Altered Expression in Squamous Carcinoma Cells of an Orientation Restricted Epithelial Antigen Detected by Monoclonal Antibody A9

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ABSTRACT

The monoclonal IgG2a antibody A9 was raised to the UM-SCC-1 human squamous cell carcinoma cell line. Hemadsorption assays using monolayer cultures as target cells revealed a restricted range of A9 reactivity with human cell lines. The A9 antibody was reactive with 29 of 34 squamous cell carcinoma cell lines and with 5 epithelial cancer cell lines of non-squamous origin. In contrast, no antibody binding was detected with twelve malignant melanoma, two fibrosarcoma, two malignant lymphoid, two transitional cell carcinoma, or four adenocarcinoma cell lines. Similarly, normal lymphoid cells, RBC, and fibroblasts from multiple donors were negative. The relative expression of the A9 antigen varied greatly among positive squamous cancer lines such that the 50% endpoint titer of A9 ascites fluid ranged from less than 104 for low antigen expressor lines to 106 for strong antigen expressers. Hemadsorption tests with secondary passage cultures of normal squamous cells failed to detect A9 binding to the cell surface. However, in experiments with intact colonies in primary cultures of normal squamous cells, antibody binding was observed at the periphery of individual colonies. Mechanical detachment of such colonies revealed A9 antigen bound to the plastic surface underneath the cells, but the newly exposed surface of the cells, like the upper surface, was negative. In contrast, when cells of similar cultures were detached by trypsinization prior to testing no antigen remained on the plastic, but approximately 30% of the trypsinated cells were positive. Similar experiments with the five surface-negative squamous cell carcinoma cell lines revealed that cryptic A9 antigen could also be detected underneath mechanically detached cells and on the surface of 100% of trypsinated cells. Trypsinization of melanomas and fibroblasts did not reveal A9 antigen. Immunoperoxidase assays on frozen sections of normal epidermis localized A9 antigen to the basal cells and the basement membrane region. In frozen sections of squamous cancers, individual tumor cells and particularly the growing edge of the tumors were strongly stained by A9, while in basal cell cancers only very slight staining could be detected. Thus, in normal epithelial cells the A9 antigen appears to be largely specific for SCC. On further investigation, it was found that A9 antigen on normal epithelial cells is not immediately apparent because it is topologically restricted to the basal surface of individual cells. This restriction is lost in most squamous cancer lines. The stratified squamous epithelia of epidermis and mucosa from which squamous cell cancers arise are highly organized tissues in which there is a distinct sidedness or asymmetry such that the cells have a down side that is different from the up side. In our analysis of membrane antigens of squamous cell carcinomas we found that this sidedness and its possible disorientation in cancer must be considered in interpreting the binding and absolute specificity of antibodies to human squamous cell cancers.

INTRODUCTION

To characterize the cell surface antigens that distinguish squamous cancers from normal squamous epithelial cells, we have established an extensive panel of cell lines from patients with SCC (1–3). Cells from these lines have been tested serologically for the expression of widely distributed antigens such as the polymorphic class I and II histocompatibility antigens (HLA and HLA-DR), β2-microglobulin, and the AB and H blood group antigens, as well as squamous cell specific antigens, including those recognized by autoantibodies from patients with pemphigus vulgaris and bullous pemphigoid (1, 4). By testing these antibodies against squamous and non-squamous carcinoma cell lines, we can now define the mucosal squamous carcinoma phenotype to be HLA+, β2-microglobulin+, HLA-DR+, blood group+ (usually), pemphigus+, pemphigoid+ (usually) (5). Serological analysis of cultures of normal epithelial tissue indicates that this phenotype is probably also characteristic of normal mucosal squamous cells.

To search for antigens unique to cancer cells, we have used two approaches. The first is to use each patient's serum as a potential source of anti-tumor antibody (2, 6); the second is to raise and select monoclonal antibodies specific for squamous cancer cells. In this report, we describe the murine monoclonal antibody A9. This antibody, which was raised to the UM-SCC-1 cell line, bound to most SCC lines and initially appeared to be largely specific for SCC. On further investigation, it was found to define an antigen that is present in normal tissue sections in the basement membrane zone and on the stem cells of the epithelium. In culture, the expression of the A9 antigen on normal epithelial cells is not immediately apparent because it is topologically restricted to the basal surface of individual cells. This restriction is lost in most squamous cancer lines. The stratified squamous epithelia of epidermis and mucosa from which squamous cell cancers arise are highly organized tissues in which there is a distinct sidedness or asymmetry such that the cells have a down side that is different from the up side. In our analysis of membrane antigens of squamous cell carcinomas we found that this sidedness and its possible disorientation in cancer must be considered in interpreting the binding and absolute specificity of antibodies to human squamous cell cancers.

MATERIALS AND METHODS

Cell Lines. All cell lines designated UM-SCC were established in our laboratory from tumor specimens of patients with squamous carcinoma of the head and neck region as described previously (1–3). Other cell lines used include melanomas SK-MEL-1, 5, 8, 9, 13, 22, 26, 27, 28, 35, 37, and MeWo (6, 7); colon carcinoma HT29; ovarian carcinoma SK-OV-3; duodenal carcinoma HuTu 80; breast carcinoma AlAb; squamous carcinomas of the uterine cervix ME-180 and HT3; cervical adenocarcinoma HeLa; transitional cell carcinomas of the bladder J82, T24, and RT4; Burkitt's lymphoma Daudi; T-cell leukemia Jurkat; and the human diploid fibroblast line WI-38. These cell lines were obtained from the human tumor cell line bank of the Memorial Sloan-Kettering Cancer Center (Rye, NY) through the courtesy of J. Fogg (8). The human fetal lung fibroblast line HFLF was established by T. Carey. Renal adenocarcinoma UM-RC-6 and squamous cell carcinoma of the prostate UM-SCP-1 were obtained from H. B. Grossman of The University of Michigan, Ann Arbor, MI (9). Pancreatic adenocarcinoma UM-Pad-1, lung adenocarcinoma UM-Lad-1, and fibrosarcomas UM-FS-1A and IB were established in our laboratory.

Cell lines with the same numerical designation followed by A or B were derived from the same patient. Cell lines UM-SCC-10A, 10B,
cells and fibroblasts were cultured from normal skin or mucosa tissue and metastasis. Tumor tissue obtained at the same time from different sites, i.e., primary different times. Cell lines 17A, 17B, 22A, and 22B were derived from cultures were often free of fibroblasts. Such primary epithelial cell experiments in which the A9 expression of fibroblasts and express higher levels of HLA reactivity than do the squamous epithelial cells. For experiments in which the A9 expression of fibroblasts and normal epithelial cells were found in clusters or colonies. Fibroblasts were broadly defined as bipolar spindle-shaped and widely spreading cells that were readily detached by trypsin and EDTA in contrast to the epithelial cells, which detached only after prolonged exposure to trypsin and EDTA. Secondary cultures of the readily detached cells contained nearly 100% bipolar spindle cells or spreading cells. Cells undetached by short trypsin-EDTA exposure were mostly of epithelial morphology. In addition, in other experiments we have shown that the epithelial cells but not the fibroblasts express squamous cell specific antigens such as the pemphigus antigen, the bullous pemphigoid antigen, and blood group antigens AB and H, depending on the donor's blood type. Similarly the fibroblasts express higher levels of HLA reactivity than do the squamous epithelial cells. For experiments in which the A9 expression of fibroblasts and normal squamous epithelial cells were to be compared, fibroblasts were separated from mixed cultures by brief trypsin treatment, and a second more prolonged trypsinization was used to harvest the epithelial cells. The separate fibroblast-enriched and the epithelial cell-enriched populations were then either washed and tested in suspension or were plated in microtest wells and allowed to adhere for later serologic testing as monolayer cultures. Culturing tissue fragments in Petri dishes proved to be relatively selective for the growth of epithelial cells, and these cultures were often free of fibroblasts. Such primary epithelial cell colonies were tested without subculturing. For some experiments, keratinocyte cultures established from normal adult skin (10) were provided by R. C. Grekin (University of Michigan, Ann Arbor, MI).

All cultures were maintained in Eagle's minimal essential medium containing 15% FBS, 2 mM glutamine, 1% nonessential amino acids, penicillin (100 units/ml) and streptomycin (100 μg/ml).

Hemadsorption Assays. Antibody binding to target cells was detected with either the protein A or anti-mouse immunoglobulin hemadsorption assay (11) adapted for use in microtest plates. Three to 5 days prior to the assay, target cells were plated in 72-well microtest plates (Nunc 1-36526; Grand Island Biological Co., Grand Island, NY). At the time of testing, the plates were rinsed with phosphate-buffered saline containing 5% γ-globulin-free FBS (PBS-FBS); then target cells were incubated at room temperature for 1 h with serial dilutions of antibody or appropriate controls. Unbound antibody was removed with several PBS-FBS washes. Target cells were then incubated for 1 h at room temperature with indicator cells, prepared by coupling rabbit anti-mouse immunoglobulin (Dako Z109, Piscataway, NJ) to human type O erythrocytes with 0.01% CrCl₃. The plates were washed again with PBS-FBS, and the binding of indicator cells was determined by light microscopy. Antibody titer is expressed as the reciprocal of the dilution above.

To test antibody binding to target cells in suspension the hemadsorption assay was slightly modified. Cells from lymphoid cultures were harvested by centrifugation. Cell suspensions from monolayer cultures were prepared by trypsinization with a solution consisting of trypsin (135 units/ml) (TRL Trypsin, Worthington, Freehold, NJ) and 0.02% EDTA in Puck's Saline A. Cells were washed in PBS-FBS and resuspended to a concentration of 4 × 10⁶ cells/ml, and 0.05 ml of each cell suspension was added to 12 × 75 mm glass tubes. Appropriate antibody dilutions (0.100 ml) were added to each tube and the mixture was incubated for one hour at room temperature. Unbound antibody was removed by washing target cells twice with PBS-FBS. Indicator cells (0.05 ml) were then mixed with the pellet and the tubes were incubated for one hour at room temperature. Antibody binding was determined by counting the percentage of rosetted cells in hemocytometer chambers.

In experiments designed to test for antibody binding to antigen underneath monolayer cultures, cell lines were grown in 35-mm 6-well plates (Falcon 3046; Becton-Dickenson, Oxnard, CA). Some of the cells were then stripped from the plates by scraping through the monolayer with forceps or rubber scrapers, or some of the cells were removed enzymatically by incubation with trypsin-EDTA for 1–5 min. In each case, the treatment was designed to remove only a portion of the cells. Each well was then tested for the presence of antigen on stripped areas and on remaining cells. Undiluted hybridoma culture supernatant or an appropriate dilution of ascites fluid was added to each well, and the hemadsorption assay was performed as described above.

Monoclonal Antibodies. Monoclonal antibody A9 was produced by immunizing a BALB/c mouse with whole cells from the UM-SCC-1 cell line, which was derived from tumor tissue of a patient with recurrent squamous cell carcinoma of the floor of the mouth. Immune spleen cells were fused to NS-1 myeloma cells with 30% polyethylene glycol 1000 (Koch-Light Laboratories Ltd., Berks, England), and hybrids were selected in Iscove's modified Dulbecco's medium (catalogue no. 430-2200; Grand Island Biological Co.) containing 20% FBS, 1 × 10⁻⁴ M hypoxanthine, 1.6 × 10⁻⁸ M thymidine, and 4 × 10⁻⁷ M aminopterin (12). Hybridomas were first screened for production of antibody to UM-SCC-1. Positive hybrids were subcloned by limiting dilution and re-screened against a panel of human cell lines consisting of UM-SCC-1, UM-SCC-13, SK-MEL-28, MeWo, SK-OV-3, UM-PAd-1, and WI-38. Selected hybrids were subcloned a second time by limiting dilution. Monoclonal antibody stocks were collected from spent culture medium or pooled from ascites fluid of hybridoma tumors grown in pristane-primed BALB/c mice. Ascites fluid from tumors induced with NS-1 cells was used for control purposes in some experiments. The A9 monoclonal antibody is of the IgG₂a class as determined by Ouchterlony double diffusion using subclass specific rabbit antibody purchased from Litton Bionetics (Bethesda, MD). All experiments were originally performed with A9 culture supernatant and with A9 culture supernatant from hybridoma stocks grown in serum free culture medium (13). To insure that low levels of expression of the A9 antigen were not missed, a pool of ascites fluid with high A9 antibody content was used for most experiments with cell lines. All immunoperoxidase experiments were performed with supernatant from A9 hybridoma cells grown in Iscove's modified Dulbecco's medium. No differences in specificity or reactivity were detected in separate batches of ascites fluid or supernatant. A9 antibody partially purified from supernatant or ascites fluid by ammonium sulfate precipitation and by affinity chromatography on protein A columns retained the same specificity and reactivity patterns as the ascites fluid and supernatants.

The IgM monoclonal antibodies G10 and E7 were produced from the same fusion as A9 and were used routinely as positive controls in the assays. The E7 antibody binds to a cell surface antigen controlled by a locus on human chromosome 11 and is expressed by most human cells (14). The G10 antibody recognizes the H type 2 blood group determinant which is expressed on normal and malignant squamous cells (15). The purified IgG₂a myeloma protein LPC-1 used as a subclass-matched negative control in some experiments was kindly provided by Dr. L. Claflin (University of Michigan, Ann Arbor, MI).

Immunoperoxidase Assays. Fresh tissues were snap frozen in liquid nitrogen. Sections were cut at 6 to 8 μm and mounted on ovalbumin-coated slides. Antibody binding to tissue sections was determined with the immunoperoxidase staining method using the Vectastain avidin-biotin complex kit for mouse IgG (Vector Laboratories, Inc., Burlingame, CA) as described in the kit instructions. Sections were incubated sequentially with normal horse serum (20 min), undiluted A9 culture supernatant or Iscove's modified Dulbecco's medium (30 min), bioti-
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Fig. 1. Phase contrast photomicrograph showing an anti-immunoglobulin hemadsorption assay using a 1/1000 dilution of A9 ascites fluid with cells of human squamous carcinoma cell lines. A, UM-SCC-1 showing binding primarily to the edges of colonies in a non-confluent culture (original magnification, 1:320); B, UM-SCC-10B showing A9 binding over the surface of a confluent monolayer (original magnification, 1:100).

nylated horse anti-mouse IgG (30 min), 0.3% hydrogen peroxide in methanol (30 min), and avidin biotin complex reagent containing avidin and biotinylated horseradish peroxidase (60 min). Incubations were performed at room temperature, and sections were rinsed with PBS, pH 7.6, between each step. Finally, sections were incubated for 5 min with freshly prepared substrate solution consisting of 0.05% 3,3'-diaminobenzidine tetrachloride and 0.03% hydrogen peroxide, washed in distilled water, counterstained with hematoxylin, and mounted in Permount.

RESULTS

Reactivity With Cell Lines. Serial 10-fold dilutions of A9 ascites fluid were tested by hemadsorption against human cell lines plated in microtest plates (Fig. 1). For each cell line, NS-1 ascites was included as a control for nonspecific binding. Antibody A9 reacted with 29/34 SCC lines and 5 non-SCC epithelial lines (Table 1). The non-SCC tumor lines positive with A9 were four adenocarcinomas (HeLa, A1Ab, UM-PAD1, UM-RC-6) and one transitional cell carcinoma (RT-4). Antibody A9 did not bind to twelve melanoma lines, four adenocarcinoma cell lines, two transitional cell carcinoma lines, two fibrosarcoma lines, two long-term fibroblast lines, or to Daudi (a Burkitt’s lymphoma) or Jurkat (a T-cell leukemia).

Major differences in relative antigen expression among the A9 positive monolayer cell lines were observed. The end point titer with positive cell lines ranged from $10^4$ to $10^8$ (Table 1). Of particular interest were those cases in which two SCC cell lines were available from a single donor. Two patterns of reactivity were observed. For 10A and 10B, 11A and 11B, and 14A and 14B, the A cell line was less reactive with antibody A9 than was the B cell line (Fig. 2, top). In the other two examples (17A and 17B, 22A and 22B), both cell lines had nearly identical titration curves, with 17A and 17B being poor A9 expressors and 22A and 22B being high A9 expressors (Fig. 2, bottom). A variable between the two patterns of expression that may be significant is the time interval between establishment of the A and B cell lines. The A and B cell lines from UM-SCC patients 17 and 3616
Antibody reactivity with cell lines was determined with the anti-mouse immunoglobulin hemadsorption assay. Serial dilutions of A9 ascites fluid were tested on monolayer cultures of cell lines, in microtest plates. After incubation with the antibody the cells were rinsed, and bound antibody was visualized by incubating the monolayers with rabbit anti-mouse immunoglobulin-coated erythrocytes. After rinsing, each well was scored for percentage of target cells binding indicator erythrocytes. Positive cell lines are grouped according to the reciprocal of the antibody dilution at which 50% of the target cells were positive.

### Table 1 Reactivity of antibody A9 with cultured cell lines

<table>
<thead>
<tr>
<th>Squamous cell carcinoma</th>
<th>Adenocarcinoma</th>
<th>Transitional cell carcinoma</th>
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<tr>
<td>UM-SCC-5</td>
<td>UM-SCC-1</td>
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<td>UM-SCC-4</td>
<td>UM-SCC-12</td>
<td>UM-SCC-16</td>
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<tr>
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<td>UM-SCC-11B</td>
<td>UM-SCC-20</td>
<td>AlAb</td>
</tr>
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<td>UM-SCC-22A</td>
<td>UM-RC-6</td>
</tr>
<tr>
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<td>UM-SCC-3</td>
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</tr>
<tr>
<td>UM-SCC-2</td>
<td>UM-SCC-15</td>
<td>FD-1</td>
</tr>
<tr>
<td>UM-SCC-8</td>
<td>ME-180</td>
<td>(&lt;10^4)</td>
</tr>
<tr>
<td>UM-SCC-14A</td>
<td>UM-SCC-13</td>
<td>ND*</td>
</tr>
<tr>
<td>UM-SCC-17A</td>
<td>UM-SCC-18</td>
<td></td>
</tr>
<tr>
<td>UM-SCC-17B</td>
<td>UM-SCC-24</td>
<td></td>
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**Positive**

**Negative**

*ND, cell lines were positive with A9 culture supernatant, but the titration curve with ascites was not determined.

* Antibody reactivity with lymphoid cell lines was determined with the anti-mouse immunoglobulin hemadsorption assay modified for use in 12 x 75 mm tubes rather than microtest plates (see Table 2).

22 were obtained in each case during one surgical operation in which tumor tissue was removed from different sites (primary sites 17A and 22A and metastases to cervical lymph nodes, 17B and 22B).

**Reactivity With Normal Cells.** RBC from multiple donors of all ABO blood groups were tested for reactivity with A9 antibody in slide and tube hemagglutination tests. No hemagglutination activity with A9 antibody was found.

Lymphocytes and fibroblasts from multiple donors were negative in hemadsorption tests with A9 antibody. Normal epithelial cells, when trypsinized and subcultured in microtest plates before testing, were also negative for A9 binding. However, because of the low plating efficiency of these secondary cultures and also because it was difficult to subculture normal squamous epithelial cells completely free of the more rapidly growing fibroblasts, these results were considered inconclusive. Therefore, primary epithelial cell colonies were cultured in Petri dishes and tested for antigen expression in situ. Control antibody G10, which is directed against the H type 2 blood group antigen (15), exhibited strong surface binding to intact colonies (Fig. 3A). In contrast, no A9 binding was seen on the surface of the cells. However, some binding of antibody A9 was observed on the peripheral growing edge of each colony (Fig. 3, B and C). This pattern was consistently found on cultures from different individuals (both adult and neonatal), both with A9 ascites at dilutions of 1/10 and 1/100 and with serum free and serum containing A9 culture supernatants, but it was never seen in control cultures incubated with NS-1 ascites (diluted 1/10 or 1/100), PBS, Iscove's medium with 20% FBS, or the irrelevant IgG2a monoclonal antibody LPC-1 (Fig. 3, D and E).

**Detection of A9 Underneath Cultured Cells.** The peripheral distribution of the A9 antigen in intact colonies suggested that it might be associated with an extracellular substance secreted as a substrate material by dividing cells. By gently lifting the edge of the colony with a hypodermic needle or by grasping the central tissue fragment with forceps, the cells could be removed from the Petri dish in intact sheets. Areas of the plate to which cells had been attached were strongly reactive with antibody A9 (Fig. 4, A and B). Binding was not detected on the underside of the monolayer cells; rather, all of the binding activity was confined to the substrate. No binding was observed when PBS, NS-1 ascites fluid, or the LPC-1 hybridoma protein were tested (Fig. 4C).

To determine whether A9 was present under SCC cells as well as normal epithelial cells, cells from the UM-SCC-17A line (a low A9 expressor) were grown in 35-mm culture dishes. Although the tumor cells could not be reflected in sheets as was done with normal cells, some of the cells on each dish were removed mechanically by scraping. Binding of indicator cells was observed on scraped areas and was greater than that observed on the top of the cells (Fig. 5A). In other dishes, the cells were briefly treated with trypsin and EDTA. In these
ALTERED EXPRESSION OF A9 ANTIGEN IN SCC

Fig. 3. Phase contrast photomicrographs showing anti-immunoglobulin and protein A hemadsorption assays of monoclonal antibodies with primary monolayer cultures of normal epithelial cells. A, positive control antibody G10 (ascites fluid diluted 1/10). Shows strong binding to the upper surface of cells. Original magnification, 1:100. B, antibody A9 (ascites fluid diluted 1/10). Shows binding of anti-immunoglobulin indicators only along the edge of the monolayer. Original magnification, 1:100. C, antibody A9 (undiluted serum-free culture supernatant). Shows binding of protein A indicator cells to edge of monolayer. Original magnification, 1:320. D, negative control NS-1 (pooled ascites fluid diluted 1:10). Shows no reactivity with protein A indicator cells. Original magnification, 1:100. E, negative control LPC-1 antibody (100 μg/ml) (anti-immunoglobulin indicator cells). Original magnification, 1:100.

Disks, the antigen was not retained on areas from which the cells had detached. However, 100% of the remaining cells gave a positive hemadsorption reaction (Fig. 5B). The same result was obtained with other low expressor cell lines. This was not due to nonspecific binding of indicator cells to scraped or trypsinized monolayers, since PBS, NS-1 ascites, and LPC-1 controls remained negative.

UM-SCC-9 did not bind antibody A9 when tested in microtest plates (Table 1). However, when these cells were grown in larger wells we noted that peripheral binding, like that observed
with normal epithelial cells, could be detected around untrypsinized monolayers at high antibody concentration (1/10 dilution of ascites) (Fig. 6A). As before, no reactivity was observed on the surface of the cells. After exposure to trypsin and EDTA for 5 min, the UM-SCC-9 cells were approximately 90% positive even at a 1/10^6 dilution of ascites (Fig. 6B).

Expression of A9 on Trypsinized Cells. Since partial trypsinization increased the amount of detectable A9 antigen, we confirmed the results of Petri dish experiments by assaying for A9 expression on cells detached completely by trypsinization. Normal epithelial cells and fibroblasts from six individuals (two adults, four newborns) were separated from primary cultures by sequential trypsinization and tested for A9 expression in suspension. One-fourth to one-third of the cells in the epithelial cell enriched populations were found to be positive. As shown in the representative experiment presented in Table 2, there was no loss of specific reactivity over a 100-fold dilution range, showing that this binding was unlikely to be due to irrelevant antibodies contributed by the mice used for ascites production. In agreement with the microplate assays, only 2 to 4% of the fibroblast-enriched population was reactive with A9. The squamous carcinoma UM-SCC-12 was also tested for A9 expression after complete trypsinization and was found to remain strongly A9-positive. No reactivity was observed with the NS-1 myeloma ascites fluid, while all cell populations were reactive with the positive control antibody E7. In other experiments (not shown) the melanoma cell line SK-MEL-8, negative for A9 binding in microplate assays, was also found to remain negative after trypsinization.

Localization of A9 Antigen in Tissue Sections. In immunoperoxidase assays on frozen sections of normal epidermis, undiluted A9 culture supernatant produced strong local staining of basal cells and the basement membrane zone (Fig. 7A). Keratinocytes in the upper layers of skin were not stained. When frozen tumor sections from six patients with SCC were treated, strong A9 staining was observed in and around the invading tumor cell islands (Fig. 7C). In addition, the tumor cells appeared to stain more strongly than did the corresponding normal basal cells. A distinct band of staining was observed around each island of SCC tumor. Frozen sections of BCE tumor tissue from six individuals were also tested; four BCE were weakly positive and two were negative. As with SCC sections, the staining was found as a distinct but faint band around the periphery of each island of tumor cells (Fig. 7E). In cases in which normal skin and BCE sections were available from the same patient, the tumor cells were less strongly stained than were the normal basal cells. No staining with A9 was found in formalin fixed tissue sections, indicating that the fixation process denatured the antigen.

DISCUSSION

The A9 antibody was selected for study based on its restricted pattern of reactivity with human cancer cell lines. Initially, A9 appeared to bind only to SCC lines and a few other tumor lines of non-squamous epithelial origin. Secondary passage cultures of epithelial cells derived from normal tissue were apparently unreactive with this antibody, which indicated that A9 might be a tumor-specific reagent. However, when intact primary colonies of normal epithelial cells were tested, the A9 antigen was found to be weakly expressed at the periphery of each colony. Additional experiments showed that the antigen was present on areas of culture dishes underneath primary colonies after the cells had been lifted away. The failure to demonstrate the A9 antigen on secondary cultures may be due to their entry into a more differentiated state. After primary colonies have been trypsinized, the normal epithelial cells have a low secondary plating efficiency on plastic. Those cells that do attach usually do not resume dividing and often appear to be more differentiated than the cells in the primary culture, as evidenced by a thickened, more refractile appearance under the microscope. This entry into a more differentiated state may coincide
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with a loss of the ability to produce detectable amounts of A9 antigen. It is likely that the A9 antigen can be detected on peripheral cells of growing primary colonies, because antigen synthesis is functionally related to continued cell growth and division.

Immunoperoxidase staining of frozen skin sections revealed that expression of the A9 antigen was confined to cells of the basal layer and to the acellular basement membrane region. Non-dividing cells of the upper squamous cell layers were negative for A9. This finding was consistent with results of hemadsorption assays on live cultured cells, showing that the expression of A9 was confined largely to the basal areas underneath growing cells. Thus, in normal squamous epithelial cells the expression of the A9 antigen is dependent on orientation and possibly on the degree of differentiation of the individual cells.

The orientation restriction of A9 in normal epithelial cells did not apply to the expression of the antigen on SCC lines. In fact, A9 antigen was easily detected on the upper surface of most monolayer SCC cultures. Thus, a major difference between normal and malignant cells is the orientation of display of the A9 antigen. In addition, there appears to be a quantitative difference in the amount of A9 antigen produced by malignant cells in comparison to normal cells. This suggests that relative A9 antigen expression may be related to the biological behavior of malignant cells such that A9 expression increases with tumor progression, eventually leading to a complete loss of orientation restriction in advanced cancers. Support for this hypothesis comes from our observations with A and B cell lines derived from the same donor. In each case in which the B cell line was derived at a later time than the A cell line, the B cell line expressed greater amounts of surface A9 antigen than did the A cell line. In each of these cases, the B cell lines demonstrated greater aggressiveness in that the cells grew more rapidly in vitro and in general were more likely to produce tumors in nude mice (16). In fact, 10B and 11B, but not 10A or 11A, grew in nude mice; 14A grows slowly, while 14B grows more rapidly. The 17A and 17B cell lines both fail to produce tumors. The 22A and 22B cell lines have not yet been tested. These observations are consistent with an in vivo adaptation or progression of the tumors. With regard to this, it is interesting to note that patient 17, whose A and B cell lines were both established at the same time and which are both low A9 expressors, had no recurrence of disease since surgery 4 years ago. In contrast, UM-SCC-22A and 22B, also derived simultaneously from different sites (primary and metastasis), express high amounts of A9 and grow rapidly in culture. These lines probably represent an aggressive cancer, since the patient died of recurrent disease within a year of diagnosis. It will be interesting to see whether the quantitative expression of A9 antigen by cell lines will also vary with time in culture.

Further evidence that A9 expression increases in squamous cancers comes from our observations with trypsinized cell cultures. It was found that A9-negative SCC cell lines could be converted to A9-positive by exposure to trypsin and EDTA. Thus, these cells are negative only in that the A9 antigen is masked in some way. There are two explanations for this finding. Either proteolysis cleaves peptide fragments that mask cryptic A9 determinants, or the A9 determinant is revealed following membrane rearrangement undergone by a cell when it is detached from its solid support. Our experiments do not discriminate between these possibilities. However, we favor a rearrangement mechanism, since the A9 antigen could be found on the plastic substrate underneath “negative” SCC lines following mechanical detachment. Also, in retrospective review, we noted that in non-confluent cultures the A9 antigen was most strongly represented at the borders of the tumor cell islands. In fact, at high concentrations of antibody, we were able to show weak A9 binding at the periphery of even those SCC cultures originally scored as negative (Fig. 6), a pattern that closely resembled that found with normal epithelial monolayer cultures. However, when normal epithelial cells were trypsinized only 30% were positive, while even the “negative” SCC lines became 90–100% positive after trypsinization. This indicates that there is a major quantitative difference in A9 antigen expression between normal and malignant epithelial cells.

Immunoperoxidase tests for A9 antigen in BCE and SCC also indicated that squamous cancers express increased levels of A9 antigen compared to normal skin. All of the SCC tumors that have been tested were positive for A9 and all showed a strong outline stain around nearly all of the cells and around the invasive or growing edges of tumor islands. In contrast, the basal cell epitheliomas expressed less A9 than did either squamous cell cancers or normal basal cells and showed only a faint outline of staining around the tumor islands. This contrasting pattern of A9 expression between BCE and SCC suggests that there may be a correlation between A9 expression and metastatic behavior. Basal cell cancers express little A9 and usually do not metastasize, whereas squamous cell cancers express A9 strongly and have a high frequency of metastases.

Fig. 5. Phase contrast photomicrographs showing binding of undiluted A9 culture supernatant in anti-immunoglobulin hemadsorption assays on UM-SCC-17A cells. A, binding pattern after some of the cells were removed by scraping; B, binding pattern after the cells were given a brief exposure to trypsin and EDTA before the antibody was added. (Original magnification, 1:320).
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Fig. 6. Phase contrast photomicrographs showing A9 binding in anti-immunoglobulin hemadsorption assays on UM-SCC-9 cells. In A, antibody binding (1/10 dilution of ascites) is limited to the periphery of the monolayer when tested against undisturbed cells. B, antibody binding (1/10° dilution of ascites) to nearly all cells of a replicate culture when the assay was performed after treatment of the monolayer with trypsin and EDTA (magnification, 1:320). In the absence of antibody there was no binding to either undisturbed or trypsin-EDTA treated control monolayers (not shown).

Table 2 Antibody binding to trypsinized cells in suspension

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Epithelial cell-enriched</th>
<th>Fibroblast-enriched</th>
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<tr>
<td>A9</td>
<td>23</td>
<td>2</td>
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<td>1/1000</td>
<td>28</td>
<td>4</td>
<td>90</td>
</tr>
<tr>
<td>E7*</td>
<td>98</td>
<td>98</td>
<td>86</td>
</tr>
<tr>
<td>1/10</td>
<td>100</td>
<td>90</td>
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<tr>
<td>1/1000</td>
<td>64</td>
<td>98</td>
<td>96</td>
</tr>
<tr>
<td>NS-1†</td>
<td>5</td>
<td>3</td>
<td>7</td>
</tr>
</tbody>
</table>

* The E7 antibody is directed against a common antigen of human cells and serves as a positive control (2, 14).
† NS-1 ascites fluid was pooled from mice inoculated with the NS-1 non-secretor myeloma that was used as the fusion partner for creating the A9 hybridoma.

This possible association of A9 expression and metastatic propensity for epithelial tumors is reminiscent of other systems in which extracellular matrix components such as laminin have been associated with metastatic behavior (17). The localization of the A9 antigen is similar to that of laminin (18, 19); however, A9 does not appear to be laminin because there is no correlation between A9 antibody binding and anti-laminin antibody reactivity in hemadsorption assays. Furthermore, laminin has been shown to be sensitive to proteases (20), while the A9 determinant is stable to limited trypsinization. The laminin receptor has been revealed by exposing cells to protease to remove laminin (20), and this receptor is a possible target for A9 binding. Our initial experiments in which A9 antibody did not inhibit SCC cells from binding to laminin coated plates have not supported this hypothesis. The orientation of the A9 antigen and its restriction to basal cells is also similar to that of the pemphigoid antigen (21); however, pemphigoid expression as detected with autoimmune serum does not always correlate with A9 expression on cell lines. In comparison to monoclonal antibodies raised to epithelial cells in other laboratories, A9 does not exhibit the distribution of binding reported for anti-keratin or anti-vimentin antibodies (22-24). Several groups have reported antibodies that bind to the basement membrane region or to epidermal basal cells (25-29). Although the A9 antibody appears to differ from these in that it binds to both basal cells and the underlying basement membrane region, the different assay methods used preclude exact comparison.

We have attempted to characterize biochemically the antigen defined by A9 using standard techniques for metabolic labeling and precipitation of proteins from detergent extracts but have thus far been unsuccessful. Possible reasons for this include a resistance of the A9-bearing molecule to the relatively mild extraction procedures used or an inability of the A9 antibody to precipitate the antigen. Alternatively, the antigenic determinant may be carried on lipid rather than protein. Future experiments will address these possibilities in order to better interpret the relationship of A9 expression to squamous cancer cell biology.

ACKNOWLEDGMENTS

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Fig. 7. Photomicrographs showing immunoperoxidase staining after incubation with undiluted A9 culture supernatant or complete Iscove's modified Dulbecco's medium culture medium on frozen tissue sections. In A, antibody binding is localized to basal cells and the basement membrane zone of normal epidermis (solid arrows). Note similar distribution in basal cells of the hair follicle. Dark areas in the stratum corneum have no brown stain. In B, a section from the same patient without A9 antibody shows no staining at open arrows. In C, antibody A9 strongly demarcates the invading tumor cells in a section of tissue from a patient with squamous carcinoma of the tongue. D, control section from the same patient. E and F, sections of a basal cell epithelioma of the same individual whose normal skin is shown in A and B. E shows A9 antibody binding as a faint outline around nests of tumor cells (solid arrows). F, control section showing (at open arrows) no outlining in absence of A9 antibody. Original magnification: A, B, C, D, and F 1:160; E, 1:100.

for confirming our experimental results on cultures of neonatal foreskin tissue. The authors also wish to thank Delisa Ervin and Dr. Roy Grekin for providing frozen sections of normal and malignant epithelial tissues. Special thanks are also extended to the physicians of the Departments of Otolaryngology/Head and Neck Surgery, and Pathology for providing tissue samples for tissue culture studies. We are also grateful to Francine Hume for her expert assistance in the typing of this manuscript.

REFERENCES


ALTERED EXPRESSION OF A9 ANTIGEN IN SCC


Altered Expression in Squamous Carcinoma Cells of an Orientation Restricted Epithelial Antigen Detected by Monoclonal Antibody A9

Kathryn A. Kimmel and Thomas E. Carey