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Extraction and Preliminary Characterization of Tumor Antigens in Human Bronchogenic Adenocarcinoma and Squamous Cell Carcinoma*

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Abstract—*Guinea pigs were immunized with extracts (Pfr) prepared from the insoluble fractions of either human bronchogenic adenocarcinoma (BAC), or squamous cell carcinoma (BSC). After suitable absorption, the anti-BAC-Pfr serum reacted with* 6 *of* 6 *BAC-Pfr by immunodiffusion and the anti-BSC-Pfr serum with* 7 *of* 7 *BSC-Pfr. These absorbed antisera failed to react with adult organ extracts, fetal organ homogenates, or extracts from tumors of other histologic types. The possibility that the reaction could be related to previously known antigens was ruled out. These antigens were detected in sera from patients with the corresponding tumor, but not in sera from post-operative patients or in control sera. Initial characterization of the antigens showed them to be cell membrane-associated proteins with β-electrophoretic mobility and to be insoluble in* 50% *saturated ammonium sulfate. Further purification of the antigens by reverse immunoabsorption has been successfully undertaken. Both purified antigens were estimated to have mol. wts close to* 63,000.

INTRODUCTION

AN INCREASING number of reports of lung cancer antigens are appearing [1–16]. It has been established that oat-cell carcinoma cells possess an antigen highly associated with this tumor on their plasma membrane [1, 2]. The presence of antigens associated with alveolar cell carcinoma, apparently tumor-specific though of viral origin, has also been reported [3, 4]. The tumor-related antigens which have so far been identified in the two main histologic types of lung cancer, bronchogenic adenocarcinoma (BAC) and squamous cell carcinoma (BSC), have not had such a high degree of specificity [5–16]. Such antigens have either been present in low levels in normal lung or have been detected in different types of tumors or in sera from patients with the other histologic types of lung cancer. The method of preparation of antigens and their antisera by immunization of animals may be responsible for the failure to demonstrate antigens highly associated with these tumors.

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We describe in this paper two distinct, apparently tumor-specific antigens, a BAC- and a BSC-antigen, in human lung cancers and report studies on their characterization and purification.

MATERIALS AND METHODS

Tissues

Tumor specimens of six BAC (five differentiated and one less differentiated) and seven BSC (four differentiated and three less differentiated), and in all cases corresponding normal lung tissue were obtained from surgery. Normal organ specimens were obtained at autopsy from several noncancer patients. Fetal organs were obtained from several 6-month fetuses. A variety of tumor specimens were also obtained from surgery or autopsy material. All the tissues except that for living cell membrane immunofluorescence were stored at −20°C until used. The interval between surgery and/or death and freezing of the specimens ranged from 3 to 12 hr. The histologic diagnoses of tumors were carefully reviewed and verified.

Preparation of antigens

The methods employed were similar to those used for the extraction and solubilization of several organ-specific antigens and a nephro-blastoma antigen by Kurata and colleagues [17–19]. Briefly, the insoluble cellular lipoprotein fraction (LP) was prepared individually from all tumor and normal tissue specimens and stored at −20°C until used. The LP preparation was homogenized with 0.2% desoxycholate, stirred for 48 hr at 4–8°C, and centrifuged at 10,000 *g* for 30 min at 4°C. Two additional extractions of the residue were carried out with 0.2% desoxycholate in the same manner. To the combined extracts, about 10 volumes of chilled acetone was added, and the mixture was allowed to stand overnight at −20°C. The precipitate which formed (LPsol) was separated by centrifugation and dissolved in a small amount of water. The solution then underwent gel filtration on Sepharose 4B using distilled water as the eluent. The LPsol separated on Sepharose 4B yielded two main peaks of material in monitoring by u.v. absorption at 280 nm. Figure 1 shows typical examples of the elution profiles. The included fractions (Pfr) were concentrated by pervaporation and stored at −20°C until studied. Approximately 10 mg protein of Pfr was obtained per 10 g wet weight of BAC or BSC.

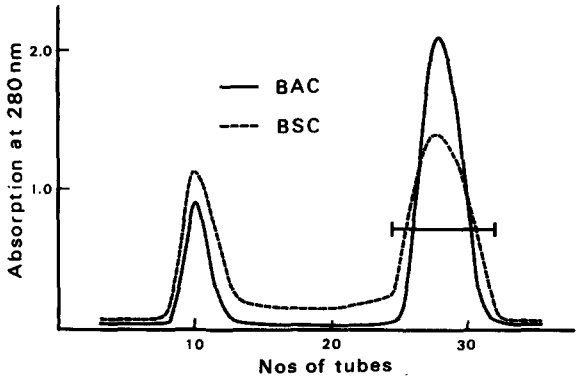

Fig. 1. Sepharose 4B chromatography of the fractions solubilized from the insoluble lipoproteins of a BAC and a BSC.

Additionally, a 20% w/v homogenate in saline was prepared from fetal organs as material for absorption and immunodiffusion.

Immunization

Five guinea pigs weighing 400–500 g were each immunized with the Pfr from different lung cancers. Five Pfr were used: two BAC-Pfr and three BSC-Pfr. Each animal received 7–11 mg protein of Pfr emulsified with 1 ml of Freund's complete adjuvant by injection into the subscapular region on day 0. Similar injections were given on days 7, 14 and 28 after the initial injection. All the animals were bled 7 days after the last injection and the sera were stored at −20°C until used. In addition, control sera were obtained from guinea pigs injected with adjuvant alone on the same inoculation schedule.

Absorption of antisera

All anti-BAC-Pfr and anti-BSC-Pfr sera were absorbed as follows. One ml of antiserum was homogenized with 100 mg wet weight each of the LP from lung (corresponding, normal lung), spleen and liver, 20 mg of lyophilized whole human serum, 0.5 ml of a 20% w/v fetal liver homogenate, and a crude carcinoembryonic antigen (CEA) preparation (containing 1 mg of CEA), incubated for 1 hr at 37°C, and left overnight at 4–8°C with constant agitation. The insoluble material was removed by centrifugation at 3000 *g* for 20 min. Three absorptions were always performed. The serum was concentrated to the original volume and used.

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. Antigen concentration used was from 3 to 5 mg protein/ml. The precipitin patterns were allowed to develop in a moist chamber at 4°C for 7 days. Some plates were washed, dried, and stained with thiazine red R for protein, *p*-phenylenediamine oxidation reaction or alcian blue 8GS for carbohydrate, or oil red 0 for lipid.

For living cell membrane immunofluorescence (LCMF), cells obtained from finely minced fresh tissue without trypsinization by pressing on the slide glass were treated as described by Baldwin and Barker [20]. An Olympus microscope BH-RFL equipped with FITC-interference filters was used.

Immunological reagents

Commercially available antisera used were: antisera to A and B group human erythrocytes (Orth Pharmaceutical Corp., Raritan, N.J., U.S.A.); anti-Forssman antigen serum (hemolysin, Difco Laboratories, Detroit, Mich., U.S.A.); anti-α_1-fetoprotein serum

(Behring Institute, Hoechst Pharmaceuticals Inc., Germany); anti-lactoferrin and anti-CRP serum (Behringwerke AG, Marburg/Lahn, Germany); anti-CEA and anti-ferritin serum (DAKO-immunoglobulins Ltd., Denmark); and anti-β_2-microglobulin serum (Seikagaku Kogyo Co., Tokyo, Japan).

A crude CEA preparation was prepared from a fresh human adenocarcinoma of the colon according to the method of Krupey *et al.* [21], and its CEA content was measured by the CEA-Roche kit (Roche Diagnostics Division of Hoffman-La Roche Inc., Nutley, N. J., U.S.A.).

Patients' sera

Sera were taken from the hospital patients with BAC or BSC in stage III and the postoperative patients during 5–12 months after an operation. Control sera were collected from patients with other cancer, patients with tuberculosis, and normal donors. All sera were stored at −20°C and studied for the presence of antigens by gel diffusion using the absorbed anti-BAC-Pfr and anti-BSC-Pfr serum.

Chemical analyses

Protein concentration of Pfr was determined by the method of Lowry *et al.* [22]. Carbohydrate content was determined by the orcinol–sulfuric acid procedure and lipid content by the method of Fings and Dunn [23].

The solubility of the antigens in 50% saturated ammonium sulfate was investigated as follows. To 1 ml (4.5 mg protein) of BAC-Pfr or BSC-Pfr, 1 ml of saturated ammonium sulfate was added dropwise at 4°C with constant stirring. After allowing to stand at 4°C for 30 min, the mixture was centrifuged at 3000 × for 10 min, yielding a pellet. The pellet was dissolved in a small amount of distilled water and the solution was dialyzed against two changes of distilled water for 24 hr, adjusted to a volume of 1 ml, and tested by gel diffusion. In a similar manner, the supernatant was dialyzed, adjusted to 1 ml, and tested.

Disc electrophoresis was performed on 10% polyacrylamide gel in Tris–glycine buffer of pH 8.3. During the run, a current of 4 mA per tube was used. The gel was stained with amide black 10B.

For mol. wt determination, the purified fraction, after desalting with a Column PD-10 (Pharmacia Fine Chemicals, Uppusala, Sweden), was dansylated and migrated by dodecylsulfate (SDS)-polyacrylamide gel electrophoresis according to the method of Talbot and Yphantis [24]. Calibration was achieved by simultaneous separate runs of the standards from a kit of proteins for SDS-electrophoresis (Combithek, Boehringer and Sohne GmbH., Mannheim, Germany).

Purification studies

In some experiments, BAC-Pfr and BSC-Pfr were further purified by reverse immunoabsorption. Antisera for absorption were prepared as follows. Rabbits were injected in the subscapular region with 100 mg wet weight each of the LP preparation from lung, spleen, liver, and kidney, 1 ml of whole serum, and a CEA preparation (1 mg of CEA) emulsified with 1 ml of Freund's complete adjuvant. Injections were given on days 7, 14, 21 and 35 after the first injection. The antisera were collected 7 days after the last injection and their IgG fractions were separated by ammonium sulfate precipitation and gel filtration on Sephadex G-100.

An aliquot of the Pfr containing about 15 mg of protein was applied to a 1.5 × 45 cm column of Sepharose 4B which had been linked with about 700 mg of the rabbit IgG fraction according to the method of Axén *et al.* [25] and eluted with 0.1 M bicarbonate at a flow rate not exceeding 7 ml/hr. A single peak, in the included volume, was obtained. The peak fractions, after suitable concentration by pervaporation, were rechromatographed twice more on the same column to ensure maximum efficiency of absorption.

RESULTS

The unabsorbed anti-BAC-Pfr and anti-BSC-Pfr serum produced precipitin lines in double diffusion with normal organ Pfr, whole serum, CEA, and fetal organ homogenates, as well as with BAC-Pfr and BSC-Pfr, while adjuvant control sera failed to react with any of these antigens. The anti-BAC-Pfr serum absorbed as described in Materials and Methods produced a single precipitin line with the Pfr used for its preparation and all Pfr obtained from the other five BAC (five different patients) tested but not with any of the Pfr from normal adult organs, including lung, liver, spleen, kidney, brain, heart, thyroid gland, thymus, stomach, small intestine, colon, esophageal mucosa, urinary bladder mucosa, testis, muscle and prostate gland, or with homogenates of fetal organs, including lung, liver, spleen, kidney, brain, stomach, gut, muscle and adrenal gland. Similarly, the

absorbed anti-BSC-Pfr serum reacted with seven of seven BSC-Pfr (seven different patients) tested but not with any normal adult organ Pfr or fetal organ homogenates. Incorporating the Pfr from normal lung (100 mg protein/ml) in agar by pre-diffusion did not alter the patterns of the precipitin line between the absorbed antiserum and the BAC-Pfr or BSC-Pfr. Some of the results are seen in Figs. 2 and 3. We have not found a close relation between the concentration of the antigen and the size or the degree of differentiation of the tumor. The absorbed anti-BAC-Pfr and anti-BSC-Pfr serum were tested against the Pfr from a variety of malignancies. The results are shown in Figs. 2d and 3d, and Table 1. Any of the Pfr from lung cancers of differing types of from various non-pulmonary tumors tested did not react against these antisera. Both these absorbed antisera failed to react with CEA (0.5–10 mg/ml) (Figs. 2d and 3d).

The anti-α_1-fetoprotein serum, anti-CEA serum, anti-lactoferrin serum, anti-ferritin serum, anti-CRP serum, anti-β_2-microglobulin serum, as well as antisera to A and B group human erythrocytes, and to Forssman antigen did not react with BAC-Pfr or BSC-Pfr even when concentrated to one half the original volume.

Immunoelectrophoresis of BAC-Pfr against the absorbed anti-BAC-Pfr serum showed a single arc with β_2-mobility while that of BSC-Pfr against the absorbed anti-BSC-Pfr serum showed a single arc with β_1-mobility (Figs. 4a and 4b).

With the LCMF test, tumor cells liberated from BAC showed a bright green–yellow fluorescence outlining clearly the cell surface while normal lung cells from the same patient were negative (Figs. 5a and 5b). Similarly, the surface of BSC cells was stained with the absorbed anti-BSC-Pfr serum (Fig. 5c).

In an attempt to detect circulating antigens, the sera from patients with BAC, BSC, or tumors of different types, as well as from non-tumor bearing control subjects were tested by double gel diffusion using the antiserum which, after absorption, had been ensured to lack reaction with the absorbing material. The antigen was detected in the

Table 1. Reactivity of Pfr preparations from a variety of tumors with absorbed anti-BAC-Pfr and anti-BSC-Pfr serum by immunodiffusion

Tumor histologic type	No. positive/No. tested	
	Anti-BAC-Pfr serum	Anti-BSC-Pfr serum
Bronchogenic carcinoma:		
Adenocarcinoma	6/6	0/6
Squamous cell carcinoma	0/7	7/7
Large cell carcinoma	0/2	0/2
Small cell carcinoma	0/3	0/3
Gastric adenocarcinoma	0/3	0/2
Colonic adenocarcinoma	0/5	0/3
Pancreatic adenocarcinoma	0/1	0/1
Hepatoma	0/1	0/1
Renal cell carcinoma	0/4	0/5
Squamous cell carcinoma of cervix	0/1	0/1
Cystadenocarcinoma of ovary	0/1	0/1
Adenocarcinoma of prostate	0/1	0/1
Rhabdomyosarcoma	0/1	0/1
Leiomyosarcoma	0/1	0/1
Lymphosarcoma	0/1	0/1
Reticulum cell sarcoma	0/1	0/1

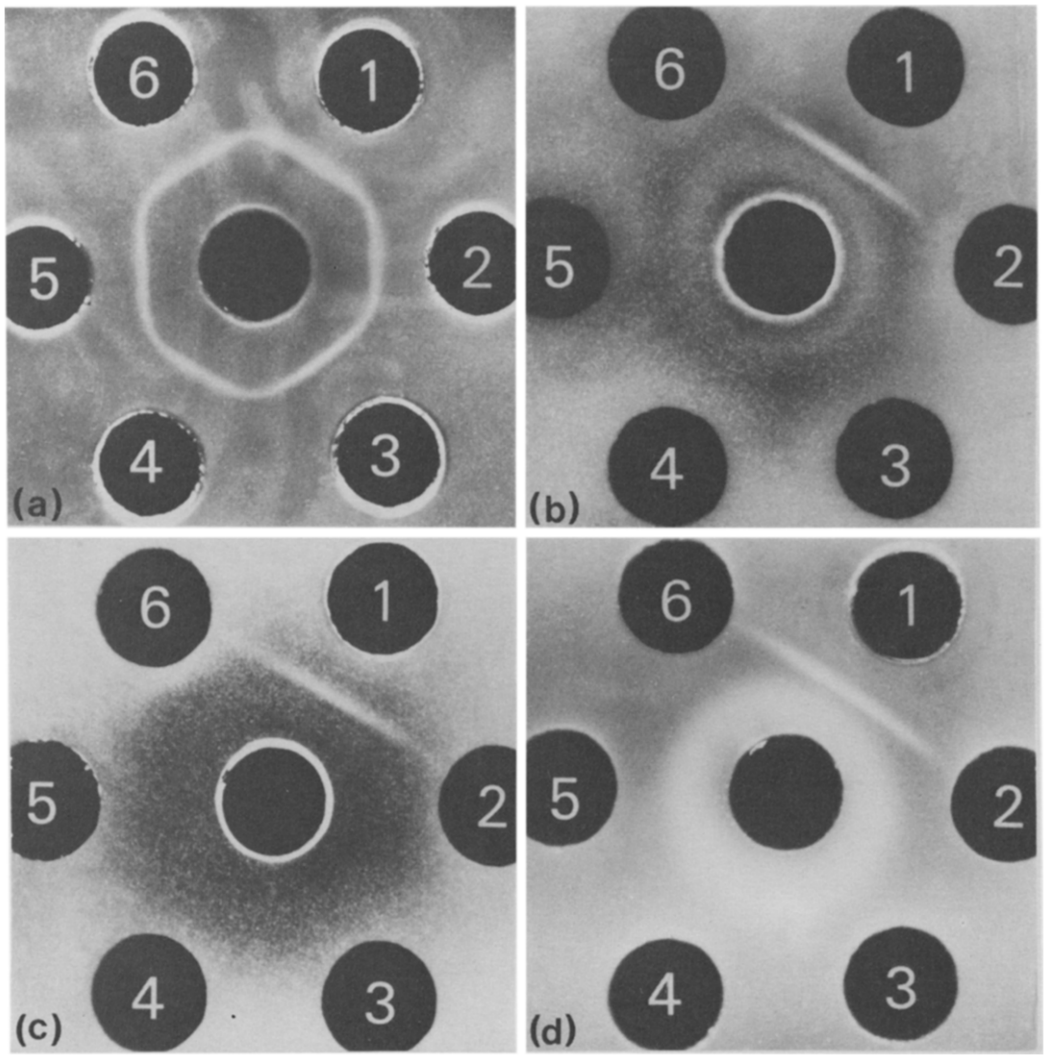

Fig. 2. Examples of immunodiffusion patterns of absorbed anti-BAC-Pfr serum in center well against BAC-Pfr and various control samples. (*a*) 1–6: *P-fr of* BAC_1–BAC_6, *respectively.* (*b*) 1: *BAC-Pfr*, 2: *lung-Pfr*, 3: *liver-Pfr*, 4: *kidney-Pfr*, 5: *spleen-Pfr*, 6: *whole serum.* (*c*) 1: *BAC-Pfr*, 2: *fetal lung*, 3: *fetal liver*, 4: *fetal kidney*, 5: *fetal spleen*, 6: *fetal gut.* (*d*) 1: *BAC-Pfr*, 2: *gastric adenocarcinoma-Pfr*, 3: *hepatoma-Pfr*, 4: *renal cell carcinoma-Pfr*, 5: *colonic adenocarcinoma-Pfr*, 6: *CEA* (3 mg).

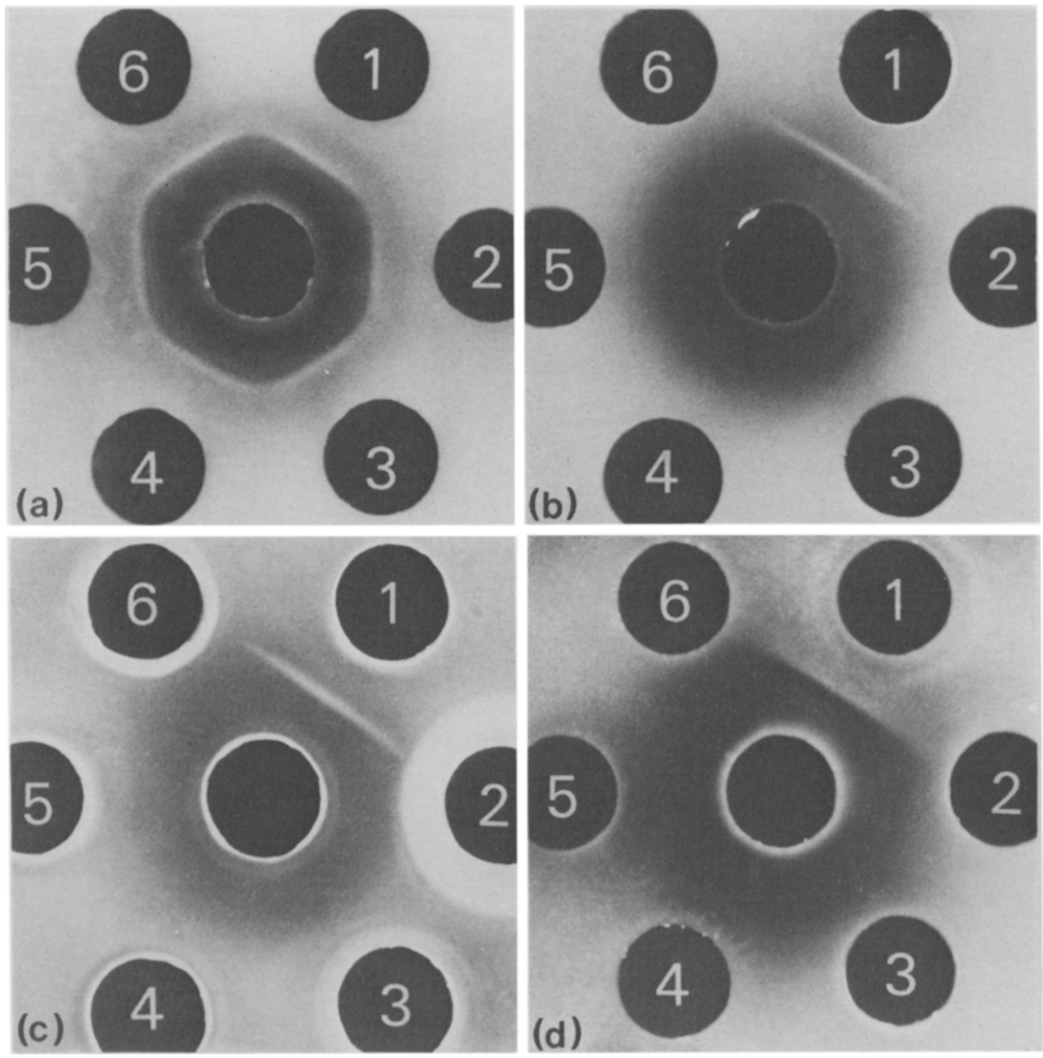

Fig. 3. Examples of immunodiffusion patterns of absorbed anti-BSC-Pfr serum in center well against BSC-Pfr and various control samples. (*a*) 1–6: *Pfr of* BSC_1–BSC_6, *respectively.* (*b*) 1: *BSC-Pfr*, 2: *lung-Pfr*, 3: *liver-Pfr*, 4: *kidney-Pfr*, 5: *spleen-Pfr*, 6: *whole serum.* (*c*) 1: *BSC-Pfr*, 2: *fetal lung*, 3: *fetal liver*, 4: *fetal kidney*, 5: *fetal spleen*, 6: *fetal gut.* (*d*) 1: *BSC-Pfr*, 2: *bronchogenic large cell carcinoma-Pfr*, 3: *bronchogenic small cell carcinoma-Pfr*, 4: *BAC-Pfr*, 5: *colonic adenocarcinoma-Pfr*, 6: *CEA* (3 mg).

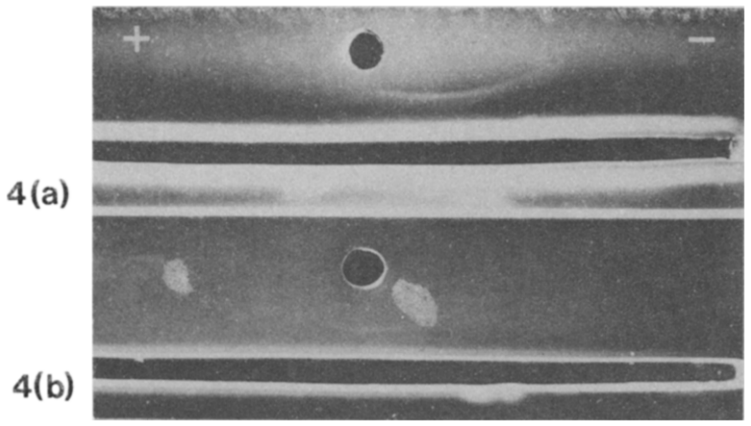

Fig. 4. Immunoelectrophoretic analysis of (a) BAC-Pfr and (b) BSC-Pfr, reacting with absorbed anti-BAC-Pfr and anti-BSC-Pfr serum, respectively.

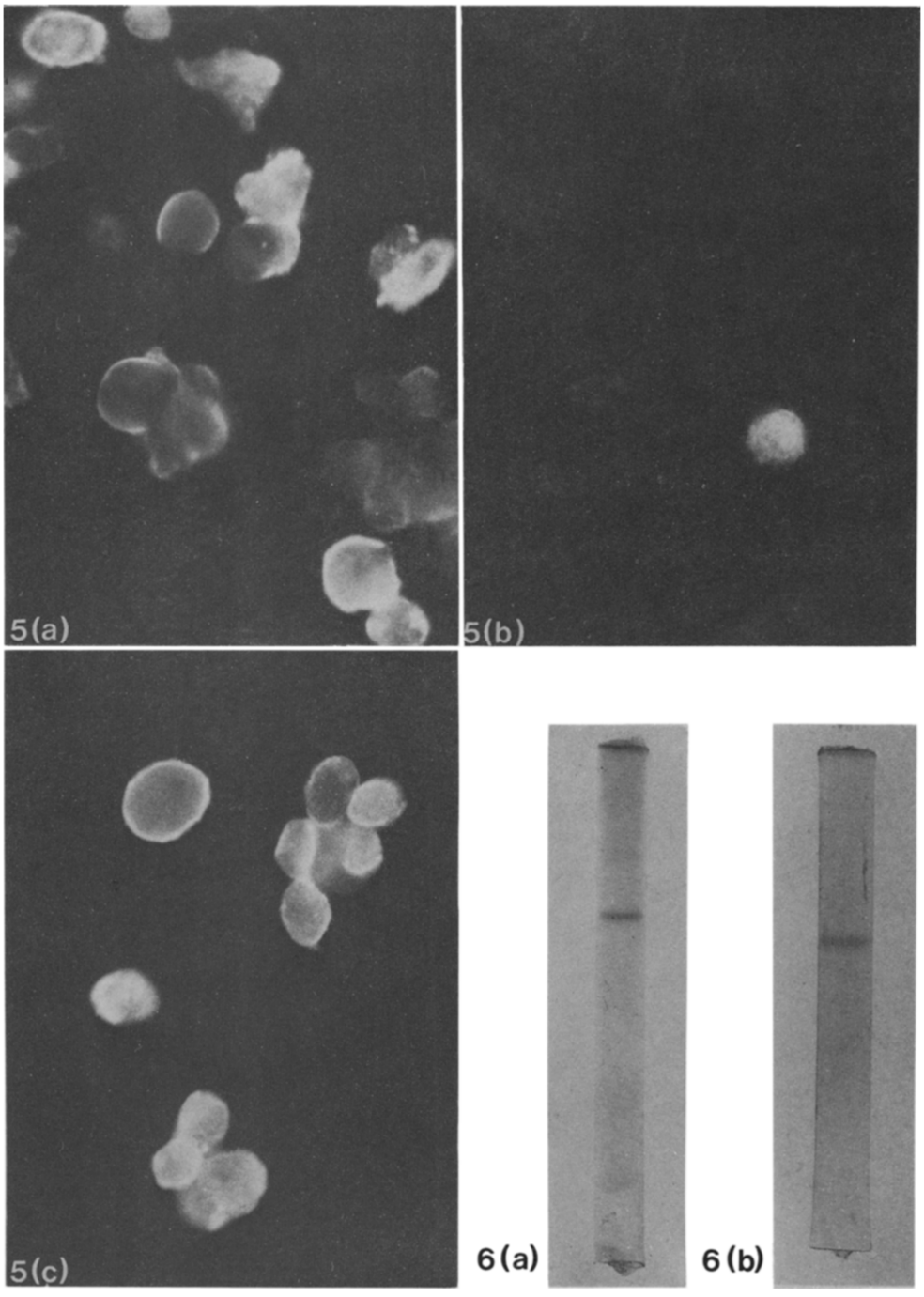

Fig. 5. *Living cell membrane immunofluorescence of* (*a*) *BAC cells,* (*b*) *normal lung cells, and* (*c*) *BSC cells.* ×300.

Fig. 6. *Disc electrophoresis of the purified* (*a*) *BAC-antigen and* (*b*) *BSC-antigen. Approximately* 100 μg *protein each of the purified fractions prepared from BAC-Pfr and BSC-Pfr were separated.*

serum from patients with the corresponding, apparent tumor but not in the serum from post-operative patients with no apparent tumor. Sera from patients with lung tumors of other histologic types, non-pulmonary tumors, or tuberculosis, as well as from normal persons were negative (Tables 2 and 3). In addition, absorption experiments showed the identity of the antigen detected in patients' sera with that in BAC-Pfr or BSC-Pfr.

In order to gain information about the antigens, some preliminary experiments were performed. Gross chemical analysis revealed that the BAC-Pfr and the BSC-Pfr contained protein, carbohydrate (5–7% of protein), and virtually no lipid. The specific precipitin line formed between the absorbed antiserum and BAC-Pfr or BSC-Pfr was positive for protein stain but not for carbohydrate or lipid stain. When the precipitate and the supernatant obtained from BAC-Pfr or BSC-Pfr by 50% saturation with ammonium sulfate were tested for the presence of the antigen by gel diffusion, the absorbed antiserum produced a precipitin line with the solution of the precipitate but not with the supernatant.

Table 2. Reactivity of sera from bronchogenic adenocarcinoma patients against absorbed anti-BAC-Pfr serum by immunodiffusion

Sera tested	Positive	Negative	Total of sera tested
From patients with bronchogenic adenocarcinoma			
—Apparent tumor, pre-op	6	0	6
—Apparent tumor, recurrence	2	0	2
—No apparent tumor, post-op	0	8	8
Controls			
From patients with:			
—bronchogenic squamous cell carcinoma	0	9	9
—bronchogenic large cell carcinoma	0	1	1
—gastric cancer	0	4	4
—colonic cancer	0	3	3
—tuberculosis	0	20	20
Healthy volunteers	0	29	29

Table 3. Reactivity of sera from bronchogenic squamous cell-carcinoma patients against absorbed anti-BSC-Pfr serum by immunodiffusion

Sera tested	Positive	Negative	Total of sera tested
From patients with bronchogenic squamous cell carcinoma			
—Apparent tumor, pre-op	7	3	10
—Apparent tumor, recurrence	2	0	2
—No apparent tumor, post-op	0	5	5
Controls			
From patients with:			
—bronchogenic adenocarcinoma	0	11	11
—bronchogenic large cell carcinoma	0	1	1
—gastric cancer	0	4	4
—colonic cancer	0	3	3
—tuberculosis	0	20	20
Healthy volunteers	0	30	30

When the antigen fraction purified from BAC-Pfr or BSC-Pfr by reverse immunoabsorption, after concentration, was tested against the absorbed and unabsorbed antiserum by gel diffusion, a single precipitin line was produced. By disc electrophoresis in acrylamide gel, the purified fraction gave rise to a single band (Figs. 6a and 6b). When a comparable region was sliced from an unstained gel in which more than 200 μg protein of BAC-Pfr or BSC-Pfr was migrated and reacted against the antiserum by gel diffusion, a single though somewhat broad line was produced. By SDS-polyacrylamide gel electrophoresis, the fraction purified from BAC-Pfr, after dansylation, showed a single zone of apparent mol. wt 62,000–63,000 and that from BSC-Pfr, a zone of mol. wt 63,000–64,000. When the protein concentration was doubled, the electrophoretic patterns of both fractions showed no additional components.

DISCUSSION

We have demonstrated an antigen associated with human BAC and BSC, respectively, using hyperimmune xenoantisera against the extracts prepared from insoluble fractions of these tumors and extensively absorbed with a number of human materials. The antigens, a BAC- and a BSC-antigen, were detected in all BAC and BSC samples tested, respectively, but none in extracts of various tumors of histopathologically different types or in normal adult organ extracts and fetal organ homogenates. They appear to differ from the previously known antigens, such as CEA, α_1-fetoprotein, ferritin, lactoferrin, C-reactive protein, major blood group antigens, and Forssman antigen. They appear to be also different from the previously described antigens in lung cancers. In contrast to the antigens described by Yachi *et al.* [5], Schlipköter *et al.* [7], Sega *et al.* [8], Frost *et al.* [12] and Veltri *et al.* [16], our antigen are not soluble in 50% saturated ammonium sulfate. The antigens described by Veltri *et al.* [16] cross-reacted with anti-ferritin serum or anti-lactoferrin serum while our antigens failed to react with these antisera. The antigens can further be distinguished from the antigens described by other investigators [6, 9–11, 13–15] by their absence from normal tissues or tumors of other histopathological types. They seem to be not merely present in higher concentrations in tumor tissue than in normal tissue since pre-diffusion in agar of a large quantity of normal lung Pfr did not alter the pattern of the specific precipitin line formation. The possibility that the antigens could be related to histocompatibility antigens is ruled out in the present investigation since each antiserum which was absorbed with the Pfr preparation from normal lung associated with the tumor used for its preparation reacted with the extract or living cells of the tumor but not with those of the normal lung from the same patient. The absence of β_2-microglobulin in the Pfr preparation and the fact that all the Pfr from BAC and BSC tested react with the corresponding antiserum also reduce the above possibility. Based on these criteria, the BAC- and BSC-antigen appear to be tumor-specific neoantigens. In this respect, however, further studies by more sensitive methods will be required.

As can be seen in Tables 2 and 3, the detection of circulating antigens in the serum of patients appears to have potential diagnostic usefulness although the state of the antigens in the serum of patients is as yet not clear. However, the high percentage of positive sera presented in BAC and BSC patients could be due to that the sera were collected from the hospital patients with advanced clinical disease. The amount of serum circulating antigen may vary depending on the clinical staging of the tumor; consequently, the value of this assay as a primary screening procedure for carcinoma may remain to be proven. Rather than concentrating on studies on circulating antigens, efforts should be made to comprehensively analyse cell-mediated responses to these antigens. Further work along this line is under way in this laboratory.

The present antigens were shown to be cell membrane-associated by the LCMF test. It is at present not decided whether these molecules are also distributed in the intracellular membranes of tumor cells. The antigens could be purified by reverse immunoabsorption. The obtained fractions were shown to be homogeneous as judged by polyacrylamide gel electrophoresis in the present and absence of detergent. The antigens have mol. wts of about 63,000 which are different from those of the BAC-associated antigen recently described by Braatz *et al.* [26] and the BSC-associated antigen of glycoprotein nature described by Wolf [27]. Efforts in our laboratory are also directed toward further characterization of these antigens.

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