Europ. J. Cancer Vol. 16, pp. 1451–1458

0014–2964/80/1101–1451 $02.00/0

Demonstration and Preliminary Characterization of an Antigen in the Insoluble Extracts of Human Transitional Cell Carcinoma*

SHUJI OKADA,† YORIAKI KURATA,† KIYOTAKA KITAGAWA‡ and MITSUO OOKAWA‡

†*Department of Pathophysiology, Cancer Research Institute, Kanazawa University, Kanazawa, 920, Japan and*
‡*Department of Urology, School of Medicine, Kanazawa University, Kanazawa, 920 Japan*

Abstract—*The insoluble extract of human transitional cell carcinoma has been solubilized by the aid of desoxycholate and the soluble product, after partial purification by gel filtration, used to raise a hyperimmune antiserum in guinea pigs. The antiserum, after suitable absorption, allowed the characterization of an antigen common to TCC by immunodiffusion. This antigen was undetectable in preparations of normal adult organs, fetal organs, or other malignancies and was distinguishable from other antigens, including histocompatibility antigens, carcinoembryonic antigen, and α-fetoprotein. The immunofluorescence tests suggested that it was a cell membrane-associated component.*

INTRODUCTION

IDENTIFICATION of the antigen closely associated with human tumor as an entity will be a major problem in tumor immunology. In studies concerning human urinary bladder carcinoma (transitional cell carcinoma, TCC), detection of tumor specificity in assays for cellular immunity and of antibody against tumor-associated antigens in serum of patients has been performed by a number of investigators. However, the results are conflicting (e.g. [1, 2]), possibly indicating the immunological complexity of the antigenic pattern of the TCC cells. A more direct way of establishing the immunological basis of such assays may be offered by antigen analysis of the TCC cells themselves. Previous work in this laboratory was directed toward determining the presence of a tumor-specific antigen in desoxycholate-solubilized fractions from insoluble extracts of human nephroblastoma using hyperimmune xenogeneic antisera to them [3]. The studies described below represent preliminary experiments designed to identify a possible tumor-specific or associated antigen in human TCC by similar techniques, and to offer the basis for its purification and precise characterization.

Accepted 2 April 1980.
*This work was partially supported by a grant from the Ministry of Education, Science and Culture.

MATERIALS AND METHODS

Tissues

Seven primary TCC tumors of grades 1–3, six from the renal pelvis and one from the urinary bladder, were obtained within 3 hr after surgery. All grossly normal and necrotic tissue were dissected free from each tumor, and the specimens were stored at −20°C until used. Two other TCCs from the bladder were used in living cell membrane immunofluorescence (LCMF), immediately after surgery. Normal adult organs were obtained at autopsy within 6 hr after death from several patients with non-malignant diseases. Normal urinary bladder mucosa was obtained at autopsy from several patients dying with non-malignant, non-septic causes. Mucosa was carefully excised from underlying muscle and submucosal tissue. Fetal organs were obtained from several fetuses at the second trimester of gestation. We also used various tumors of the other histologic types obtained from surgery or autopsy. All diagnoses were confirmed by histopathologic examination. Additionally, small blocks from two TCCs, one normal

adult bladder, and one fetal bladder were quick frozen in dry ice–acetone and stored at −80°C for fixed cell immunofluorescence.

Preparation of antigens

The extraction and solubilization methods similar to those described in detail for nephroblastoma in a preceding paper [3] were used. The cellular insoluble lipoprotein of each TCC, of normal adult organs, and of various other tumors was prepared by the method of Smith *et al.* [4], with modifications, as follows.

A freeze-thawed tissue was cut into small pieces and homogenized in a Waring blendor with 3–5 vol of "Solution I" [4] for 10 min at 4°C. The homogenate was strained successively through one, two, four and eight layers of gauze, and centrifuged at 10,000 ***g*** for 30 min at 4°C. The pellet was homogenized for 5 min at 4°C by a teflon glass homogenizer in 5–10 vol of Solution I and the homogenate was centrifuged at 10,000 ***g*** for 30 min at 4°C. The pellet was homogenized in 5–10 vol of "Solution II" [4] and centrifuged at 10,000 ***g*** for 30 min at 4°C. Subsequently, the homogenization of the pellet with Solution II and the centrifugation in the same manner were repeated seven times. The resulting precipitate (LP) was stored in a glass-stoppered bottle at −20°C until used.

A portion of each LP was homogenized at 4°C with 3–5 vol of 0.2% sodium desoxycholate (DOC) until a creamy homogenate was obtained. The homogenate was stirred for 48 hr at 4–8°C and centrifuged at 10,000 ***g*** for 30 min at 4°C. To the supernatant, 10 vol of cold acetone were added and the mixture was left overnight at −20°C. The precipitate formed was collected by centrifugation, dissolved in a small amount of distilled water and the solution was applied to column chromatography on Sepharose 4B at 4–8°C, using distilled water as eluent. The included fractions, called Pfr, were pooled and concentrated by pervaporation in air stream at 4°C or Aquacide II-A (Calbiochem., La Jolla, U.S.A.) hydration. This material was stored at −20°C, and subsequently used as antigen for immunization and immunodiffusion experiments. The protein concentration of the Pfr was determined by the method of Lowry *et al.* [5].

In some experiments, the 20% w/v homogenates in saline prepared from each TCC and from fetal organs were used as antigens for immunodiffusion.

Antiserum production

Four guinea pigs weighing about 400 g were each injected in the subscapular region with 4–5 mg protein of the Pfr from a TCC of grade 1 (wet weight 110 g) emulsified in complete Freund's adjuvant on day 0; this was followed by injection of the same composition on days 7, 14 and 35. Two guinea pigs were further given an injection of the same composition on days 42. The antisera were obtained by cardiac puncture 1 week after the last injection. Control sera were obtained from guinea pigs injected as follows: a guinea pig injected with 10 mg protein of a nephroblastoma Pfr in complete Freund's adjuvant 3 times during 31 days; a guinea pig injected with 10 mg protein of a renal cell carcinoma Pfr in complete Freund's adjuvant 4 times during 42 days; and a guinea pig which received 5 injections of adjuvant alone on the same schedule as described for TCC Pfr.

Absorption

One milliliter of the antiserum to TCC Pfr was sequentially absorbed once with 100 mg wet weight each of spleen, liver, kidney and pooled urinary bladder mucosa and twice with 100 mg wet weight of pooled urinary bladder mucosa. The antiserum and freeze-thawed tissues were homogenized and left for 2 hr at room temperature, followed by leaving at 4–8°C overnight with constant agitation. After each absorption, the precipitate was removed by centrifugation at 10,000 ***g*** for 30 min at 4°C. In addition, antiserum to a renal cell carcinoma Pfr was absorbed once with 100 mg each of spleen, liver, kidney, lyophilized whole serum and fetal liver in a similar manner.

Immunological tests

Double diffusion studies were performed by Ouchterlony's method, in 1.3% agar in phosphate buffer at pH 7.6. Immunoelectrophoresis was carried out in pH 8.2 veronal buffer. The antigen concentration was 4–5 mg protein/ml. The precipitin patterns were allowed to develop in a moist chamber for 7 days at 4–8°C. Some of plates were washed, dried, and stained by amido black 10B, *p*-phenylenediamine oxidation, alcian blue 8GS or oil red 0.

For fixed cell immunofluorescence, cryostat sections of tissue were air-dried, incubated for 1 hr at room temperature with the absorbed antiserum at a final dilution of 1:32–1:64 (including the dilution factor resulting from absorptions with tissues), and then rinsed with phosphate-buffered saline (PBS). The sections were treated with nitrite as described [6], coupled with a 1:80 dilution of FITC-tagged anti-guinea pig γ-globulin serum (Hoechst Pharmaceuticals Inc., Germany) for 1 hr at room temperature, washed in PBS, and mounted in buffered glycerin.

For LCMF, tumor cells liberated from a finely minced tumor without trypsinization by pressing on the slide glass were treated as described [7]. Observations were made with an Olympus Microscope BH-RFL equipped with FITC-interference filters.

Commercially available antisera used were: anti-carcinoembryonic antigen (CEA) serum (DAKO-immunoglobulins Ltd., Denmark); anti-α_1-fetoprotein serum (Hoechst); antisera to A and B group erythrocytes (Orth Pharmaceutical Corp., Raritan, U.S.A.); anti-Forssman antigen serum (Hemolysin, Difco Laboratories, Detroit, U.S.A.); anti-ferritin serum (Hoechst); anti-lactoferrin serum (Hoechst); anti-β_2-microglobulin serum (DAKO); and anti-κ-light chains serum (DAKO). The immuno-plate test kit for β_2-microglobulin (Seikagaku Kogyo Co., Tokyo, Japan) was also used. A partially purified preparation of CEA was prepared from a colonic adenocarcinoma by the method of Krupey *et al.* [8] and its CEA content was determined by the CEA-Roche kit (Roche Diagnostics, Hoffman-La Roche Inc., Nutley, U.S.A.).

Enzymic degradations

Enzymes used were: trypsin (from bovine pancreas, Boehringer Mannheim GmbH, Mannheim, Germany); neuraminidase (Type V, Sigma Chemicals, St. Louis, U.S.A.); and phospholipase A (from *Crotalus t. terrificus*, Sigma). TCC Pfr preparations were reacted for 2 hr at 37°C with and without trypsin (0.02 mg/ml, in phosphate buffer of pH 8.0), neuraminidase (1.0 mg/ml, in citrate–phosphate buffer of pH 5.3), or phospholipase A (0.5 mg/ml, in phosphate buffer of pH 7.0). The reaction with trypsin was stopped by adding 1 mg/ml of soybean trypsin inhibitor (Type 1-S, Sigma). After incubation, these preparations were added to immunodiffusion wells to assess the remaining antigen activity.

RESULTS

Preparation of antigens

When DOC-solubilized fractions from insoluble extracts of TCCs and other malignancies as well as normal organs were chromatographed on Sepharose 4B, two peaks of material were usually observed in monitoring at 280 nm. Figure 1 shows the typical elution profiles of the TCC fraction. The second peak, in the included volume, labelled 'Pfr', was used as antigen while the first peak, in the excluded volume, was discarded because of its low protein content and variable yield.

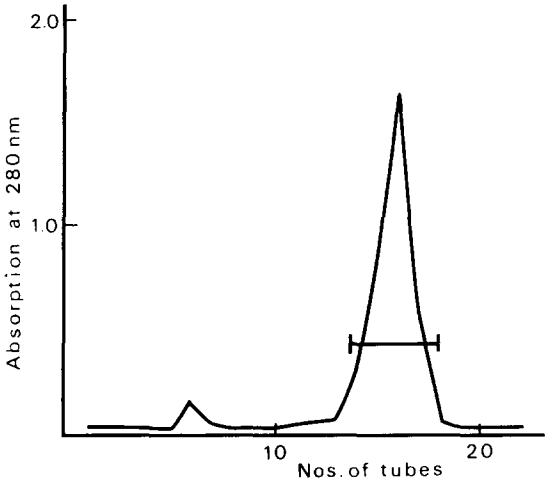

Fig. 1. Gel filtration of the DOC-solubilized fraction from the insoluble extract of a TCC on a column of Sepharose 4B (40 × 1.5 cm) *eluted with distilled water at* 4°C. *Sample:* 20 mg *protein; flow rate:* 7.0 ml/hr; *fraction volume:* 4.4 ml. *The horizontal bar indicates pooled fractions 'Pfr'.*

Five to 10 mg protein of Pfr was obtained from 10 g wet weight of a TCC. Preliminary gross chemical analysis revealed that TCC Pfr contained protein and carbohydrate (several % of protein) but virtually no lipid. The concentrated Pfr preparations do present problems in handling because they aggregate slowly on storage at 4°C or when thawed after storage at −20°C. When the preparation contained visible precipitate, it was rechromatographed on Sepharose 4B and the included fractions were pooled, concentrated, and soon used as an antigen for immunodiffusion.

Antigen analysis

Guinea pig antisera to TCC Pfr produced several precipitin bands against TCC Pfr (Fig. 2) on double gel diffusion while adjuvant

control serum did not react. Since the antisera strongly reacted also against urinary bladder mucosa Pfr and more or less against normal organ Pfr, fetal organ homogenates, and Pfr from other cancers, various absorption methods were examined with the use of normal tissues. When the antiserum was extensively absorbed with urinary bladder mucosa in addition to spleen, liver, and kidney as described in Materials and Methods and retested, only the band closest to the antigen well was formed. The antiserum AT44, obtained from a guinea pig immunized five times with 4 mg protein of TCC Pfr during 49 days, was selected and used in subsequent experiments because it gave a definite precipitin band against TCC Pfr even after extensive tissue absorptions. Other antisera also produced a single precipitin band against TCC Pfr after absorption but the bands were very weak. Various Pfr preparations from normal organs, including spleen, liver, kidney, pancreas, stomach, heart, brain, testis, thyroid, muscle, placenta and urinary bladder, or homogenates of various fetal organs showed no sign of reaction with the absorbed AT44 antiserum. Some of the results are seen in Figs. 3a and 3b. Additionally, the absorbed antiserum did not react with the Pfr prepared from the normal kidney area associated with TCC. When the Pfr preparations or homogenates of the six different TCCs from the renal pelvis (including the immunizing tumor) were tested against the absorbed antiserum, each of them formed a single precipitin band (Fig. 4a and 4b). The six bands thus formed showed reactions of identity between themselves. Another experiment showed a reaction of identity between the Pfr of a TCC from the renal pelvis and that from the urinary bladder.

The absorbed antiserum AT44 was then assayed against Pfr preparations of various tumors to assess its specificity with regard to malignant tissue (Table 1). It failed to react with Pfr from two types of renal cancer (nephroblastoma and renal cell carcinoma). In addition, an unabsorbed anti-nephroblastoma Pfr serum and an absorbed anti-renal cell carcinoma Pfr serum produced no precipitin bands with any of TCC Pfr although they produced a single precipitin band with the Pfr of corresponding tumor. All Pfr preparations derived from carcinomas of the stomach, colon, liver, ovary, cervix and testis, as well as from two sarcomas failed to react with the absorbed AT44 antiserum.

The absorbed antiserum AT44 was tested in gel diffusion against dilutions of a preparation of CEA (0.1, 0.5, 1.0, 5.0 mg CEA/ml) and no reaction was observed. Anti-α_1-fetoprotein serum produced no precipitin band against TCC Pfr even when concentrated to one fourth the original volume. Antisera to A and B group human erythrocytes and to Forssman antigen, anti-ferritin serum and anti-lactoferrin serum did not react with TCC Pfr. Both anti-β_2-microglobulin serum and anti-κ-light chains serum which is cross-reactive with β_2-microglobulin [9] also failed to react. When the kit for β_2-microglobulin was used, no detectable reaction was demonstrated in its reaction to the preparation (5 mg protein/ml) of TCC Pfr.

The immunoelectrophoresis in which the electrophoretically separated TCC Pfr was tested against the absorbed antiserum revealed a single precipitin arc in the β_2–γ region (Fig. 5).

Table 1. Reactivity of Pfr preparations from various tumors with absorbed antiserum AT44 by immunodiffision

Tumor histologic type	No. positive/ No. tested
Transitional cell carcinoma from renal pelvis	6/6
Transitional cell carcinoma from urinary bladder	1/1
Renal cell carcinoma	0/6
Nephroblastoma	0/1
Adenocarcinoma of stomach	0/6
Adenocarcinoma of colon	0/3
Hepatoma	0/2
Squamous cell carcinoma of lung	0/1
Adenocarcinoma of lung	0/1
Small cell carcinoma of lung	0/1
Squamous cell carcinoma of cervix	0/1
Cystadenocarcinoma of ovary	0/2
Seminoma	0/1
Lymphosarcoma	0/1
Rhabdomyosarcoma	0/1

Immunofluorescence data

With fixed cell immunofluorescence, the absorbed AT44 antiserum stained a TCC from the urinary bladder but virtually no staining was observed using a normal and a fetal urinary bladder (Figs. 6a, 6b and 6c). The appearance of staining in TCC cells seemed to be brighter in the periphery than in the center of cells. The same staining

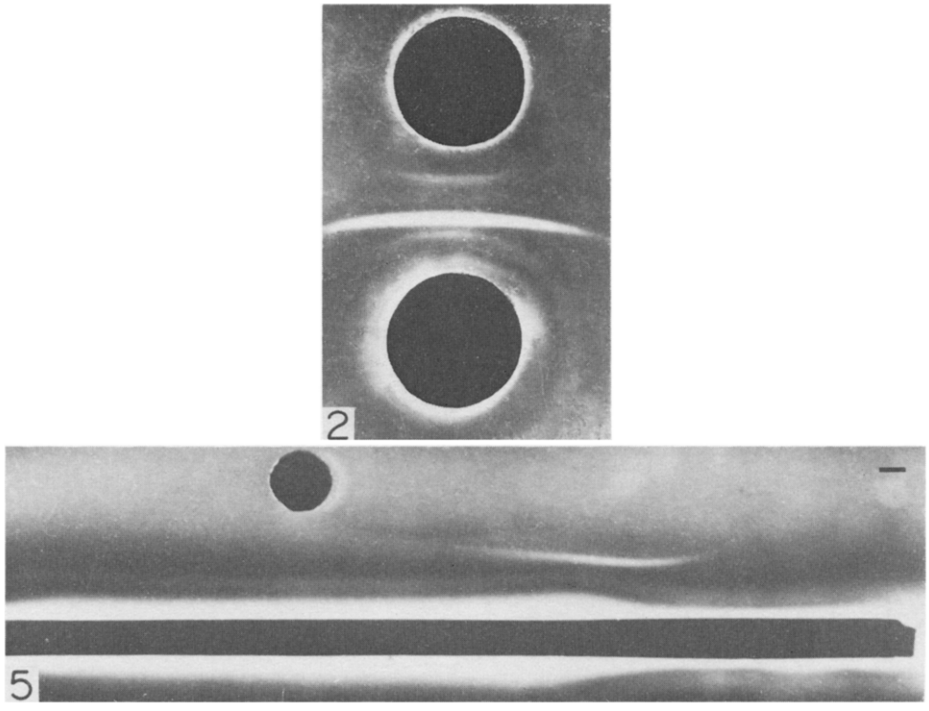

Fig. 2. Immunodiffusion: reaction of unabsorbed anti-TCC Pfr serum in lower well with TCC Pfr in upper well.

Fig. 5. Immunoelectrophoresis: TCC Pfr, developed with absorbed antiserum AT44.

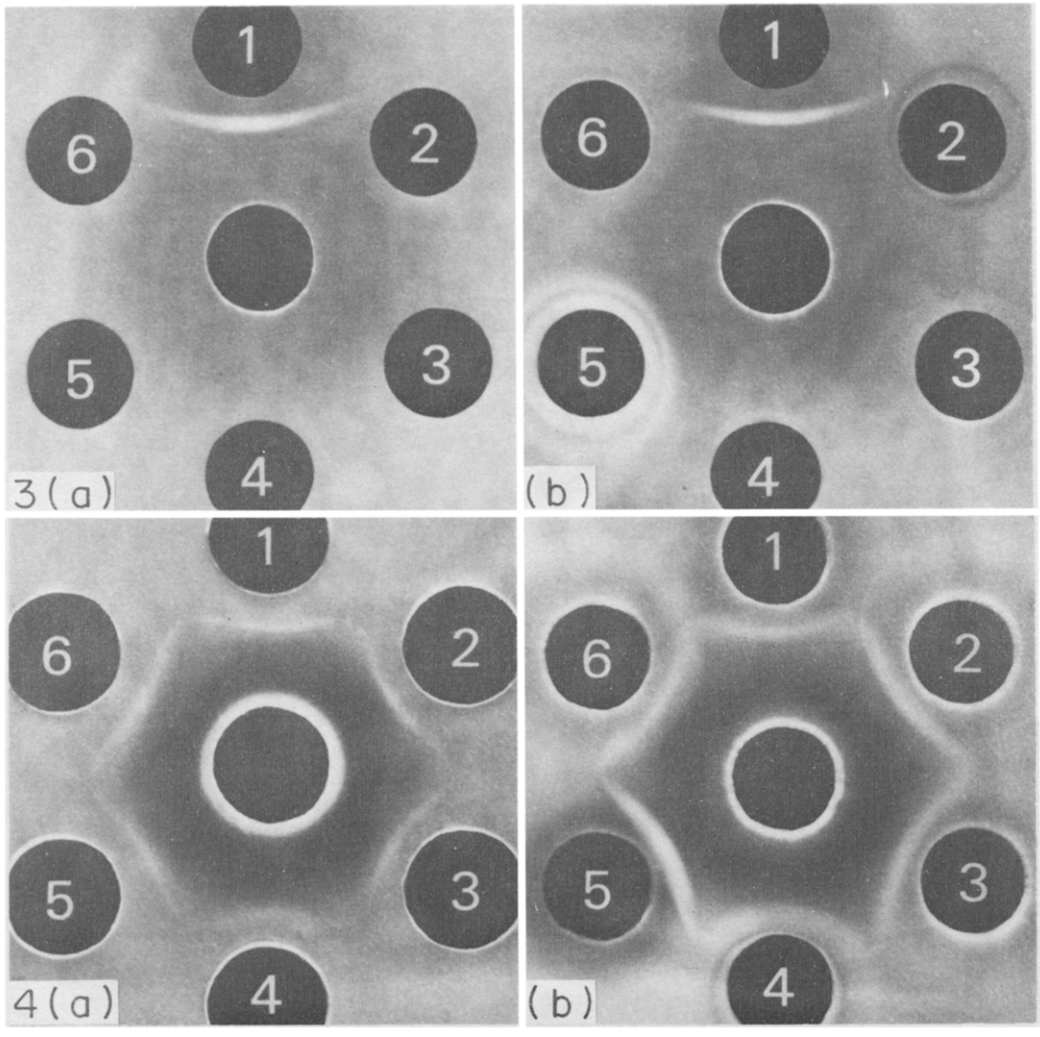

Fig. 3. Immunodiffusion: reaction of absorbed antiserum AT44 in center well with TCC Pfr, normal adult organ Pfr, and fetal organ homogenates in peripheral wells. (a): (1) TCC Pfr; (2) liver Pfr; (3) kidney Pfr; (4) spleen Pfr; (5) lung Pfr; (6) pancreas Pfr. (b): (1) TCC Pfr; (2) fetal liver homogenate; (3) fetal kidney homogenate; (4) fetal spleen homogenate; (5) fetal lung homogenate; (6) fetal gut homogenate.

Fig. 4. Immunodiffusion: reaction of absorbed antiserum AT44 in center well with 6 different (a) TCC Pfr and (b) TCC homogenates in peripheral wells.

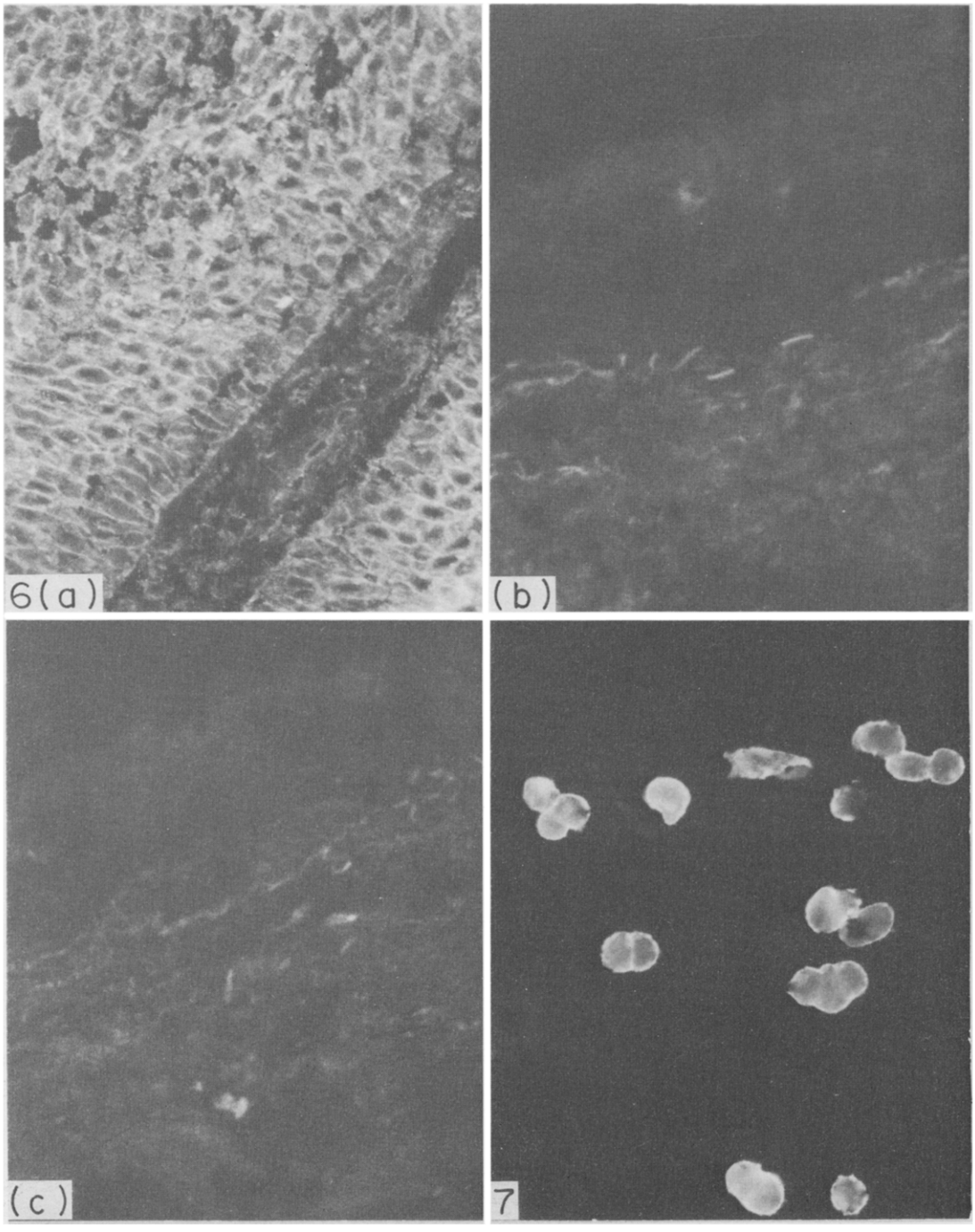

Fig. 6. Immunofluorescence of absorbed antiserum AT44 on section of TCC (a), adult urinary bladder (b), and fetal urinary bladder (c). ×315.

Fig. 7. Immunofluorescence of absorbed antiserum AT44 on living TCC cells. ×315.

pattern was obtained with another TCC from the urinary bladder.

With LCMF, all tumor cells from a TCC showed a bright green–yellow fluorescence clearly outlining the cell surfaces (Fig. 7). TCC cells from another individual were also similarly stained.

Other properties

Preliminary information concerning the chemical nature of the antigen was obtained by staining the precipitin band in agar and by treating the TCC Pfr with enzymes, followed by assessment of the antigenic activity. The precipitin band formed between the absorbed antiserum and TCC Pfr was positive for protein stain but not for carbohydrate or lipid stain. Trypsin destroyed the precipitating activity of the antigen, whereas neuraminidase and phospholipase A left the antigen intact.

DISCUSSION

Detection of a sought-for antigen in tissue demands either elimination of reactions with unimportant antigens or techniques which will discriminate among the many antigens which are present. The use of 'Pfr' (previously called LPfr or LPfr2) of tissue as an antigen for immunization, and for immunodiffusion techniques seems to be satisfactory in this respect [3, 10, 11]. The Pfr preparation from TCC tumors used here eliminates the problem of contamination with a large number of irrelevant cell antigens. It contains at least one tumor-associated antigen which appears to be absent from samples of normal adult and fetal organs, as well as from various tumors of other origin. It is, furthermore, capable of producing sufficient antibody to be detectable by immunodiffusion. Although immunodiffusion is not sensitive, it is the unrivalled method of being able to identify a definite number of antigen–antibody systems in reacting material. We reduced the disadvantage of this technique by the use of hyperimmune antiserum and of concentrated antigen fractions.

The results obtained suggested the existence of an apparently tumor-specific antigen which was common within TCC and had β_2–γ mobility. This antigen is distinct from the well-known antigens, such as CEA, α-fetoprotein, ferritin and lactoferrin. The possibility that the antigen could be related to histocompatibility antigens is ruled out by its absence in the Pfr prepared from normal kidney area associated with TCC, and the antigenic identity of all seven TCC tested. Absence in the TCC Pfr of β_2–microglobulin which is the distinctive fragment of the HL-A antigen molecule [12] may also reduce the above possibility. On the basis of the findings described above, we call the present antigen a transitional cell carcinoma antigen or TCCA.

Preliminary information concerning the chemical nature of the TCCA was obtained by staining the precipitin band and by enzymic degradation studies. The results suggested that the protein portion was of importance for the integrity of the antigen. The view that tumor antigens are integral components of the cell plasma membrane protein or glycoprotein determinants is widely held [16, 17]. Immunofluorescence studies and the procedure used for the extraction of the antigen would also suggest that the TCCA is an integral protein of the plasma membrane. If so, it will be possible that this antigen is responsible for some of TCC-specific cytotoxicity reactions described by a number of investigators [18–22].

Various procedures for antigen extraction of experimental and human tumors have thus far been employed in an attempt to identify tumor-associated or specific antigens. With respect to TCC, although membrane antigenic extracts prepared by a method similar to that of Oren and Heberman [13] were used to raise antisera in rabbits by Gozzo *et al.* [14] and Monaco *et al.* [15], their antisera obtained appeared to be not adequately potent to detect the tumor-specific antigens. These results could be due to the antigen preparations and the immunization methods.

The antigenic simplicity provided by the Pfr preparations of tumor, together with the capability of their mass production, make them attractive as starting materials for further antigen isolation and purification. To determine whether or not the TCCA is truly tumor-specific, further studies with more purified antigens and more sensitive techniques will be required. Studies for further purification of the antigens by reverse immunoabsorption are in progress.

Acknowledgements—We wish to thank Prof. A. Mukawa, Dept. of Pathology, Kanzawa Medical University, and Drs. M. Tateno and T. Shitano, Dept. of Gynecology, Toyama Prefectural Central Hospital, for providing the human material and Mrs., K. Kitagawa and T. Fujita for their technical assistance.

REFERENCES

1. W. J. Catalona, R. K. Oldham, R. B. Herberman, J. Y. Djen and G. B. Cannon, Lack of specificity of lymphocyte-mediated cytotoxicity against the bladder cancer cell line, T24. *J. Urol.* **118,** 254 (1977).
2. M. Vilien and H. Wolf, The specificity of the microcytotoxicity assay for cell-mediated immunity in human bladder cancer. *J. Urol.* **119,** 338 (1978).
3. S. Okada, K. Itaya and Y. Kurata, Identification of a tumor-specific antigen in the insoluble fraction of human nephroblastoma. *Europ. J. Cancer* **15,** 1085 (1979).
4. J. T. Smith, A. J. Funckes, A. J. Barak and L. E. Thomas, Cellular lipoproteins. I. The insoluble lipoprotein of whole liver cells. *Exp. Cell. Res.* **13,** 96 (1957).
5. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193,** 265 (1951).
6. P. Burtin and M. C. Sabine, Inhibition of the non-specific fixation of fluorescent globulins. *Rev. Europ. clin. Biol.* **17,** 76 (1972).
7. R. W. Baldwin and C. R. Barker, Demonstration of tumor-specific humoral antibody against aminoazo dye-induced rat hepatoma. *Brit. J. Cancer* **21,** 793 (1967).
8. J. Krupey, T. Wilson, S. O. Freedman and P. Gold, The preparation of purified carcinoembryonic antigen of the human digestive system from large quantities of tumor tissue. *Immunochemistry* **9,** 617 (1972).
9. A. B. Gottlieb, M. Engelhard, H. G. Kunkel, N. Tanigaki and D. Pressman, A cross-reaction between β_2-microglobulin and κ-light chains. *J. Immunol.* **119,** 2001 (1977).
10. Y. Kurata, Y. Watanabe, S. Okada and Y. Fukuyama, Immunological studies of insoluble lipoproteins. II. On the salivary gland-characteristic antigens. *Int. J. Allergy* **35,** 392 (1969).
11. S. Okada, Y. Kurata, K. Konishi and T. Matsuda, Immunological studies of insoluble lipoproteins. III. Characterization of the lipoprotein-bound thyroid gland-specific antigen. *Int. J. Allergy* **39,** 6 (1970).
12. N. Tanigaki, M. Katagiri, K. Nakamuro, V. P. Kreiter and D. Pressman, Common antigenic structures of HL-A antigens. II. Small fragments derived from papain-solubilized HL-A antigen molecules. *Immunology* **26,** 155 (1974).
13. M. E. Oren and R. E. Herberman, Delayed cutaneous hypersensitivity reactions to membrane extracts of human tumor cells. *Clin. exp. Immunol.* **9,** 45 (1971).
14. J. J. Gozzo, R. M. Schlesinger and A. P. Monaco, Detection of bladder-cancer-associated antigens in urine by micro-complement fixation. *Surg. Forum* **25,** 117 (1974).
15. A. P. Monaco, J. J. Gozzo, R. M. Schlesinger and S. D. Codish, Immunological detection of human bladder carcinoma. *Ann. Surg.* **182,** 325 (1975).
16. M. R. Price and R. W. Baldwin, Shedding of tumor cell surface antigens. In *Cell Surface Reviews.* (Edited by G. Poste and G. L. Nicolson) Vol. III, p. 423. North-Holland, Amsterdam (1976).
17. M. R. Price and R. A. Robins, Circulating factors modifying cell-mediated immunity in experimental neoplasia. In *Immunological Aspect of Cancer.* (Edited by J. E. Castro) p. 155. MTP Press, Lancaster (1978).
18. J. Bubenik, P. Perlmann, K. Helmstein and G. Moberger, Cellular and humoral immune responses to human urinary bladder carcinomas. *Int. J. Cancer* **5,** 39 (1970).
19. E. T. Bloom, R. C. Ossorio and S. A. Brosman, Cell-mediated cytotoxicity against human bladder cancer. *Int. J. Cancer* **14,** 326 (1974).
20. M. M. Elhilali and S. Nayak, Immunologic evaluation of human bladder cancer: *in vitro* studies. *Cancer (Philad)* **35,** 419 (1975).
21. M. Troye, P. Perlmann, A. Larsson, H. Biomgren and B. Johansson, Cellular cytotoxicity *in vitro* in transitional cell carcinoma of the human urinary bladder: ^{51}Cr-release assays. *Int. J. Cancer* **20,** 188 (1977).
22. C. O'Toole, A ^{51}Cr-isotope release assay for detecting cytotoxicity to human bladder carcinoma. *Int. J. Cancer* **19,** 324 (1977).