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UNME/K1: an IgG_{2a} monoclonal antibody specific to cytokeratin of human urinary bladder squamous cell carcinoma

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Summary. The main distinctive feature of carcinoma in schistosomal bladder is keratinized squamous cell carcinoma. Keratins/cytokeratins constitute a multigeneic family of structurally related polypeptide markers for the malignant state of epithelial cells. A monoclonal antibody (UNME/K1) regognizing keratins associated with squamous cell carcinoma of the human urinary bladder was generated at the Urology and Nephrology Center, Mansoura, Egypt (UNME), by fusion of spleenocytes from a BALB/c mouse immunized with a keratin extract (K1) of human squamous cell carcinoma and P3X63Ag8/U1 syngeneic myeloma cells. UNME/K1 was purified by a protein-A affinity column and was of the IgG2a type, as determined by immunoelectrophoresis and gel diffusion techniques. When tested against keratins of different types of urinary bladder tumors using enzym linked immunosorbent assay (ELISA), UNME/K1 reacted only with the high molecular weight keratin of squamous cell carcinoma and showed selectivity towards specific histopathological grades of tumors.

Key words: Monoclonal antibodies – Keratin – Bladder cancer

Intense research in cell biology has provided new approaches to cell and tissue characterization. One of them is based on the finding that different cell types in mammalian tissue differ in their content and type of components of the cytoskeletal complex. Intermediate filaments (IF), described as a separate entity of cytoskeletal complex [8], are so named because their diameter (7–11 nm) is intermediate between those of the actin microfilaments and of the microtubules [12]. Keratin-type intermediate filaments (10 nm in diameter) are present in almost all epithelial cells but not in any nonepithelial cell types. Biochemical analysis indicated that epithelial keratin composition varies depending on cell type, stage of histologic differentiation, and disease state. It is therefore perhaps not surprising that numerous keratin species have been described in the literature [21].

Human carcinoma, derived from different types of ephithelia, display different cytokeratin polypeptide patterns that are characteristic of certain groups of tumors. Epithelium-derived tumors appear to maintin the expression of many cytokeratin polypeptides typical of the specific nontransformed cells. The cytokeratin patterns of many other types of tumors display a far-reaching similarity to those of normal tissue. Such differences may reflect selection, during cell transformation and tumor development, of a cell type that is not the quantitatively predominant one in the tissue. Moll and coworkers [14] regarded cytokeratins as having been produced by a multigene family coding for polypeptides, expressed differently during different routes of epithelial differentiation. They considered the majority of these cytokeratin polypeptides as genuine products of translation and not as fragments proteolytically derived from precursor molecules. The complex keratin polypeptide pattern has become a characteristic antigenic component for bladder cancer [22].

Most studies concerning the use of antikeratin antibodies as tools in tumor diagnosis have made use of conventional polyclonal antisera. Nearly all tumors with epithelial differentiation have been positive for the antisera used. In the past, these considerations limited what could have been achieved by conventional serology. In 1975, individual clones of normal antibody-secreting cells were immortalized by fusion with myeloma cells [11]. With the resulting hybridoma approach the results of serological examination became much more precise and the major problems of specificity and reproducibility became solvable. Virtually unlimited quantities of homogenous specific antibodies could be produced even for impure immunogens.

Because monoclonal antibodies have only a fraction of the properties of conventional antisera, a number of them may have to be generated to find the one most suitably tailored for a particular antigen. The importance of specific keratin antibodies has become apparent in view of

the growing interest in the use of such antibodies for tumor diagnosis [1–3]. Production of a monoclonal antibody specific for keratin associated with human urinary bladder squamous cell carcinoma – associated with schistosomiosis which is endemic to the Middle East and Africa – could be useful for immundiagnosis of such type of bladder cancer.

Materials and methods

Tumor samples

Keratin proteins of human tumors were extracted from 12 squamous cell carcinoma, 3 transitional cell carcinoma, and 2 adenocarcinoma of the urinary bladder. Squamous cell tumors were classified histologically [16] into GI, GII, and GIII (eight, two and two extracts, respectively).

Keratin extraction

Keratin proteins were extracted using the method previously described [22]. Briefly, tumor tissue was minced to cubes, rinsed in phosphate-buffered saline (PBS), and extracted with high salt buffer [1.5 *M* KCl, 10 m*M* NaCl, 2 m*M* dithioerythritol (DTE), 0.5 m*M* phenylmethylsulfonylfouride (PMSF), 0.5% Triton X-100, 10 m*M* Tris HCL, pH 7.5]. Suspension was homogenized for 15 min in a tissue homogenizer (Cole Parmer Instrument Co., USA) and stirred overnight at room temperature. The insoluble proteins collected by centrifugation for 1 h at 30,000 × *g* and 15°C (J2-21 high-speed centrifuge, Beckman, Switzerland) were dissolved by stirring overnight in keratin dissociation buffer (8 *M* urea, 50 m*M* Tris HCL, pH 9.0) followed by centrifugation. Supernatent was dialyzed overnight against reconstitution buffer (2 m*M* Tris HCL, pH 5.5) to precipitate keratin filaments. Finally, keratins were gradually dissolved in urea dissociation buffer and their protein content determined [13], aliquoted, and stored at −20°C.

Animal immunization

Five female BLAB/c mice, 6–8 weeks old, were intraperitoneally (IP) injected each with 40 µg of keratin extract [9]. Keratin FCA (Freund's complete adjuvant) emulsion was used in priming immunization and keratin FIA (Freund's incomplete adjuvant) emulsion as booster via the same route twice at 1-week intervals.

Monoclonal antibody

Cells from the myeloma line P3X63Ag8/U1 (PU) were fused at a 1:10 ratio with immune spleen cells of BALB/C mice 3–4 days after the last booster dose. Cell funsion was performed using 50% polyethylene glycol (Sigma Chemical Company, St. Louis, MO, USA, MW 1500) in RPMI-1640 medium containing 5% dimethylsulfoxide (DMSO) following previously described protocol [6], with minor modifications.

The cells were suspended to a final concentration of 1×10^6 cells/ml and seeded (0.5×10^6 cells/well) in 24-well tissue culture plates (Flow Laboratories, Irvine, Scotland). The plates had been prepared a day before with 0.25×10^6 normal syngeneic spleenocytes per well as feeder cells.

Hybridoma cells were cultured in HEPES-buffered RPMI-1640 complete medium supplemented with 20% heat-inactivated fetal calf serum, 2 m*M* *L*-glutamine, 4×10^7 hypoxanthine, 1.6×10^{-5} thymidine, 100 IU penicillin, and 100 µg/ml streptomycin (HT medium). Cultures were grown at 37°C in a humidified atmosphere with 5% CO_2 in air. On the next day selective HAT medium (containing 4×10^{-7} aminopterin) was added to each well. HAT medium was used for the first 2 weeks and HT medium for another 3 weeks. Supernatants were screened for antibody production in an enzyme-linked immunosorbent assay (ELISA) system after 2 weeks of culture.

A selected hybridoma was cloned by limiting dilution at a theoretical density of 0.5 cell per well over a layer of feeder cells. Spent media from wells with colonies originating from a single cell were ELISA assayed. A selected clone was expanded and cells preserved frozen in liquid nitrogen. Cloned cells (2×10^6 cells/animal) were implanted IP in pristane (0.5 ml/animal) primed syngeneic mice for production of monoclonal antibody as ascites fluid.

Antibody purification

The ascitic monoclonal antibody was purified utilizing protein A according to Vidal and Conde [21]. Protein A-Sepharose CL-48 (Pharmacia Fine Chemicals, Sweden) was swollen in PBS, pH 7.2 and packed in 1.14 × 13 cm column (LKB, Bromma, Sweden). The monoclonal antibody (5 mg/ml of gel) was applied in PBS, pH 7.2, to the column at a rate of 5 ml/h and eluted at the same rate. Afterwards 0.1 *M* sodium citrate buffer, pH 4.5, was passed through. Fractions of 0.5 ml each were collected over 60 µl of 2 *M* Tris buffer, pH 8.5, to equilibrate the pH value.

Enzyme-linked immunosorbent assay

According to the direct ELISA [5] polystyrene 96-well flatbottom plates (Flow) were coated with 100s µl/well (50 µg/ml) of keratin extract. Coating was performed overnight at +4°C in carbonate buffer, pH 9.6. After washing with PBS containing 0.05% Tween-20 (PBS-T), the coated plates were blocked for 2 h at 37°C with 1% bovine serum albumin [(BSA) fraction V, Sigma] in carbonate buffer. Materials under antibody investigation were applied at 100 µl aliquot to each microtiter well and incubated for 2 h at 37°C. The plates were washed three times with PBS-T, and antimouse immunoglobulin whole molecule (IgG, IgM, or IgA, when desired) peroxidase conjugate (diluted 1:800 in PBS-T containing 4% BSA) was dispensed at 100 µl volumes to each well. After 2 h incubation at 37°C, plates were washed as previously mentioned. To develop the reaction, plates were incubated at room temperature in complete darkness for 20 min with 100 µl/well of peroxidase substrate [0.034% orthophenylenediamine (OPD) in citrate phosphate buffer, pH 5.0, and 20 µl of 30% H_2O_2]. The absorbance was measured at 492 nm using Titertek MC Multiskan (Flow).

Immunoelectrophoresis

Glass slides were covered with 1% agarose in barbiturate buffer pH 8.6 [21]. Five µl of monoclonal antibody were placed in a well and separated electrophoretically for 1 h at 10 *V*/cm and 10°C. The through was then filled with 100 µl of goat antimouse class-specific antiserum. Plates were incubated overnight at 4°C and then at room temperature for 8 h in a humidified atmosphere. The gels were stained using amido black (Sigma).

Immunodiffusion

The immuoglobulin isotype was determined by monoclonal antibody diffusion (5 µl at 1:100 and 1:1,000 dilutions in PBS) in 1%

Table 1. Stability of antibody-secreting hybrids in relation to culture volume as measured against immunizing keratin extract

Hybrids	Immunoreactivity in ELISA (Abs ± SE)		
	1-ml culture (3.4-ml well)	10 ml culture (50-ml flask)	50-ml culture (250-ml flask)
A2	0.07 ± 0.003	0.20 ± 0.004	0.34 ± 0.004
A3	0.53 ± 0.020	0.31 ± 0.010	0.18 ± 0.004
B3	0.53 ± 0.030	0.80 ± 0.030	1.30 ± 0.010
B4	0.51 ± 0.003	0.20 ± 0.020	0.00
B5	0.25 ± 0.002	0.40 ± 0.010	0.13 ± 0.000
C4	0.64 ± 0.010	0.00	ND
C5	0.21 ± 0.010	0.30 ± 0.010	0.85 ± 0.003
PU	0.10 ± 0.010	0.08 ± 0.020	0.08 ± 0.020

PU, P3X63Ag8/U1, BALB/c mouse myeloma; ND, not done; Abs, absorbance at 492 nm

agarose gel (Pharmacia) against 5 µl or class$^-$ or subclass$^-$ specific goat antimouse antisera (Sigma, 1:1,000 dilution in PBS) [17]. Precipitin lines were stained with amido black (Sigma).

Results

We produced a monoclonal antibody raised against keratin extracted from human urinary bladder squamous cell carcinoma. In order to confirm the keratin content of prepared keratin extracts, we raised antiserum against standard keratin (Sigma). In direct ELISA one extract prepared form squamous cell carcinoma (K1) showed the highest level of reactivity with this antiserum (data not included). This extract was used as immunogen in cell hybridization.

Thirty out of the 40 plated wells showed growing hybridomas (75% success). After 2 weeks of fusion the supernatant of wells showing cells growth were tested by ELISA using antimouse immunoglobulin peroxidase conjugate to detect the presumably secreted antikeratin antibodies. Supernatants from seven wells (representing 17.5% of the total number of wells) showed detectable levels of antikeratin antibodies when tested against immunizing extract. Hybrids producing specific antibodies were studied for their stability in secreting immunoglobulins throughout the cell propagation. Three stable hybrids (A2, B3, and C5) gave increasing levels of antibody secretion up to 50 ml culture stage (Table 1). Since two hybrids, B3 and C5, had higher levels of antibody secretion than A2 we neglected cultures with a low level of antibody secretion.

Table 3. Classification of antibodies in hybrid supernatants

Hybrids	Immunoreactivity with different immunoglobulin classes (Abs ± SE)		
	Anti-IgG	Anti-IgM	Anti-IgA
B3	2.00[a]	1.86 ± 0.200	0.070 ± 0.000
C5	2.00[a]	0.01 ± 0.000	0.000
PU	0.10 ± 0.005	0.08 ± 0.010	0.016 ± 0.000

[a] Maximum range of absorbance; Abs, absorbance at 492 nm

The two hybrids were tested to detect specificity against keratins prepared from different types of urinary bladder tumors (namely, squamous cell carcinoma, transitional cell carcinoma, and adenocarcinoma) of the same histological grade (grade I). The B3 hybridoma supernatant reacted only with the immunizing extract (K1), but C5 hybridoma supernatant reacted with the two squamous cell carcinoma extracts (K1 and K2) with different levels of reactivity and did not react with K3 or K4 (Table 2). The two hybrids were tested using ELISA against three peroxidase conjugated antimouse immunoglobulin classes (anti-IgG, anti-IgM and anti-IgA). The B3 hybridoma supernatant gave positive reactivity with both anti-IgG and anti-IgM, but the C5 hybridoma supernatant showed positive reactivity with anti-IgG only (Table 3). Cloning of the C5 hybridoma cells, performed selectively at a dilution of 0.5 cell/well, resulted in 4 IgG secreting clones out of 12 showing growth (8.5% yield of stable monoclonal hybridomas, 34%). In studying the stability of antibody production by the four monoclonal hybridomas obtained, three were found to be unstable. They showed a declined level of specific antibody production.

Table 2. Specificity of immunoglobulins in supernatants of stable antibody-secreting hybrids

Hybrids	Immunoreactivity in ELISA with keratin extracts (Abs ± SE)			
	SqCC		TrCC	AdC
	K1	K2	K3	K4
B3	1.92 ± 0.010	0.18 ± 0.010	0.07 ± 0.010	0.08 ± 0.060
C5	1.21 ± 0.000	0.48 ± 0.010	0.02 ± 0.010	0.13 ± 0.040
PU	0.11 ± 0.020	0.15 ± 0.000	0.14 ± 0.030	0.14 ± 0.005

SqCC, Squamous cell carcinoma; TrCC, transitional cell carcinoma; AdC adenocarcinoma; K, keratin extract; Abs, absorbance at 492 nm

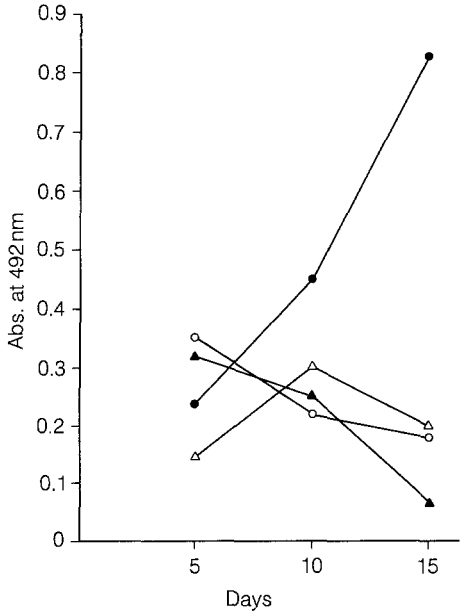

Fig. 1. Stability versus instability of produced clones. Only one clone expressed increasing level of specific antikeratin antibody reactivity

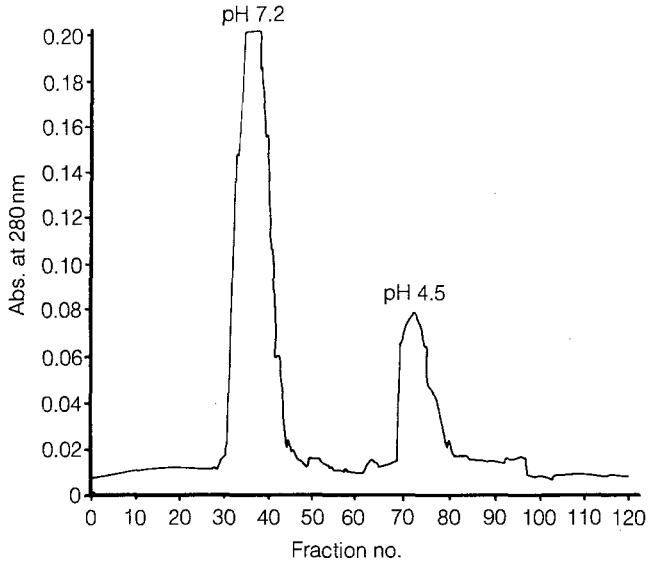

Fig. 2. Chromatogram of UNME/K1 monoclonal antibody purification. Ascitic monoclonal antibody (1 ml) was chromatographed on the Protein-A Sepharose CL-4B packed column, absorbance range 0.2 and chart speed 0.5 mm/min. The *first peak* represents unbound proteins eluted at pH 7.2, The *second peak* bound protein of UNME/K1 monoclonal antibody eluted at pH 4.5

The fourth clone represented was clearly stable with a high level of antibody secretion (Fig. 1).

For large scale production of the monoclonal antibody (UNME/K1), we expanded the growth of the monoclonal hybrid cell line (C5) as an intraperitoneal ascites tumor. The monoclonal antibody as ascitic fluid was purified utilizing Protein-A Sepharose CL-4B packed column (Fig. 2). The unbound protein and eluted fractions were tested against squamous cell carcinoma extracted keratin

Table 4. Immunoreactivity of purified ascitic monoclonal antibody fractions eluted at different pH values

pH	Immunoreactivity in ELISA (Abs)	
	UNME/K1 eluant	PU eluant
7.2	0.26	0.20
6.0	0.22	0.18
4.5	0.97[a]	0.23
3.5	0.23	0.22

UNME/K1, Monoclonal antibody; PU, P3X63Ag8/U1, BALB/C mouse myeloma; Abs, absorbance at 492 nm

[a] Indicates the highest level of reactivity of UNME/K1 monoclonal antibodies eluted at pH 4.5

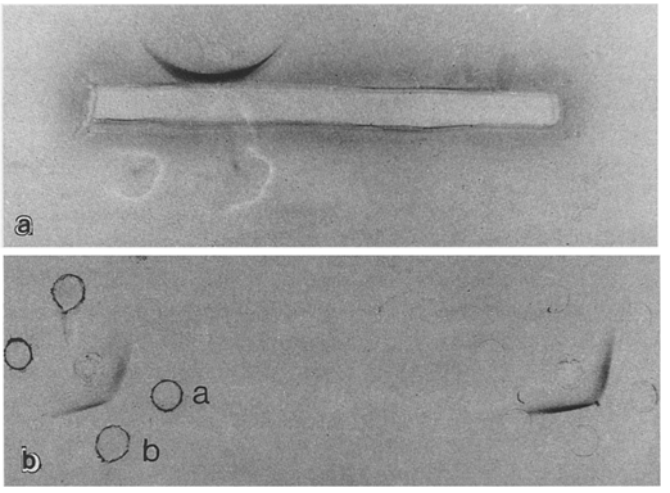

Fig. 3. a Immunoelectrophoretic characterization of purified UNME/K1 monoclonal antibody showing electrophoretic patterns of reaction between the antibody nand anti-igG. **b** Characterization of purified UNME/K1 monoclonal antibody using gel diffusion. Patterns of reaction of both crude (*left*) and purified (*right*) antibody at 1:100 (*a*) and 1:1,000 (*b*) dilutions with antimouse IgG2a antibody

(K1). The activity was found to be concentrated in the bound fraction eluted at pH 4.5 as compared to PU ascitic fluid processed the same manner (Table 4).

At immunoelectrophoresis no precipitin line was observed between the purified monoclonal antibody and antimouse IgM. There were precipitin lines between purified antibody and anti-mouse IgG (Fig. 3a). To identify the immunoglobulin subclass, gel diffusion was performed using antimouse IgG subclasses. A precipitation reaction was observed only between the UNME/K1 antibody and the antimouse IgG2a (Fig. 3b). Results also showed the reaction between crude or purified antibody (1:100 and 1:1,000 dilution of each) and antimouse IgG2a. The reaction was more intense with purified antibody.

The purified monoclonal antibody was tested by ELISA to determine its specific reactivity with different types of urinary bladder tumors (Table 5). There was

Table 5. Reactivity pattern of purified UNME/K1 monoclonal antibody towards different types of bladder carcinoma keratin extracts

Extracts	Immunoreactivity in ELISA	
	Ratio	Percentage
SqCC	12/12	100
TrCC	0/ 4	0
AdC.5	0/ 3	0

SqCC, Squamous cell carcinoma; TrCC, transitional cell carcinoma; AdC, adenocarcinoma

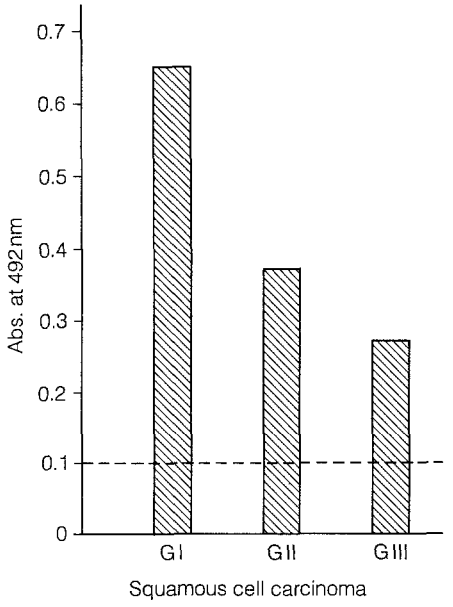

Fig. 4. Grade-related specific reactivity of UNME/K1 monoclonal antibody towards bladder squamous cell carcinoma keratin extracts. *GI, GII* and *GIII* represent the histopathological grade of tumors. Background level of reaction is indicated by *discontinuous line* at 0.1 absorbance units

100% antibody-specific reacivity with the squamous cell carcinoma type but no antibody reactivity was detected with either transitional cell carcinoma or adenocarcinoma keratin extracts. Within the squamous cell carcinoma type grade related levels of ractivity were recorded (Fig. 4). The higher the grade of tumor, the lower the specific reactivity, indicating a reciprocally inversed relationship.

Discussion

The development of hybridoma technology has already revolutionized many areas of basic and applied immunology and has provided an opportunity to reevaluate the role of immunological methods in the diagnosis of bladder cancer. Few monoclonal antibodies have been raised against keratins extracted from human normal epidermis, tumor cell lines, and urinary epithelia [4, 18, 20].

As has been mentioned in the literature, keratins can be defined as the subunit proteins constituting the tonofilaments that are recognizable in the cytoplasm of almost all epithelial cells or tissues [7] as intermediate-sized filaments. Recent studies have indicated that such proteins can be identified by their water insolubility, high molecular weight range (40–70 kDa), ability to reconstitute into 10-nm filaments, epithelial tissue specificity, reactivity with antisera prepared against certain authentic keratins, and reactivity with highly specific monoclonal antikeratin antibodies [10, 19].

To our knowledge, this is the first report of the production of hybridomas by fusion of P3X63Ag8/U1 myeloma cells and spleen cells of BALB/c mouse immunized with keratin extracted from GI squamous cell carcinoma of the human urinary bladder associated with schistosomiasis. Seven hybridomas have been investigated for their stability in antibody secretion, and three stable hybridomas were obtained. This level has been also obtained by other investigators [6]. The instability of antibody secretion as a major problem of hybridoma technology has been attributed to the initial instability in rearranging the genetic material coding for the desired immunoglobulin in the hybrids [15].

The actively growing hybridoma, secreting a remarkable amount of antikeratin antibody of the IgG class, was cloned. Only one out of four of the monoclonal hybrids obtained has been remarkably stable in antibody secretion. This monoclonal hybrid was propagated as an ascites tumor and large amounts of antibody (UNME/K1) were produced. Purification of UNME/K1 antibody was performed using Protein-A Sepharose CL-4B affinity column. The purified UNME/K1 antibody was confirmed to be of the IgG2a subclass of immunoglobulins as determined by immunoelectrophoresis and gel diffusion.

The observation of diversity of expression of cytokeratins in different epithelium-derived tumors [14], together with the general tendency towards conserved cytokeratin polypeptide patterns during malignant growth and metastasis, might provide the basis for a novel approach to tumor characterization, with cytokeratins as differentiation markers [23].

A high level of specificity of UNME/K1 antibody was expressed towards keratin extracted from squamous cell carcinoma. This level of specific reactivity decreased as the histopathological grade of tumor increased. This was to be expected since by the increase in histopathological grade, the keratinization of tumor decreases. By contrast, no level of reactivity was observed with keratin extracts of transitional cell carcinoma or adenocarcinoma which inidicated the monospecificity of the produced monoclonal antibody. Both the cell and tissue type-specific expression of cytokeratins and its possible modulation in response to stage of differentiation or state of disease is now well documented.

In summary, for the first time a monoclonal antibody of the IgG2a subclass of immunoglobulins has been generated that is directed against keratin expressed by human urinary bladder squamous cell carcinoma. Urinary bladder squamous cell carcinoma secondary to schistosomiasis is a major cause of death in Egypt. Only in

recent years has interest in its control reached a level equal to its medical and economic importance. This UNME/K1 monoclonal antibody has a monospecific reactivity inversely related to the histopathological grade of tumor. Such characterization of tumors, with respect to cytokeratin polypeptide patterns, could contribute to tumor diagnosis and classification. Experiments are in progress to characterize the antigenic target and to assess the potential of this antibody as a diagnostic tool for urinary bladder squamous cell carcinoma.

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