble fraction of tumor even at high salt concentrations and solubilized only by the prolonged use of detergent.

Therefore, these molecules seem to fulfil the operational criteria of integral proteins of cell membranes [26].

The presence of the NPSA in 3 nephroblastomas or in sera of 3 patients with this tumor suggest that the antigen is a common antigen within nephroblastomas. Though the state of the antigen in the patients' sera is as yet not clear, its presence allows speculation for some diagnostic usefulness.

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