Specific Antigens in Human Alveolar Cell Carcinoma

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SUMMARY

Heterologous antiserum was made in rabbits against a virus-like particle pellet obtained from a human alveolar cell carcinoma tumor cell line. This antiserum was used for detection of similar antigens in other alveolar cell carcinoma tumor cell lines, original uncultured tumor, and infected “normal” cells. The methods used were immunodiffusion and immunofluorescence. The results indicate that similar antigens are present in original tumors from different patients and in the tumor cell lines derived from these tumors. It is also shown that the same antigen is induced following infection of continuous commercial cell lines (WI-38 and BHK-21).

INTRODUCTION

An ultrastructural study of ACC by Coalson et al. (2) reported the presence of virus-like material in the nucleus of tumor cells. Subsequent in vitro studies by Coalson et al. (3) on alveolar cell carcinoma demonstrated that a biologically active agent obtained from ACC could induce cytopathic effects on a variety of cell lines. More recently, ultrastructural studies by Nordquist et al. (R. E. Nordquist, R. E. Coalson, J. A. Mohr, and J. J. Coalson. Observations on a Virus-like Particle Associated with Aveolar Cell Carcinoma, submitted for publication) have shown that the tumor cell cultures of Coalson et al. (3) have an associated virus-like particle. This work further showed that the particles incorporated uridine-3H and could be partially separated by centrifugation. The availability of continuous tumor cultures, which were productively infected, prompted an immunological approach since the identification of such agents by morphological means alone is unreliable.

The purpose of this study was to determine whether the cultures from different ACC tumors contained similar antigens and to compare these antigens with those in the original uncultured tumor tissue and those induced in “normal” cells. Some of the techniques used in this study made possible the localization of these antigens.

MATERIALS AND METHODS

Cell Cultures. Continuous tumor cell cultures from 3 alveolar cell carcinomas and indicator cell lines (WI-38, BHK-21) were cultivated at 37° with 5% CO₂ on Eagle’s minimal essential medium with the addition of 1% glutamine, 10% heat-inactivated fetal calf serum, and a variety of antibiotics. Methods of establishing these continuous tumor cultures have been reported elsewhere (3).

Production of Antiserum. Tumor cultures that supplied supernatant known to induce cytopathic effects in indicator cells were subcultured to increase medium yields. Pools of culture medium were then spun at 800 × g, followed by 20,000 × g and 100,000 × g. The final pellet was resuspended in the smallest possible volume of PBS and emulsified in an equal volume of complete Freund’s adjuvant. The resulting mixture was injected into the footpads of young New Zealand White rabbits; this procedure was followed by giving the animals a booster injection in each flank every 45 days. The rabbits were bled by cardiac puncture 21 days after the initial inoculation and every 30 days thereafter.

The serum was pooled and the IgG was separated by ammonium sulfate precipitation and DEAE-Sephadex A-50 column chromatography. Purity was assayed by immunoelectrophoresis and double diffusion against goat anti-rabbit IgG.

Fluorescein isothiocyanate was coupled to 10 mg of IgG using the method of Clark and Shepard (1). The conjugated IgG was applied to a DEAE-Sephadex A-50 column and eluted with a gradient of PBS and 1 M NaCl; 0.001 M phosphate, pH 7.2. The fluorescein:protein ratio of the various fractions was determined and the conjugate was concentrated to 1 mg/ml. For staining, a conjugate fraction with a fluorescein:protein ratio of 1 to 2 (on a mole:mole basis) was selected and diluted to a protein concentration of 0.02 mg/ml.

Absorption for Normal Cellular Antigens. Absorption of purified IgG was accomplished with lyophilized calf serum, lyophilized tissue culture medium, normal human white cells, normal human serum, and normal human lung powder. Each of the absorbing powders were mixed 1:1 with IgG (1 mg/ml) in PBS and allowed to absorb at room temperature for 2 hr. Following this incubation period, the mixture was spun for 1 hr at 50,000 × g, and the supernatant was recovered.

Antigens. Antigens were obtained from: (a) PBS extracts of original tumor tissue, (b) supernatant from tumor cell cultures, (c) supernatant from inoculated indicator
cells and, (d) T-E extraction of original tumors and virus pellets. Control antigens were normal human lung, normal human leukocytes, and supernatant from uninfected indicator cell lines.

**Preparations for Immunofluorescence.** Tumor cell cultures, infected indicator cells and control cells were grown on coverslips in Leighton tubes with standard growth medium. At various intervals, coverslip preparations of infected indicator cells and controls were fixed in acetone, air dried, and stored at −70° for immunofluorescent studies. Before staining, the coverslips were warmed to room temperature and rinsed in PBS. The fluorescent antiserum (protein concentration, 0.02 mg/ml) was applied to the tissue surface and incubated in a moist chamber for 30 min at room temperature. The coverslips were then rinsed in frequent changes of PBS for 1 hr, mounted in buffered glycerin, and viewed on an Olympus UV microscope with Wratten No. UG-1 or BG-12 exciter filters and Y-52 or O-54 barrier filters. Photographs were taken using a Wild automatic camera system and Ektachrome X color film.

Tumor tissue for immunofluorescence was frozen sectioned to 8 to 10 µm and fixed for 10 min in acetone; the staining sequence was the same as described above.

**Immunodiffusion Studies.** The method for gel diffusion was essentially that of Ouchterlony (4), using various percentages of Noble agar, plate thickness, and hole patterns; however, all results herein were obtained with 1% agar in a layer 4 mm thick. Diffusion plates were allowed to run for 7 days at room temperature and washed in PBS for a minimum of 24 hr. Photographs were taken with a Polaroid camera on black and white film.

**RESULTS**

**Immunodiffusions.** Gel diffusion plates were performed with rabbit IgG anti-ACC antigens. Antigens for diffusion were obtained from original tumors, tumor cell cultures, and infected indicator cell lines. It was found that absorption of the antiserum was essential because of the presence of antibodies directed against normal cellular antigens and components of the tissue culture medium. However, sequential absorption with normal human lung and leukocytes, lyophilized calf serum, culture medium, and normal human serum reduced the number of precipitation lines to 1. If the fully absorbed antiserum was then exposed to ACC tumor cells, the precipitation line was abolished.

This precipitation line could be found against original tumors, tumor cell cultures, and infected indicator cell cultures but was never observed against normal tissues or control tissue culture cells (Fig. 1, Well h; Fig. 2, Wells a and e; Fig. 3, Well d; Fig. 4, Wells a and e; Fig. 5, Wells a and e).

Diffusion detectable antigens were readily recovered from the medium of tumor cell cultures and infected indicator cells but were not present in medium recovered from normal lung cultures or uninfected indicator cell cultures (Fig. 2, Wells a and e). Antigens were also undetectable in the medium recovered from the 1st and 2nd passages of tumor cells in culture (Fig. 1, Well g; Fig. 2, Wells f and h; Fig. 4, Wells f and h; Fig. 5, Wells b, f, and h) but appeared gradually in the 3rd and 4th passages of all tumor cell cultures.

Original tumor tissue from Patient Sch. was frozen at −70° immediately after biopsy and extracted with PBS or with T-E during homogenization at room temperature. Fig. 1, Well a, demonstrates the absence of a line to the PBS extract of this tumor. However, when the same tumor is extracted with T-E, a line is formed (Fig. 3, Well f) that shows identity with a T-E extract of a high-speed pellet of particles recovered from the medium of ACC tumor cell cultures (Fig. 3, Well a). These T-E extracts form a line of identity with unextracted medium from tumor cell cultures (Fig. 3, Well b) and is continuous with the line formed by infected indicator cells (Fig. 3, Well c). Yet Wells d and e demonstrate that the antigen is not present in uninfected indicator cells (Fig. 3, Well d) or in normal human serum (Fig. 3, Well e).

With medium from Patient Sch. tumor culture as a standard, it may be seen in Fig. 2 that tumor cultures from other ACC patients contain identical cross-reacting antigens (Fig. 2, Well b, Patient Car.; Well c, Patient Ruz.; Well d, Patient Hix.). It is also shown in Fig. 2 that this antigen is not present in medium from normal human lung cultures (Fig. 2, Well e).

**Immunofluorescence.** Four ACC tumors from different patients were stained with antiserum directed against antigens from tumor cell cultures of a single patient. These studies on frozen sections of original ACC tumors indicate that specific staining occurs in malignant cells that lie in the alveolar space. The fluorescence was primarily found in the cytoplasm as uniform staining; however, one specimen exhibited fluorescence limited to discrete cytoplasmic bodies. Figs. 6 to 8 show fluorescent staining of 2 original tumors, Fig. 6 (Patient Sch.) demonstrates uniform cytoplasmic staining, while Figs. 7 and 8 (Patient Hal.) demonstrate particulate fluorescence.

Controls consisted of normal human lung, other lung neoplasms, and nonneoplastic diseased lung. No specific fluorescence was observed in these controls.

Immunofluorescent studies on early-passage ACC tumor cell cultures indicated that cytoplasmic staining could be found in 70 to 80% of the tumor cells in vitro. However, after 25 passages, only 4 to 5% of the tumor cells contained detectable antigens (Figs. 9 and 12). These figures show fluorescent staining of Patient Sch. tumor cell culture after 35 passages. It appears that most of the specific staining occurs in very large cells (Fig. 9), some of which are multinucleate (Fig. 11). Some cells contain large cytoplasmic bodies, which stain very brightly (Figs. 10 and 12). Also present in some cells are small, bright-staining nuclear bodies with much greater intensity than normal nucleolar fluorescence (Fig. 10).

Monolayers of human fetal lung (Wistar-38) were infected with medium from ACC tumor cell cultures and coverslip preparations were taken at various intervals. At 48 hr PI, the fluorescence was limited to a small number of cells and was quite dim, but at 8 days PI, approximately 95% of the cells showed brilliant cytoplasmic fluorescence.
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(Figs. 13 to 16). It was noted at this time that a small number of cells exhibited nuclear staining (Fig. 17). After 15 days PI, most of the cells had detached; however, those cells which remained affixed to the coverslip showed no staining at all. Control coverslips of noninfected Wistar-38 were completely negative for fluorescence (Fig. 18).

DISCUSSION

This study was designed to investigate the immunological properties of a biological agent associated with ACC. Although Coalson et al. (3) reported that the in vitro behavior of the agents from ACC tumors from different patients was identical, it was necessary to determine whether they also were antigenically similar. Moreover, if the agents from the in vitro studies were similar, how did they compare with the antigens of the original tumor? The finding that the cytopathic effect could be induced in normal cell cultures also raised the possibility of induction of specific antigens in cells which were previously considered normal and contained no detectable similar antigen. It may be seen in the results of these studies that the antigens from tumor cultures, original tumors, and infected "normal" cells do indeed share a single antigen, which is not present in normal tissue, uninfected tissue cultures, or other types of lung tumors.

Coalson et al. (3) reported that the tumor cultures ceased production of the agent responsible for cytopathic effect after 18 passages, but this study indicates that cytoplasmic antigens are still present in a small number of cells. This may be analogous to the findings of animal tumor virus systems in which cells are nonproductive but retain viral antigens. Coalson et al. (3) also showed that light microscopic inclusions were primarily associated with giant cells, many of them multinucleate. This directly correlates with the present fluorescent studies of ACC tumor cell cultures, where most fluorescence was confined to uniform cytoplasmic staining in large cells or nuclear and cytoplasmic inclusions in a few others.

Fluorescent staining of "normal" tissue culture cells after infection indicates that not all cells in a culture are receptive to infection and that a small percentage are completely resistant, even after long-term exposure to the agent. Fluorescent staining also shows that the antigen is intracellular and not completely plasma membrane-associated, ruling out the possibility that the antigen is a specific tumor surface membrane antigen. Fluorescence also revealed the presence of nuclear antigens in a small number of cells PI.

The results herein, which show that T-E extracts of original tumors cross-react with supernatant from ACC tumor cell cultures, lends credence to the hypothesis that the observed antigen is truly associated with this tumor and not induced by culturing or contamination.

The sum of the data in this communication suggests that biological agents with similar antigens may be found in ACC from different patients and that this agent can induce cytopathic effects and specific cellular antigens in "normal" cell lines in vitro.

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REFERENCES

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Fig. 1. Immunodiffusion (center well) antiserum (fully absorbed). All culture fluids were taken between the 5th and 35th passage unless denoted otherwise. a, Patient Sch., ACC original tumor, PBS extract; b, Patient Sch., ACC tumor cell culture; c, Patient Bun., adenocarcinoma, PBS extract; d, Patient Hix., ACC tumor cell culture; e, blank; f, Patient Sch., ACC tumor cell culture; g, Patient Sch., ACC tumor culture, 1st passage; h, normal human lung culture.

Fig. 2. Immunodiffusion (center well) antiserum (fully absorbed). All culture fluids were taken between the 5th and 35th passage unless noted otherwise.

Arrow, nonspecific line, which is a scratch on the Petri dish below the gel. a, Wistar-38 control; b, Patient Car., ACC tumor cell culture; c, Patient Ruz., ACC tumor cell culture; d, Patient Hix., ACC tumor cell culture; e, normal human lung culture; f, Patient Has., ACC tumor cell culture, 2nd passage; g, Patient Sch., ACC tumor cell culture; h, Patient Whi., ACC tumor cell culture, 1st passage.

Fig. 3. Immunodiffusion (center well) antiserum (fully absorbed). All culture fluids were taken between the 5th and 35th passage. a, Patient Sch., virus pellet, T-E extract; b, Patient Sch., ACC tumor cell line, unextracted supernatant from the cultures; c, Wistar-38, infected with supernatant from tumor cell line (Patient Sch.); d, Wistar-38 control; e, normal human serum; f, Patient Sch., ACC original tumor, T-E extract.

Fig. 4. Immunodiffusion (center well) antiserum (fully absorbed). All culture fluids were taken between the 5th and 35th passage unless noted otherwise. a, Wistar-38 control; b, baby hamster kidney, infected with supernatant from tumor cell culture (Patient Sch.); c, Patient Hal., ACC tumor cell line; d, Patient Sch., ACC tumor cell line; e, normal human lung culture; f, Patient Has., ACC tumor cell line, 2nd passage; g, Patient Sch., ACC tumor cell line; h, Patient Whi., ACC tumor cell line, 1st passage.

Fig. 5. Immunodiffusion (center well) antiserum (fully absorbed). All culture fluids were taken between the 5th and 35th passage unless noted otherwise. a, Wistar-38 control; b, Wistar-38, infected with 1st passage tumor cell line from Patient Sch.; c, Patient Ruz., ACC tumor cell line; d, Patient Sch., ACC tumor cell line; e, normal human lung culture; f, Patient Has., ACC tumor cell line, 2nd passage; g, Patient Sch., ACC tumor cell line; h, Patient Whi., ACC tumor cell line, 1st passage.
Fig. 6. Immunofluorescence of patient Sch., original tumor. Arrows, staining of individual cells in the alveolar space; note the surrounding cells which are unstained. The intensely staining areas on each side are elastic fibers which autofluoresce strongly. × 200.

Fig. 7. Immunofluorescence of Patient Hal., original tumor. Arrows, malignant cells hanging on the alveolar walls; elastic fibers are visible in the septum. × 400.

Fig. 8. Immunofluorescence of Patient Hal., original tumor. These cells are free in the alveolar space; some neighboring cells are not stained. × 400.

Fig. 9. Immunofluorescence of Patient Sch. tumor cell culture, 35th passage. Note the large cell which demonstrates uniform cytoplasmic fluorescence. The surrounding area contains many unstained cells, which are of normal size. × 400.

Fig. 10. Immunofluorescence of Patient Sch. tumor cell culture, 35th passage. The cell in the center has a large fluorescent body in the cytoplasm and a smaller one of equal intensity in the nucleus. Arrow, normal nucleolar fluorescence. × 400.

Fig. 11. Immunofluorescence of Patient Sch. tumor cell culture, 35th passage. This field shows the center of a giant multinucleate cell, where intense fluorescence is localized in the area surrounding the many nuclei. × 200.

Fig. 12. Immunofluorescence of Patient Sch. tumor cell line, 35th passage. The cell in the center of the plate contains an intensely fluorescent cytoplasmic inclusion. In the surrounding field many unstained cells are present. × 200.
Figs. 13 to 17. Immunofluorescence of Wistar-38, 8 days PI with supernatant medium from Patient Sch. tumor cell line. Figs. 13 to 16 demonstrate cytoplasmic fluorescence; Fig. 17 shows fluorescence of the total nuclear area with no discernible cytoplasmic staining. Fig. 13, × 100; Figs. 14 and 16, × 200; Figs. 15 and 17, × 400.

Fig. 18. Wistar-38 control. This photomicrograph shows that uninfected cells exhibit no fluorescence. × 200.
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