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) recognizing 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 epithelia, display different cytokeratin polypeptide patterns that are characteristic of certain groups of tumors. Epithelium-derived tumors appear to maintain 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 antiserum 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 schistosomiasis which is endemic to the Middle East and Africa - could be useful for immunodiagnosis 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 mM NaCl, 2 mM dithioerythritol (DTE), 0.5 mM phenylmethylsulfonylfouride (PMSF), 0.5% Triton X-100, 10 mM 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 30,000Xg and 15°C (J2-21 high-speed centrifuge, Beckman, Switzerland) were dissolved by stirring overnight in keratin dissociation buffer (8 M urea, 50 mM Tris HCL, pH 9.0) followed by centrifugation. Supernatant was dialyzed overnight against reconstitution buffer (2 mM Tris HCL, pH 5.5) to precipitate keratin filaments. Finally, keratins were gradually dissolved in urea against reconstitution buffer (2 mM Tris HCL, pH 7.5) to precipitate centrifugation for l h at 30,000Xg and 15~ (J2-21 high-speed polyethylene glycol (Sigma Chemical Company, St. Louis, MO, USA, MW 1500) in RPMI-1640 medium containing 5% dimethyl sulfoxide (DMSO) following previously described protocol [6], with minor modifications. The cells were suspended to a final concentration of 1X10^6 cells/ml and seeded (0.5 X 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 X 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 mM L-glutamine, 4X10^-5 hypoxanthine, 1.8X10^-5 thymidine, 100 IU penicillin, and 100 µg/ml streptomycin (HT medium). Cultures were grown at 37°C in a humidified atmosphere with 5% CO2 in air. On the next day selective HAT medium containing (4X10^-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 (2X10^6 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-4B (Pharmacia Fine Chemicals, Sweden) was swollen in PBS, pH 7.2 and packed in 1.14 X 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 100 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% orthophenylene diamine (OPD) in citrate phosphate buffer, pH 5.0, and 20 µl of 30% H2O2]. 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 monochol 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 immunglobulin isotype was determined by monoclonal antibody diffusion (5 µl at 1:100 and 1:1,000 dilutions in PBS) in 1%