A monoclonal antibody recognizing a keratin filament protein in a subset of transitional and glandular epithelia

Karen K. Nadakavukaren1, Ian C. Summerhayes1, Brad F. Salcedo1, James G. Rheinwald2, and Lan Bo Chen1

1 Department of Pathology, and 2 Department of Physiology and Biophysics, Dana-Farber Cancer Institute, Harvard Medical School, 44 Binney Street, Boston, MA 02115, USA

Abstract. Monoclonal antibodies were raised against the detergent-insoluble cytoskeletal fraction of the human bladder carcinoma cell line, EJ. By immunofluorescence, one of these antibodies, H10-1, localized to keratin filaments in EJ and three out of five other transitional-cell carcinoma lines. Primary normal-human urothelial cells in culture were not recognized by H10-1. Strong staining of keratin filaments was also seen in 11 human adenocarcinoma cell lines, but it was only seen in a subpopulation of cells in one out of four squamous-cell carcinoma lines and not at all in two normal diploid human epidermal keratinocyte strains. Immunofluorescence on frozen sections of mouse, human, and rabbit tissues showed that H10-1 recognized the upper layer of the transitional epithelium of mouse and rabbit bladder, and a subset of simple epithelia (mouse stomach glands and some colon mucosal glands, but not kidney tubules, small intestine, ovary germinal epithelium, or endometrium; some human colon mucosal glands and glandular breast epithelium, but not endometrium). The antigen which is recognized by this antibody was not detected in examples of stratified squamous epithelium (mouse skin, nonglandular stomach, or esophagus; human skin, esophagus, or rectum) or nonkeratinized tissue. On frozen sections of human tumors, the H10-1 antibody recognized 9 out of 14 colon carcinomas and 4 out of 6 breast carcinomas but did not recognize two sarcomas or a melanoma. This antibody apparently does not recognize its antigen in the presence of sodium dodecyl sulfate (SDS) because brief exposure of methanol-fixed cells to 0.1% SDS reversibly inhibited the binding of the H10-1 monoclonal antibody to keratin filaments by immunofluorescence, even when the binding of a rabbit polyclonal antikeratin antibody was unaffected. Two-dimensional gel-electrophoretic analysis of keratin-enriched fractions which had been isolated from various cell lines disclosed a good correlation between the presence of a 47-kdalton, pl-5.35 polypeptide and positive immunofluorescence with this antibody. These observations suggest that a certain keratin or keratin-associated protein is specifically expressed in a subpopulation of transitional and simple epithelial cells, and cultured cells derived from these epithelia. The monoclonal antibody described here may therefore be useful for increasing the precision of histological classification of epithelia, as well as the verification of the origin of certain cultured cell lines.

Introduction

Of the intermediate-filament proteins found in vertebrate cells, the keratin class, which is specifically expressed in epithelial cells, is the most complex [9, 14]. Distinctive subsets of 2–10 of the 15–20 different keratin polypeptides are expressed by different types of epithelial cells and tissues [11, 16, 17, 28, 31]. Keratin expression also changes as a function of differentiation within the same tissue, as is illustrated by the epidermal keratinocyte [6, 16, 29]. At present, the biological reason for these diverse patterns of keratin expression is unknown.

Monoclonal-antibody technology has recently become a powerful tool for the study of keratin expression. Because monoclonal antibodies recognize specific antigenic sites, it is possible to generate antibodies that recognize all the keratins, a specific subset, or even a single keratin [4, 10, 13, 29]. We report the characterization of monoclonal antibody H10-1, which recognizes a keratin-filament-associated antigen in most adenocarcinoma-derived and many transitional-cell carcinoma-derived cell lines. This antigen is not apparently characteristic of the cell lines and strain which are derived from stratified squamous epithelium. Its distribution in vivo appears to be unusually specific, being limited to a subset of simple glandular epithelia and part of the transitional epithelia of bladder; it is not found in non glandular simple epithelia or stratified squamous epithelia.

Methods

Hybridomas and monoclonal antibodies

The procedure used for producing the hybridoma lines was a modification of the method of Köhler and Milstein [12]. Balb/c mice were intraperitoneally immunized with the detergent-insoluble cytoskeletal fraction of the human-bladder carcinoma cell line, EJ, which had been prepared by extraction with a buffer containing 1% Triton X-100, 10 mM 1,4-piperazinediethanesulfonic acid, 100 mM KCl, 300 mM sucrose, 2.5 mM MgCl2, and 1 mM PMSF (phenyl methanesulfonyl fluoride) (“CSK buffer”), as has been described previously [1–3]. For each injection, two 100-mm dishes of confluent EJ cell cultures were used as starting material for the antigen. All injections were intraperitoneal and contained a suspension of the antigen in 0.4 ml complete (for the first injection) or incomplete (days 18 and
30) Freund's adjuvant. Two days later, the immunized mice were killed, and the spleen cells were isolated. NS-1 mouse myeloma cells (8 x 10^5) were fused with spleen cells (2 x 10^8) in the presence of 1 ml 50% polyethylene glycol (PEG 1,000; Koch-Light Laboratories, Berks, UK) in RPMI 1640 medium (Gibco, Grand Island, NY) and 1.5% dimethylsulfoxide for 2 min at 37°C. The cells were washed with RPMI 1640, resuspended in 200 ml complete medium (RPMI 1640 plus 15% fetal calf serum, 2 mM glutamine, 100 μg/ml garamycin), and plated in twenty 96-well plates (Flow Laboratories, McLean, Va.). The cultures were fed with HAT selection medium (13.6 μg/ml hypoxanthine, 0.18 μg/ml aminopterin, 3.9 μg/ml thymidine) for 2 weeks and then changed to the same medium without aminopterin. After screening, selected wells were weaned to complete medium without hypoxanthine and thymidine. Cloning was carried out in medium which had been made semisolid by the addition of Methocel (1.4%; Sigma, St. Louis, Mo), using a serum lot (M.A. Bioproducts, Walkersville, Md) which had been selected to give a cloning efficiency equivalent to that obtained with a 3T3-cell feeder layer.

Antibody-containing ascites fluid was obtained by intra-peritoneally injecting cloned hybridoma cells (1-10 x 10^7 per mouse) into Balb/c mice which had been primed with Pristane (2,6,10,14-tetramethylpentadecane; Aldrich Chemical, Milwaukee, Wis). The ascites fluid was stored at 4°C, either under sterile conditions or in the presence of 0.01% sodium azide. Both the conditioned culture medium and ascites fluid were stable for at least 1 year when stored this way. Each batch of ascites fluid was checked by immunofluorescence to ascertain that, at dilutions greater than 1:50, the staining pattern was the same as that of the conditioned medium.

Antibody screening

EJ cells were grown to confluence in UV-sterilized polyvinylchloride 96-well plates (Dynatech Laboratories, Alexandria, Va), fixed in methanol at -20°C for 5 min, and washed in phosphate-buffered saline (PBS). The hybridoma-conditioned medium was incubated overnight with fixed cells at 4°C. After washing in PBS, each well was tested for the presence of bound antibody with ether an alkaline phosphatase-conjugated second antibody or 125I-labeled goat antimouse mixed immunoglobins (Ig's) (New England Nuclear, Billerica, Mass). Conditioned media which were shown to be positive by this initial screening were further tested by immunofluorescence. Selected cultures were then cloned immediately.

Immunofluorescence

Cells which had been grown on glass coverslips were fixed in methanol at -20°C for 3-5 min, washed in water and PBS, and incubated with antibodies. Either undiluted conditioned medium or ascites fluid (diluted 1:100) was used as the source of the monoclonal antibody. The rabbit polyclonal antiserum raised against the total keratins of human epidermal stratum corneum has been described elsewhere [30] and was used at a dilution of 1:40. For double immunofluorescence labeling, mouse monoclonal and rabbit polyclonal antibodies were mixed. The second antibody was a mixture of fluorescein-conjugated goat antirabbit immunoglobulin G (IgG) and rhodamine-conjugated goat anti-

mouse mixed Ig's (Cappel Laboratories, West Chester, Pa). All incubations with antibodies were 30 min at 37°C in a humidified chamber, and each incubation was followed by a 10-min wash in PBS. Coverslips were mounted on glycerol-gelatin (Sigma, St. Louis, Mo) containing 0.1 M n-propyl gallate [8] to retard photobleaching.

Normal human endometrium was a kind gift from Dr. W. Welch (Peter Bent Brigham Hospital, Boston), and specimens of human normal and tumor tissue were generously provided by Drs. G. Steele (Dana-Farber Cancer Institute) and A. Bhan (Massachusetts General Hospital, Boston). Frozen sections of mouse, human, and rabbit tissues were prepared as has previously been described [21]. Unmasking experiments were performed by incubating methanol-fixed sections for 1 h at 37°C in 0.13% trypsin and 0.1% CaCl2, followed by washing in PBS and the application of antibodies. Immunofluorescence was performed as already described, except that the second antibody was usually a mixture of fluorescein-conjugated goat antimouse Ig's and rhodamine-conjugated goat antirabbit IgG (Cappel).

Fluorescence microscopy was carried out with a Zeiss Photomicroscope III equipped with epi-fluorescence optics and Planapo 63-x and 40-x objective lenses. For double-immunofluorescence experiments, exposures on Kodak Tri-X films were made for fluorescein and then for rhodamine at exposure index 3,600 and 6,300, respectively. The film was developed in Kodak HC110 (dilution B) for 12 min.

Cell cultures

Normal human kidney (J. Rheinwald, in press) and lines SCC12, SCC15, and XB2, strains A1EP and D2EP, and normal human urothelial strains have been described elsewhere [19-21, 31]. The cell lines WiDr, SW660, A549, PaCa-2, CaSkI, MDCK, PtK2, CV-1, and HT 1376 were from the American Type Culture Collection. A431 was from Drs. T. Hunter and J. Cooper (Salk Institute); A2058 was from Dr. G. Todaro (National Cancer Institute); T47D was from Dr. C. Pizak (National Cancer Institute); Rat-1 was from Dr. W. Topp (Cold Spring Harbor Laboratory); FS-2 was from Dr. R. Sager (Dana-Farber Cancer Institute); NIH-3T3 was from Dr. G. Cooper (Nina-Farber Cancer Institute); HL60 and CEM were from Dr. H. Lazarus (Dana-Farber Cancer Institute); CX-1 was from Dr. S. Bernal (Dana-Farber Cancer Institute); MCF-7 was from Dr. M. Rich (Michigan Cancer Foundation). RBC-1, MB49, MB48B, and AnAn have been previously described [20, 23, 24, 27]. EJ, T24, J82, RT4, and RT112 were from Dr. L.M. Franks (Imperial Cancer Research Fund, UK).

Radiolabeling and two-dimensional gel analysis of keratin-enriched fractions

Subconfluent cultures in 100-mm dishes were incubated for 12-15 h in Dulbecco's modified Eagle's medium (DME; Gibco) containing 10% calf serum, methionine at 20% of the normal level, and 50-100 μCi/ml 35S-methionine (400 Ci/mmol, NEN). The remaining procedures were performed in the cold room (4°C). The labeled cultures were washed three times with PBS, scraped with a rubber policeman, and pelleted in Eppendorf tubes (Sarstedt, Princeton, NJ) in an Eppendorf-5412 microfuge. The pellet was extracted with RIPA buffer (10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% Triton X-100, 1% Na deoxycholate, 0.1% SDS, 1 mM PMSF; cf. [4]) for 3 min, centrifuged,
and homogenized in 20 mM Tris-HCl (pH 7.4), 0.6 M KCl, 1% Triton X-100, and 1 mM PMSF in a Wheaton 2-ml homogenizer. The homogenate was centrifuged, and the pellet was rehomogenized in a buffer containing 20 mM Tris-HCl (pH 7.4) 3 M urea, and 1 mM PMSF. Following centrifugation, the pellet was vortexed in 20 mM Tris-HCl (pH 7.4). After the final centrifugation, the pellet was solubilized in 30 μl lysis buffer (9.5 M urea, 4% Triton X-100, 5% 2-mercaptoethanol, pH 3-10 ampholines) for two-dimensional gel analysis which involved isoelectrofocusing and sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, as has been previously described [7].
Results

Generation of hybridomas

Hybridoma cells were produced by fusing NS-1 myeloma cells with spleen cells from Balb/c mice which had been immunized with a Triton-insoluble cytoskeletal fraction prepared from the human-bladder carcinoma line, EJ. Supernatants that were positive by the preliminary screening were further screened by immunofluorescence on methanol-fixed EJ cells. Hybridoma cells grew in 432 out of 1,820 wells, and 10 of these produced antibodies that intensely labeled EJ cells by immunofluorescence. One of these displayed the staining pattern characteristic of keratin in these cells (Fig. 3b). It did not recognize any of the nonepithelial cell lines which were tested, including FS-2 (normal human foreskin fibroblast), Rat-1 (normal rat fibroblast), AnAn (RSV-induced rat sarcoma), NIH-3T3 (mouse fibroblast), CEM (human leukemia), H1.60 (human promyeloctytic leukemia), or A2058 (human melanoma). Hybridoma cells from this well were cloned in Methocel medium. All of the 35 clones which were chosen produced antibodies which gave a similar immunofluorescence staining pattern on EJ cells and were characterized as mouse IgM. One of the clones, H10-1, was arbitrarily selected for this work.

Immunofluorescence on frozen sections

To examine whether the antigen which is recognized by the H10-1 monoclonal antibody occurs in vivo, frozen sections of mouse, human, and rabbit tissues were examined by double immunofluorescence, using a rabbit anti-(total epidermal stratum corneum keratins) to provide an internal comparison of total keratin levels. The rabbit anti-(total keratins) stained all the epithelia seen in these sections, which is in agreement with observations of a similar antisera [25]. Neither antibody stained nonepithelial tissues. Figure 1(a–c) shows a cross-section of mouse dermis which was strongly stained by the rabbit anti-(total keratins; Fig. 1a) but not by H10-1 (Fig. 1b). Identical results were seen with examples of human stratified squamous epithelia: epidermis, esophagus, and rectal epithelium (Table 1). Figure 1(d–f) shows a cross-section through the ridge dividing the glandular and nonepithelial regions of the mouse stomach. Anti-(total keratins) stained both the keratinized stratified squamous epithelium of the nonepithelial region and the simple columnar epithelium of the stomach glands (Fig. 1d), whereas H10-1 only stained the epithelium of the glands. In a section through the glandular portion of the stomach, the anti-(total keratins) labeled all the epithelia (Fig. 1g), whereas H10-1 labeled the epithelium lining the stomach glands but not the simple extraglandular epithelium (Fig. 1h). H10-1-positive mucosal glands were seen in sections of both human (Fig. 2a, b) and mouse colon, but negative mucosal glands were also observed. Human breast was heterogeneous for H10-1-positive and -negative epithelia. H10-1 also recognized 9 out of 14 human colon (Fig. 2c, d) and 4 out of 6 human breast carcinomas, and did not stain nonepithelial tumors (two sarcomas and a melanoma). Human liver was heterogeneously stained, while mouse small-intestinal, kidney tubule, and mouse and human endometrial epithelium were not stained. Trypsin treatment of sections to expose "masked" antigens did not alter these results. Sections through mouse bladder were strongly stained by H10-1 monoclonal antibody in the upper (umbrella cell) but not the basal layers of the transitional epithelium, whereas the rabbit antibody stained the entire transitional epithelium (Fig. 2e–g); a similar observation was made on rabbit bladder (Fig. 2h, i). Table 1 summarizes the data. It appears that the antigen which is recognized by H10-1 monoclonal antibody distinguishes a subpopulation of cells within transitional cell epithelia and certain glandular simple epithelia.

Immunofluorescence on cultured cells

Figure 3 shows double immunofluorescence with rabbit anti-(total keratins) and the H10-1 monoclonal antibody on several human carcinoma cell lines. Keratin filaments in the bladder transitional-cell carcinoma line, EJ, were recognized by both antibodies (Fig. 3a, b). Cells in which keratin filaments were undetectable in Fig. 3a were also unlabeled by H10-1 (Fig. 3b). Cell-to-cell variation in the level of keratin expression in the EJ cell line has previously been observed with two other rabbit antikeratin antisera [22]. Both the anti-(total keratins) and H10-1 strongly labeled the keratin filaments in the lung adenocarcinoma cell line, A549 (Fig. 3d, e). However, keratin filaments in the

Table 1. Immunofluorescent staining of frozen sections of mouse, human, and rabbit tissues with the H10-1 monoclonal and rabbit antikeratin antibodies

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>H10-1 monoclonal</th>
<th>Rabbit antikeratin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse simple epithelia</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Stomach</td>
<td>+ + +</td>
<td>+</td>
</tr>
<tr>
<td>Colon (mucosal glands)</td>
<td>+, -</td>
<td>+</td>
</tr>
<tr>
<td>Small intestine</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Kidney tubules</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Uterine endometrium</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Ovary germinal epithelum</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Human simple epithelia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bile duct</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Colon (mucosal glands)</td>
<td>+, -</td>
<td>+</td>
</tr>
<tr>
<td>Colon carcinoma</td>
<td>+ (9 cases)</td>
<td>+</td>
</tr>
<tr>
<td>Breast ducts</td>
<td>+ (4 cases)</td>
<td>+</td>
</tr>
<tr>
<td>Breast carcinoma</td>
<td>- (2 cases)</td>
<td></td>
</tr>
<tr>
<td>Mouse and rabbit transitional epithe</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bladder</td>
<td>+, -</td>
<td>+</td>
</tr>
<tr>
<td>Mouse stratified squamous</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skin (keratinocyte)</td>
<td>-</td>
<td>+ + +</td>
</tr>
<tr>
<td>Stomach (nonglandular region)</td>
<td>-</td>
<td>+ + +</td>
</tr>
<tr>
<td>Esophagus</td>
<td>-</td>
<td>+ + +</td>
</tr>
<tr>
<td>Human stratified squamous</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skin</td>
<td>-</td>
<td>+ + +</td>
</tr>
<tr>
<td>Esophagus</td>
<td>-</td>
<td>+ + +</td>
</tr>
<tr>
<td>Anal</td>
<td>-</td>
<td>+ + +</td>
</tr>
<tr>
<td>Human nonepithelial</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adipose tissue</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Melanoma</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Sarcoma</td>
<td>- (2 cases)</td>
<td></td>
</tr>
</tbody>
</table>

* Both H10-1-positive and -negative epithelia detected
Fig. 2A–I. Immunofluorescence with the H10-1 monoclonal antibody on sections of normal human colon mucosal glands (A, B) and colon carcinoma (C, D). Double immunofluorescence on a frozen section of mouse bladder using rabbit antitotal callus keratins (E) and the H10-1 monoclonal antibody (F); phase (G). H10-1 staining on rabbit bladder (H); the bottom of the transitional epithelium is indicated by the black line in the phase (I). Bars, 50 μm (A, C); 25 μm (E); 100 μm (H)
oral epithelial squamous-cell carcinoma line, SCC15, were not recognized by H10-1 (Fig. 3g, h).

Of the adenocarcinoma cell lines examined, immunofluorescence staining with H10-1 of the mammary carcinoma line, MCF-7, produced the most dramatic results (Fig. 4a, b). Brightly stained, thick tonofilament bundles were seen throughout the cell. The filament bundles were also arranged in a complex, basket-like network at the nuclear region (Fig. 4b), similar to those which have previously been described [5].
Table 2. Immunofluorescent staining of human epithelial cells in culture with the H10-1 monoclonal and rabbit antikeratin antibodies

<table>
<thead>
<tr>
<th></th>
<th>H10-1 monoclonal</th>
<th>Rabbit antikeratin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenocarcinoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCF-7 (breast)</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>T47D (breast)</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>BT-20 (breast)</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>HBL-100 (breast)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>WiDr (colon)</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>SW480 (colon)</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>CX-1 (colon)</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>LS180 (colon)</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>A549 (lung)</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>LX-1 (lung)</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>PaCa-2 (pancreas)</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Transitional-cell carcinoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HT 1376 (bladder)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>T24 (bladder)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EJ (bladder)</td>
<td>+, - a</td>
<td>+, - a</td>
</tr>
<tr>
<td>J82 (bladder)</td>
<td>+, - a</td>
<td>+, - a</td>
</tr>
<tr>
<td>RT4 (bladder)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>RT112 (bladder)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Squamous-cell carcinoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A431 (vulva)</td>
<td>++++, - a</td>
<td>++++, - a</td>
</tr>
<tr>
<td>SCC12 (epidermis)</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>SCC15 (tongue)</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>CaSk (cervical)</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>Normal simple epithelial</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary urothelial</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>Primary kidney</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>Normal epidermal keratinocyte</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1Ep (foreskin)</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>D2Ep (breast)</td>
<td>-</td>
<td>+++</td>
</tr>
</tbody>
</table>

a Both positive and negative cells detected

filaments which were recognized by H10-1. Surprisingly, normal human transitional cells in culture (primary urothelial cells) were not recognized by H10-1 despite strong immunofluorescence staining by the rabbit antibody. Neither of the normal epidermal keratinocyte strains and only a subpopulation of cells in one out of the three squamous-cell carcinoma lines which were tested were recognized by the H10-1 monoclonal antibody, although keratin filaments were abundant in these cells (e.g., Fig. 3).

Several nonhuman cultured epithelial cell lines were also examined by double immunofluorescence. Consistent with the results for human cells, several cell lines which were derived from transitional-cell carcinomas expressed the keratin-associated antigen which is recognized by the H10-1 antibody (data not shown). These were RBC-1 (benzo(a)-pyrene-transformed rabbit bladder), MB488, and MB49 (dimethylbenzanthracene-transformed mouse bladder). As suggested by the frozen-section results, primary epithelial outgrowths from explants of mouse bladder yielded two populations of cells; those recognized both by H10-1 and the rabbit antibody, and those recognized by the rabbit anti-total keratins) alone. In contrast, Xb2, a mouse teratoma-derived line with keratinocyte characteristics, was entirely negative for the antigen recognized by H10-1.

**SDS-sensitivity of the antigen recognized by H10-1 antibodies**

We used immunoprecipitation and immunoblotting [29] in an attempt to identify the antigen(s) recognized by H10-1, but were unsuccessful despite success with other monoclonal antibodies and the rabbit polyclonal antikeratin. Because SDS is required to solubilize keratin for these procedures, and the immunogen for raising the H10-1 antibody had not been denatured in SDS, we suspected that H10-1 recognizes an antigenic site which is sensitive to SDS.

This possibility was investigated as follows: cells of the human squamous-cell carcinoma line, A431, were fixed by exposure to methanol at -20°C for 2 min, extracted with
Fig. 5A–L. The effect of SDS on double immunofluorescence with rabbit anti-total keratins (A, D, G, J) and the H10-1 monoclonal antibody (B, F, H, K) on the same field for methanol-fixed, Triton-extracted cytoskeletons of cell line A431. A–C Control; D–F exposed to 0.1% SDS before the application of antibodies; G–I antibodies applied before exposure to SDS; J–L washed in 100% acetone after exposure to SDS and before application of antibodies. Bars, 17 μm (C); 15 μm (F); 18 μm (I); 15 μm (L)
CSK buffer containing 1% Triton X-100, and then double labeled for immunofluorescence. The prefixation in methanol greatly reduced the tendency of the cytoskeletons to come off the coverslips, but but did not otherwise affect the results which are described here. As seen in Fig. 5(a, b), both the rabbit anti-(total keratins) and the H10-1 monoclonal antibody bound to keratin filaments in the Triton-insoluble cytoskeletons. When these preparations were further exposed to RIPA buffer containing 0.1% SDS at 4°C for 3 min and then washed in several changes of PBS before the antibodies were applied, the binding of the rabbit anti-(total keratins) was unaffected (although the organization appeared somewhat distorted). However, the binding of H10-1 antibody was almost completely abolished, as shown in Fig. 5(d, e). The results were identical when PBS containing 0.1% SDS was used instead of RIPA buffer. RIPA buffer which had been prepared without SDS had no effect on the binding of H10-1 antibody. The exposure
of the cells to 20 mM Tris-HCl, pH 7.2, containing 0.6 M KCl also did not prevent labeling with either antibody (data not shown).

The possibility that SDS was simply removing a keratin-associated protein which is recognized by H10-1 was eliminated by two experiments. First, when the sequence of the experiment was reversed by allowing both antibodies to bind to the Triton-insoluble cytoskeleton before exposure to RIPA buffer, the immunofluorescence pattern (Fig. 5g, h) was indistinguishable from that of the SDS-untreated control (Fig. 5a, b). Second, when the cells were washed in 100% acetone (2 min at 4°C) after SDS treatment in order to remove tightly bound SDS before the antibodies were applied, the result (Fig. 5k, l) was similar to that obtained with the SDS-untreated control. These results strongly suggest that the antigenic site which is recognized by the monoclonal antibody is reversibly altered by SDS.

**Two-dimensional gel analysis of keratin-enriched fractions**

Since it was not possible to identify directly the antigen which is recognized by the H10-1 monoclonal antibody, we adopted an alternative approach to the problem. A range of cell types, both positive and negative for H10-1 immunofluorescence, was metabolically labeled for 12–15 h with 35S-methionine, and keratin-enriched fractions of cellular proteins were prepared. These fractions were analyzed by two-dimensional gel electrophoresis and autoradiography. The arrow in Fig. 6 indicates the presence of a 47-kdalton polypeptide in four cell lines (MCF-7, SW480, T24, A549) that expressed an antigen which was recognized by the H10-1 antibody, and its absence in four cell lines (SCC15, PtK2, RT112, and the fibroblast line, FS-2) that did not. Not shown are the two-dimensional gel autoradiograms of CV-1 and PaCa-2, which also expressed this polypeptide and stained with the H10-1 monoclonal antibody, and Rat-1 and AnAn, which did not express either this polypeptide or stain with H10-1. The isoelectric point of this 47-kdalton polypeptide was midway between that of actin (pI 5.4) and vimentin (pI 5.3).

**Discussion**

The H10-1 monoclonal antibody recognizes a keratin filament antigen by several criteria:

1. In cultured cells, the H10-1 monoclonal antibody only recognizes intermediate filaments in epithelial cells but not in fibroblasts or other nonepithelial cells.

2. By double immunofluorescence on H10-1-positive epithelial cells with a well-characterized rabbit antikeratin antisera, the fluorescence localization of the two antibodies appears to be identical.

3. In frozen sections, this antibody recognizes a subset of epithelia but not connective, neural, or muscle tissues.

4. The antigen which is recognized by H10-1 remains associated with the cytoskeletal residue after the extraction of cultured carcinoma cells with neutral detergent followed by high-ionic-strength buffers.

Examples of three major types of epithelia were examined: transitional squamous, simple (columnar and cuboidal), and transitional. The H10-1 monoclonal antibody did not recognize mouse or human stratified squamous epithelia but did recognize a subset of glandular simple epithelia. Regions of H10-1 positive and (-)negative epithelia were found in human breast, and mouse and human colon. Carcinomas derived from these tissues tended to be entirely H10-1-positive or -negative, which suggests that these tumors reflect characteristics of the cell type of origin. The fact that the antigen which is recognized by this antibody is not present in all simple epithelia distinguishes it as being more specific than the simple-epithelial antigenic determinant which has been reported by Debus et al. [4] and Lane [13]. These observations may reflect the presence of specific keratins or keratin-associated proteins which are specific to certain simple glandular epithelia but not to stratified squamous epithelia. This would be consistent with previous reports which have suggested that the expression of certain keratin polypeptides in stratified squamous epithelia and simple (including glandular) epithelia differ significantly [16, 17, 29, 31]. The staining pattern of H10-1 on bladder is especially noteworthy, in that it differentiates between the upper and basal layers of transitional-cell epithelium.

Immunofluorescence studies with the H10-1 monoclonal antibody on a variety of cultured cell types were consistent with the frozen-section results. Adenocarcinomas are generally defined as tumors which arise from glandular epithelia; the cell lines in which the antigen recognized by H10-1 is especially abundant are derived from adenocarcinomas (Table 2; Fig. 4a, b). In contrast, with the exception of A431 which was heterogeneous for H10-1 staining, cells that were clearly derived from stratified squamous epithelia (e.g., from keratinocytes) were not recognized by this antibody (Table 2; Fig. 3h). The fact that some A431 cells possess the antigen which is recognized by the H10-1 antibody may be due to the altered keratin gene expression of the type which has already been documented for squamous-cell carcinoma lines [30, 31]. Another monoclonal antibody which recognizes simple but not stratified squamous epithelia in vitro, yet also stains A431, has been reported [4], which makes this possibility seem more likely.

The H10-1 monoclonal antibody also labels keratin filaments in several transitional-cell carcinoma lines which are derived from human bladder (Table 2), and mouse and rabbit bladder. The cytoskeletal fraction of one of these (EJ) was the immunogen for this monoclonal antibody. However, two human transitional-cell carcinoma lines (RT4, RT112) and the primary-cultured normal urothelial cells did not stain with H10-1, and explants of normal mouse bladder yielded both H10-1-negative and -positive epithelial cells. Since sections through mouse and rabbit bladder showed that H10-1 only recognizes the outer layer of transitional epithelium (Fig. 2e–i), the possibility exists that the H10-1-positive carcinoma lines (EJ, J82, T24, HT 1376) may be derived from the outer layer of bladder epithelium, whereas the H10-1-negative carcinoma lines (RT4, RT112) are derived from the basal layer. Since this possibility is without precedent, it is more likely that both kinds of transitional-cell carcinoma lines originate from the basal layer of these two sections. The expression of this antigen may be turned on in some transitional-cell carcinomas but not in others. Either possibility is consistent with the report of Wu et al. [31] that RT112 expresses a keratin pattern which is identical to that of cultured normal human urothelial cells, while EJ and J82 are very different.

The antigen which is recognized by the H10-1 monoclonal antibody appears to be very sensitive to SDS. The possibility that some monoclonal antibodies recognize SDS-
sensitive sites has been suggested [10]. This observation pre-
cluded the identification of this antigen by standard electro-
phoretic methods that employ SDS to solubilize keratins.
However, by comparing two-dimensional gel autoradi-
ograms of keratin-enriched fractions from both H10-1-posi-
tive and -negative cell lines, the presence of one polypeptide
spot (migrating at 47-kdalton, pl 5.35) was found to corre-
late perfectly with the immunofluorescence results. This ob-
ervation is striking in view of the considerable variation
in polypeptide pattern among cell lines. A keratin which
corresponds to this polypeptide on two-dimensional gels has
not previously been reported [16, 31]. However, Magin
et al. [15] have recently observed a similar, minor polypep-
tide in two-dimensional gels of in vitro MCF-7 translation
products which were enriched by co-polymerization with
added epidermal keratins. This polypeptide may be a slowly
turned-over and/or a very minor keratin or keratin-assoc-
iated protein, or may be a result of the “processing” of
another keratin. For detection on autoradiographs, 12 h
of metabolic labeling with 35S-methionine was essential.
Using the labeling times which are commonly used for other
eratins [3–6 h], we were often unable to detect this polypep-
tide. Whether the 47-kdalton polypeptide is indeed a
keratin and is recognized by H10-1 will require further
study.

In summary, the H10-1 monoclonal antibody recognizes a
keratin-filament-associated antigen which is uniquely ex-
pressed in certain simple epithelia associated with glandular
structures in vivo and is also abundant in adenocarcinoma
cell lines. This antibody also defines two types of cells with-
in transitional epithelium in vivo and in culture. Polyclonal
antibodies to specific intermediate-filament proteins have
already proven to be useful in clarifying the origins of cer-
tain cell lines (e.g. [18, 26]), and the even-greater specificity
of monoclonal antibodies such as this one should be
especially useful.

Acknowledgements. We are indebted to Dr. T-T Sun for useful
suggestions, Mr. Jong Yang and Ms. Terry O’Connell for technical
assistance, Mr. Robert Mcisaac for the running of two-dimensional
gels, Ms. Elizabeth Corkery-Walker and Mr. Douglas Partridge
for preparing frozen sections, and Dr. Sharon Rosenberg for criti-
ically reading this manuscript. This work has been supported by
grants CA 29793 and CA 22659 (awarded to LBC), and grant
CA 26856 (awarded to JGR) from the National Cancer Institute.
KKN gratefully acknowledges the support of a training grant from
the National Cancer Institute to A.B. Pardec during 1981, a post-
doctoral fellowship (PF 2105) from the American Cancer Society
in 1982, and a National Cancer Institute Postdoctoral Fellowship
(F32 CA07047-01) in 1983-4. LBC is the recipient of an American
Cancer Society Faculty Research Award.

References
1. Bernal SD, Chen LB (1982) Induction of cytoskeleton-asso-
ciated proteins during differentiation of human myeloid leuko-
cytic cell lines. Cancer Res 42:5106–5116
is translated when associated with the cytoskeletal framework
in normal and VSV-infected HeLa cells. Cell 23:113–120
containing 34,000-dalton protein in the framework of cells
transformed with Rous sarcoma virus. Proc Natl Acad Sci USA
78:2388–2392
antibodies that distinguish simple from stratified squamous epi-
thelia: characterization on human tissues. EMBO J 1:1641–
1647
5. Franke WW, Schiller DL, Moll R, Winter S, Schmidt E, Engel-
brecht I, Denk H, Krepler R, Platter B (1981) Diversity of
keratins: differentiation-specific expression of cytkeratin
polypeptides in epithelial cells and tissues. J Mol Biol 153:
933–959
during terminal differentiation of the keratinocyte. Cell 17:
573–582
7. Garrels J (1979) Two-dimensional gel electrophoresis and com-
cputer analysis of proteins synthesized by cloned cell lines. J
Biol Chem 254:7901–7977
photobleaching of rhodamine and fluorescein protein conjugates
by n-propyl gallate. Science 217:1252–1255
Cytoplasmic fibers in mammalian cells: cytoskeletal and con-
tractile elements. Annu Rev Physiol 41:703–722
10. Gown AM, Vogel AM (1982) Monoclonal antibodies to inter-
mediate filament proteins of human cells: unique and cross-
Dynamic aspects of the supramolecular organization of inter-
mediate-filament networks in cultured epithelial cells. Cell
Motil 2:197–213
12. Köhler G, Milstein C (1975) Continuous cultures of fused cells
13. Lane EB (1982) Monoclonal antibodies provide specific intra-
molecular markers for the study of epithelial tonofilament orga-
14. Lazazrides E (1980) Intermediate filaments as mechanical inte-
grators of cellular space. Nature 283:249–256
products of mRNAs coding for non-epidermal cytokeratins.
EMBO J 2:1387–1392
The catalogue of human keratins: patterns of expression
in normal epithelia, tumors and cultured cells. Cell 31:11–24
classes as molecular markers for stratified squamous epithelia:
cell-culture studies. J Cell Biol 97:244–251
K (1982) Various sympathetic derived human tumors differ in
neurofilament expression: Use in diagnosis of neuroblastoma,
ganglioneuroblastoma and pheochromocytoma. Vir-
chows Arch [Cell Pathol] 40:141–152
lines requiring anchorage and fibroblast support cultured from
human squamous cell carcinoma. Cancer Res 41:1657–1663
epithelium in culture by a cloned cell line derived from a tera-
of human epidermal keratinocytes: the formation of keratiniz-
ing colonies from single cells. Cell 6:331–344
52,000 keratin in basal epithelial cells of the mouse bladder and
expression throughout neoplastic progression. Cancer Res
42:4098–4109
neoplastic transformation of adult mouse bladder epithelium
pression of keratin and vimentin intermediate filaments in rab-
nit bladder epithelial cells at different stages of benz(a)pyrene-
induced neoplastic progression. J Cell Biol 90:63–69
25. Sun T-T, Green H (1978) Immunofluorescent staining of ker-
tin fibers in cultured cells. Cell 14:469–476


Received August 1983 / Accepted in revised form April 1984

Note added in proof

A monoclonal antibody which also recognizes the umbrella cell layer but not the basal layer of transitional epithelium has been recently reported by F. Ramackers (Laboratory Investigation 49:353–361, 1983).