Monoclonal Antibodies to Human Primary Uveal Melanomas Demonstrate Tumor Heterogeneity

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Five rat monoclonal antibodies to human uveal melanoma were produced using primary tumor tissue for immunization and screening. These monoclonal antibodies were tested by enzyme-linked immunosorbent assay against 12 uveal melanomas, one breast carcinoma, and peripheral blood lymphocytes from five healthy volunteers. One monoclonal antibody, 4A3, reacted against all the melanomas and none of the controls. On immunoblotting, this monoclonal antibody identified a protein doublet in the molecular weight range 55,000. With these monoclonal antibodies, it was possible to demonstrate significant variation in the antigenic profiles of the uveal melanomas which was not present in the controls. Invest Ophthalmol Vis Sci 27:1362-1367, 1986

Uveal melanoma is the most common primary ocular neoplasm in adult humans. Since 1978, Zimmerman and others have suggested that enucleation can contribute to the high mortality of this disease, and, more recently, studies using animal models have indicated that metastatic spread may be influenced by immunological factors. Unfortunately, the immunological study of these tumors is limited by the lack of well-defined antigens and antibodies.

The recently-developed hybridoma technology provides a means of overcoming these problems by enabling unlimited amounts of monoclonal antibodies to be produced against previously unrecognized antigens. Many monoclonal antibodies have been generated against cutaneous melanomas. Some have displayed antigenic heterogeneity in these tumors, which has been correlated with the degree of differentiation. In addition, the subclassification of cutaneous melanomas according to their differential reaction with panels of monoclonal antibodies has been shown to have some predictive value in respect of metastatic disease.

There would seem to be scope for investigating antigenic heterogeneity in ocular melanomas using monoclonal antibodies raised against primary human uveal tumors. In this preliminary report, the production of these monoclonal antibodies is described and, in addition, their potential as a tool for the investigation of antigenic heterogeneity in ocular melanomas is demonstrated.

Materials and Methods

Uveal Melanoma Antigens

Human choroidal and ciliary body melanomas were obtained minutes after surgical removal of the tumor from the eye, or, in a small number of cases, from enucleation specimens. After measurement of the dimensions of the tumor, tissue was excised from appropriate parts of the specimen so as not to interfere with pathological assessment. Shortly after this, a cell suspension was prepared by passing the tissue through a wire mesh (2 mm diameter). The cells were assessed by phase contrast microscopy to confirm that they remained intact, and the cell suspension was then used to prepare ELISA plates. If animal immunization could not be performed on the day of surgical removal of the tumor, the tissue was stored in liquid nitrogen until required.

Animals

All animal investigations conformed to the ARVO Resolution on the Use of Animals in Research, and were performed using an inbred colony of DA rats maintained and bred in the Biochemistry Department animal house at the University of Glasgow. Test bleeding was from the tail vein, and further serum (1–2 ml) was obtained from the inferior vena cava at sacrifice. Serum samples were allowed to clot, and then centrifuged at 500 g to remove the clotted material.

Immunization and Fusion

Animals were immunized intraperitoneally with approximately 2–5 × 10⁶ fresh unfixed and unwashed
uveal melanoma cells, if possible on the day of surgical removal of the tumor. The rats were boosted with a similar number of cells obtained from the tumor of a different patient 3–5 weeks later, depending on the availability of tissue. When an adequate serum immune response to uveal melanomas was demonstrated by the ELISA assay (a titre of 1/1000 or greater), a further boosting dose was given intraperitoneally using tumor cells from a third patient, and fusion was performed 3–4 days later. Fusion procedures were performed essentially by the method of Kohler and Milstein\(^6\) using 50% polyethylene glycol 4000 (Merck, Darmstadt, West Germany) (PEG) dissolved in 15% dimethyl sulfoxide (DMSO)\(^11\) with minor modifications.\(^12\) The rat myeloma Y3Ag 1.2.3 cell line\(^13\) was obtained from Dr. C. Milstein, MRC Laboratory for Molecular Biology, Cambridge. Fusions were laid down at 10\(^5\) cells/well on 24-well Costar plates. Emerging clones were visible 10–15 days after fusion and were screened by ELISA for reaction with uveal melanoma cells from allogeneic tumors. Positive wells (those with an optical density reading of greater than 0.1 units above control) were then subcloned at least twice at 0.25 or 0.5 cells/well on DA rat spleen feeder cells to ensure monoclony, and strongly positive wells were expanded to bulk tissue culture. Bulk tissue culture supernatant was concentrated tenfold by precipitation with 40% ammonium sulphate, followed by dialysis into phosphate buffered saline.

**Enzyme Linked Immunosorbent Assay (ELISA)**

ELISA assays were performed as described by Campbell.\(^12\) Briefly, 96-well flat-bottomed microtitre plates (Dynatech Laboratories Ltd., Billingham, UK) were incubated with 0.1% (w/v) poly-L-lysine (Sigma, molecular weight range 70,000–150,000) in PBS (100 \(\mu\)l/well) for 1 hr after a brief rinse in warm distilled water. The plates were washed and then treated with 10\(^3\) fresh primary uveal melanoma cells in 100 \(\mu\)l PBS or control cells, consisting of Ficoll purified peripheral blood lymphocytes or primary breast carcinoma cells, and centrifuged at 700 g for 5 min. The cells were then treated with 100 \(\mu\)l of 0.25% glutaraldehyde (Sigma London Chemical Co., Ltd., Poole, UK) for 3 min before further washing and storage in a solution containing 5% bovine serum albumin (BSA), 0.01% sodium azide and 1% heat inactivated normal goat serum at 4°C wrapped in Clingfilm.

Before the assay, the stored plates were washed and blocked with a solution of 3% BSA and 1% heat-inactivated normal goat serum in phosphate buffered saline for 4–12 hr at 4°C. After washing, the plates were treated with 100 \(\mu\)l test or control serum or hybridoma supernatant for 12–16 hr at 4°C. After further washing, 100 \(\mu\)l of rabbit anti-rat IgG (H + L) conjugated to horseradish peroxidase (Miles Laboratories Ltd., Slough, UK, diluted 1/1000 in PBS-BSA-Tween 20) were added to all the wells, incubated for 30 min at room temperature, and washed with PBS containing 0.05% Tween 20. The substrate mixture of 0.04 mg/ml freshly-prepared 0-phenylenediamine, dissolved in 0.05 M sodium citrate 0.15 M sodium phosphate buffer pH 6 containing 0.01% \(\text{H}_2\text{O}_2\), was then added at 100 \(\mu\)l/well, and the plates were incubated in the dark for 30 min. The reaction was stopped by the addition of 50 \(\mu\)l 4 N \(\text{H}_2\text{SO}_4\), and the absorbance was monitored at 492 nm on a Titretek Multiskan spectrophotometer (Flow Laboratories, Rickmansworth, UK). Positive controls consisted of immune rat sera. Negative controls were tissue culture medium from wells which contained the unfused rat myeloma cells or pre-immune rat serum.

**Class and Subclass Determination of Monoclonal Antibodies**

Class and subclass determination was by the method of Ouchterlony\(^14\) in solidified 1% agar using rabbit and goat antisera specific to the various isotypes (Miles Laboratories Ltd., Slough, UK).

**Electrophoresis and Western Blotting**

Samples of homogenized human uveal melanoma cells were analysed under reducing conditions by the discontinuous buffer system of Laemmli\(^15\) using 8.75% gels. Electrophoretic separation was by the method of Batteiger et al.\(^16\) utilizing 20 mM tris, 0.15 M NaCl, 0.05% sodium azide and 0.5% Tween 20 as blocking and washing reagent. Detection was with rabbit anti-rat IgG (H + L) (Miles Laboratories Ltd., Slough, UK, 1/100) followed by 10\(^5\) counts per minute of Protein A labeled with \(^{125}\)I at 800 \(\mu\)Ci/mg.

**Results**

One fusion utilizing the rat hybridoma system yielded 23 positive wells. The five strongest positives were selected for double subcloning and 18 were stored in liquid nitrogen. All of the five monoclonal antibodies, 4A3, 1B1, 4B4, 1B4, and 1C4, were of the IgM Class and subclass determination was by the method of Kohler and Milstein\(^6\) using 50% polyethylene glycol 4000 (Merck, Darmstadt, West Germany) (PEG) dissolved in 15% dimethyl sulfoxide (DMSO)\(^11\) with minor modifications.\(^12\) The rat myeloma Y3Ag 1.2.3 cell line\(^13\) was obtained from Dr. C. Milstein, MRC Laboratory for Molecular Biology, Cambridge. Fusions were laid down at 10\(^5\) cells/well on 24-well Costar plates. Emerging clones were visible 10–15 days after fusion and were screened by ELISA for reaction with uveal melanoma cells from allogeneic tumors. Positive wells (those with an optical density reading of greater than 0.1 units above control) were then subcloned at least twice at 0.25 or 0.5 cells/well on DA rat spleen feeder cells to ensure monoclonality, and strongly positive wells were expanded to bulk tissue culture. Bulk tissue culture supernatant was concentrated tenfold by precipitation with 40% ammonium sulphate, followed by dialysis into phosphate buffered saline.

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When the hybridoma panel was tested on 12 uveal melanomas simultaneously, it became clear that the antibodies reacted to a different extent with each tumor (Fig. 1), indicating heterogeneity in the tumors. The panel was simultaneously assayed against lymphocytes from five healthy volunteers and cells from one breast carcinoma in order to determine whether such heterogeneity could be detected on these antigens. Reaction with controls was generally much less, and there was little intersample variation. The reactivity in decreasing
Fig. 1. Monoclonal antibody binding to uveal melanomas. The results are expressed as a percentage ratio of the mean of two densitometer readings of the monoclonal antibody to four readings from medium containing 20% fetal calf serum. In each diagram, the dotted line is drawn at the mean + 2 SD of four control values. Results above this are considered positive.

order was 1B1, 1C4, 4B4, 1B4, and 4A3 in all samples (Fig. 2). When the reactivities of the monoclonals to the melanomas were compared to the control cells, 4A3 reacted significantly against all tumor specimens, whereas 4B4 and 1B4 showed preferential binding to only five and four tumors, respectively, and 1B1 and 1C4 were totally nonspecific. (Fig. 3).

The antigens were investigated to determine whether they corresponded to any of the published antigens detected by monoclonals to cutaneous melanoma. Figure 4 shows an immunoblot of the 4A3 antigen which is a 55K doublet. The doublet band was consistent in all of seven uveal melanomas tested, but was not detected in cell preparations from liver and brain. The other monoclonal antibodies did not react on immunoblotting, despite the high sensitivity of the double sandwich protein A assay employed.

Discussion

Of the five monoclonal antibodies, one (4A3) reacted significantly against all 12 primary tumors and none of the controls, two (4B4 and 1B4) reacted preferen-
Fig. 2. Monoclonal antibody binding to control cells. A–E are data from lymphocytes from healthy volunteers and F is from cells of a breast carcinoma. The results are obtained and displayed as in Figure 1.

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The majority of cutaneous melanoma-directed monoclonal antibodies have been generated by immunization with cell lines rather than primary tumor tissue and selected because they react with antigens present on primary tumor cells. Despite the recent development of uveal melanoma cell lines, we have preferred to use fresh tissue obtained from primary uveal melanomas for the purposes of this investigation. This is because neoplastic cells can rapidly undergo independent evolution in culture so that the interpretation of their relevance to primary tumor cells becomes difficult.

The rat hybridoma system was selected as it is superior to the murine one, due to the fact that the early positives generally remain stable and can be stored in liquid nitrogen prior to cloning. All of the five monoclonal antibodies produced were of the IgM class, despite selection with an anti-IgG probe. The IgG probe would, of course, have detected the antibody light chains on the IgM, but the lack of any IgG monoclonal antibodies may indicate that the selection process has also favoured epitopes which bind to IgM more strongly for reasons such as increased avidity due to IgM multivalence. This would select for closely spaced determinants.

Monoclonal antibody 4A3 was the only antibody which reacted with all 12 primary tumors and no controls. It recognizes a 55K doublet. A doublet of this
Monoclonal antibodies

Fig. 3. Binding of monoclonal antibodies to uveal melanoma cells relative to controls. For each antibody, the results are expressed as a percentage ratio of results displayed in Figure 1 to the mean of those in Figure 2. In each diagram, the dotted line is drawn at the mean + 2 SD of the six control values. Results above this are considered significant.

Heterogenous expression of tumor-associated antigens has been the subject of intense investigation during the past few years. However, the significance of this biological phenomenon is still incompletely understood. Studies such as those correlating antigenic profiles with differentiation and risk of metastatic disease provide indications of the scope of this line of research. The present study demonstrates distinct antigenic heterogeneity in uveal melanomas, which accounts for the variable results that were obtained when tumor im-
monoclonal antibody 4A3

Fig. 4. Immunoblotting identification of the antigen of one of the uveal melanoma generated monoclonal antibodies (4A3). On the left is an autoradiograph of the nitrocellulose paper containing immunoblotted antigen. On the right is the same nitrocellulose paper stained for protein with amido black after immunoblotting.

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References