

## Production of a monoclonal antibody specific for seminomas and dysgerminomas

(testicular and ovarian tumors/oncofetal antigen/glycoprotein/M, 40,000 cell surface protein/immunoperoxidase)

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**ABSTRACT** A monoclonal antibody (M2A, IgG2a) was produced against a cultured human ovarian epithelial adenocarcinoma cell line, HEY. Monoclonal antibody M2A reacted with a glycoprotein of molecular weight 40,000 on the surface of HEY cells. The affinity constant of the monoclonal antibody M2A for HEY cells was  $10^9 \text{ M}^{-1}$ , and the number of binding sites on HEY cells was  $2 \times 10^4$  per cell. The monoclonal antibody produced positive immunoperoxidase staining of fetal (but not adult) testis and of seminomas and dysgerminomas but did not stain various normal adult tissues or other gonadal or extragonadal tumors. Monoclonal antibody M2A may be useful for confirming a histological diagnosis of seminoma and dysgerminoma.

Seminomas account for approximately 40% of primary testicular tumors, whereas dysgerminomas, their counterpart in women, account for 1-2% of primary ovarian tumors (1, 2). Embryonal nests of germ cells may also give rise to extragonadal seminomas and dysgerminomas in the mediastinum (3), the retroperitoneum (4), and the suprasellar region of the brain (5). Most seminomas have a typical appearance on light microscopy and present no problem in histological diagnosis. However, attempts to confirm a diagnosis of atypical seminomas by immunohistochemical techniques have been frustrated by the lack of antibodies directed against specific antigenic markers associated with seminoma cells (6-8). In this communication we report the production of a monoclonal antibody (mAb), M2A, that reacts with fetal but not adult testis, with seminomas, and with dysgerminomas but not with other normal adult tissues or gonadal or extragonadal tumors. Potential applications of this mAb include confirmation of the histological diagnosis of primary and metastatic gonadal and extragonadal seminomas and dysgerminomas.

### MATERIALS AND METHODS

**Antibodies.** Rabbit polyclonal antibodies used were anti-mouse immunoglobulin (Ig) specific for various subclasses of mouse Ig (Litton Bionetics) and anti-mouse Ig coupled to horseradish peroxidase (Dako, Santa Barbara, CA). Mouse myeloma MOPC 315 IgA and a monoclonal anti-idiotypic antibody (AIA D10) directed against it were prepared as described (9).

**Cell Lines.** The human ovarian adenocarcinoma cell line HEY (10) was obtained from R. Buick (Ontario Cancer Institute). Primary cultures of human skin fibroblasts (ninth passage) were supplied by M. Buchwald (The Hospital for Sick Children, Toronto). Cells were grown as monolayer

cultures in minimal essential medium alpha (Flow Laboratories) supplemented with 7% fetal bovine serum and 2 mM glutamine.

**Human Normal Tissues and Tumors.** Various normal tissues and tumors were obtained from surgical biopsy specimens processed in the departments of pathology at The Hospital for Sick Children, Toronto General Hospital and Women's College Hospital.

**Production of mAb.** Eight-week-old female BALB/c mice (Canadian Breeding Laboratories, Montreal, PQ) were immunized intraperitoneally with  $2 \times 10^7$  cultured HEY cells, followed by a second, similar injection after 30 days. Three days later, splenic lymphocytes were fused with Sp2/0-Ag14 myeloma cells (11), using polyethylene glycol (PEG 4000) (9). Hybridoma colonies were selected in hypoxanthine/aminopterin/thymidine medium, using 96-well tissue culture plates (Falcon), and were screened by a cell-binding radioimmunoassay (RIA) (see below) for production of mAb against cultured HEY cells and primary cultures of human fibroblasts. Hybridomas that produced mAb reacting with HEY cells but not with fibroblasts were cloned twice by limiting dilution (9). To obtain ascitic fluid containing mAb, hybridoma cells were injected intraperitoneally into BALB/c mice primed with 2,6,10,14-tetramethylpentadecane (pristane, ICN). Immunoglobulin was prepared from ascitic fluid by affinity chromatography on protein A-Sepharose (Pharmacia) and concentrated to 1.6 mg/ml in 10 mM sodium phosphate at pH 7.2 (12).

**Cell-Binding RIA.** Cultured cells were seeded in 96-well tissue culture plates at a density of  $10^4$  cells per well and grown for 4-5 days, at which time they had formed confluent monolayers. They were incubated successively with mAb, rabbit anti-mouse Ig antisera specific for various mouse Ig subclasses, and  $^{125}\text{I}$ -labeled protein A, each for 40 min at 37°C (9). Washes with minimal essential medium alpha were performed between incubations. The cells were dissolved in 2 M NaOH, and collected with a cotton-tipped applicator, and the radioactivity was measured in a gamma counter.

**Determination of Characteristics of Binding of mAb M2A to HEY Cells.** Monolayers of HEY cells were incubated with various concentrations (7-70 nM) of mAb M2A, followed by a saturating amount of the  $^{125}\text{I}$ -labeled (Fab')<sub>2</sub> fragment of sheep anti-mouse Ig antibody (Amersham) (13). The data were plotted in a modified Scatchard form, and the affinity constant of mAb M2A and the number of binding sites per cell were calculated (13).

**Immunoperoxidase Staining of Tissue Sections.** Surgical biopsy samples were embedded in OCT compound (Miles), quick-frozen in liquid nitrogen, and stored at -70°C until use,

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Abbreviation: mAb, monoclonal antibody.

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at which time 4- to 6- $\mu$ m-thick sections were cut on an American Optical cryostat. After fixation in acetone for 15 min, the sections were rehydrated in Tris/NaCl (5 mM Tris Cl, pH 7.6/150 mM NaCl) and incubated with mAb (ascitic fluid or purified mAb diluted 1:100 in Tris/NaCl), followed by peroxidase-conjugated goat anti-mouse Ig antibody (1:50 dilution), each for 45 min, and finally with 0.05% diaminobenzidine and 0.03% H<sub>2</sub>O<sub>2</sub> for 4 min. All incubations were at room temperature and all dilutions and rinses between incubations were performed with Tris/NaCl. The sections were counterstained with hematoxylin and mounted under coverslips for viewing by light microscopy.

**Use of Immunoblot Analysis to Identify Antigen on HEY Cells That Reacts with mAb M2A.** HEY cells ( $2 \times 10^7$ ) were harvested by scraping with a rubber policeman and washed twice with phosphate-buffered saline (PBS: 140 mM NaCl/3 mM KCl/0.15 mM KH<sub>2</sub>PO<sub>4</sub>/8 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.2). The cell pellet was lysed by Vortex mixing at room temperature in 1 ml of 10 mM Tris Cl, pH 7.8/150 mM NaCl/1% (vol/vol) Nonidet P-40. The lysate was centrifuged at  $12,000 \times g$  for 10 min at room temperature. Aliquots of the supernatant, containing solubilized membrane and cytoplasmic proteins, were adjusted to 1% NaDodSO<sub>4</sub> and 2% (vol/vol) 2-mercaptoethanol (reduced proteins) or 1% NaDodSO<sub>4</sub> and 14 mg of iodoacetamide per ml (nonreduced proteins) and subjected to electrophoresis in 10% polyacrylamide slab gels containing 0.1% NaDodSO<sub>4</sub>. The separated proteins were transferred electrophoretically to Biodyne nylon membranes (Pall Ultrafine Filtration, Glen Cove, NY) (14), which then were incubated successively with 5% skim milk for 1.5 hr at room temperature, to block nonspecific binding, and then with 20  $\mu$ g of mAb M2A or D10 per ml overnight at 4°C. Protein bands reacting with the mouse antibodies were visualized by the immunoperoxidase reaction, with the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) (15).

**Metabolic and Surface Labeling of HEY Cells.** For metabolic incorporation of radioactive precursors into proteins, confluent HEY cell monolayers ( $3 \times 10^7$  cells) in 10-cm diameter tissue culture dishes were incubated with (a) 5 ml of growth medium containing 0.05 times the usual concentration of methionine, in the presence of [<sup>35</sup>S]methionine (40  $\mu$ Ci/ml, 500 Ci/mmol, New England Nuclear; 1 Ci = 37 GBq), for 20 hr at 37°C or (b) 10 ml of growth medium containing [<sup>3</sup>H]glucosamine (12  $\mu$ Ci/ml, 42.5 Ci/mmol, New England Nuclear) for 72 hr at 37°C. For enzymatic labeling of surface proteins, HEY cell monolayers were covered with 2 ml of PBS containing 200  $\mu$ l of a 1-mg/ml solution of lactoperoxidase (Sigma) and 1 mCi of carrier-free <sup>125</sup>I (Amersham). Three 50- $\mu$ l aliquots of 0.03% H<sub>2</sub>O<sub>2</sub> were added at 3-min intervals. Ten minutes after the last addition, the monolayers were washed three times with PBS containing 5 mM L-cysteine.

After metabolic or surface labeling, the monolayers were harvested and washed twice with PBS by centrifugation. The cell pellets were lysed by Vortex mixing at room temperature in 1 ml of 50 mM Tris Cl, pH 7.2/150 mM NaCl/1% (vol/vol) Triton X-100/1% deoxycholate/0.1% NaDodSO<sub>4</sub>/1 mM phenylmethylsulfonyl fluoride. The cell lysates were centrifuged at  $100,000 \times g$  for 60 min. The supernatants were incubated with 200  $\mu$ l of a 10% (wt/vol) suspension of protein A-Sepharose beads for 1 hr at 4°C. The beads were removed by centrifugation and the total radioactivity incorporated into proteins was determined by scintillation counting after precipitation in 10% (wt/vol) trichloroacetic acid at 90°C. Aliquots of the supernatants (150–300  $\mu$ l) were incubated with 30  $\mu$ g of mAb M2A or D10 and 30  $\mu$ l of protein A-Sepharose beads for 4 hr at 4°C. The beads were collected by centrifugation and washed four times with 100 mM Tris Cl, pH 7.5/200 mM LiCl/0.1% 2-mercaptoethanol and twice with 1 mM Tris Cl, pH 7.5/150 mM NaCl. The immunoadsorbed

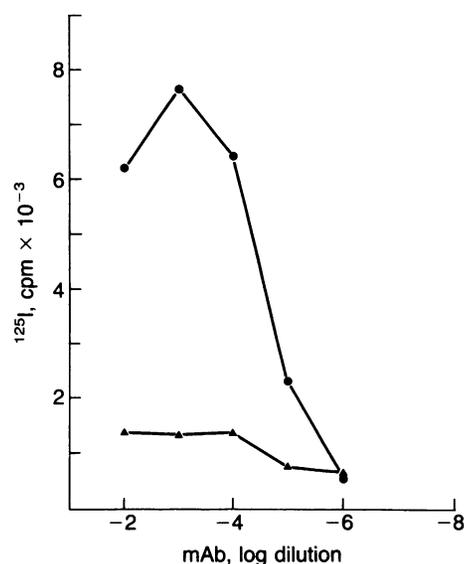


FIG. 1. Cell-binding radioimmunoassay of mAb M2A. Cultured HEY cells (●) and human fibroblasts (▲) were incubated with various dilutions of ascitic fluid containing mAb M2A, followed successively by anti-mouse IgG2a and <sup>125</sup>I-labeled protein A as described in *Materials and Methods*.

proteins were released from the protein A-Sepharose by boiling for 5 min in 100  $\mu$ l of 1% NaDodSO<sub>4</sub>/2% 2-mercaptoethanol and analyzed by polyacrylamide gel electrophoresis (PAGE) and autoradiography, as described (9).

## RESULTS

**Binding of mAb M2A to Cultured Cells.** mAb M2A was obtained in a fusion experiment using spleen cells of a mouse immunized with the HEY cell line and was selected on the basis of reactivity in a cell-binding RIA with HEY cells but not with primary cultures of human fibroblasts (Fig. 1). mAb M2A reacted with rabbit anti-mouse IgG2a antibody in an indirect cell-binding RIA but not with rabbit antibodies specific for other mouse Ig subclasses, indicating that it was of the IgG2a subclass. A modified Scatchard plot of binding of mAb M2A to HEY cells is shown in Fig. 2. The affinity constant of mAb M2A for HEY cells was  $10^9$  M<sup>-1</sup> and the number of binding sites was  $2 \times 10^4$  per cell.

**Reactivity of mAb M2A with Human Normal Tissues and Tumors.** The reactivity of mAb M2A with normal tissues and

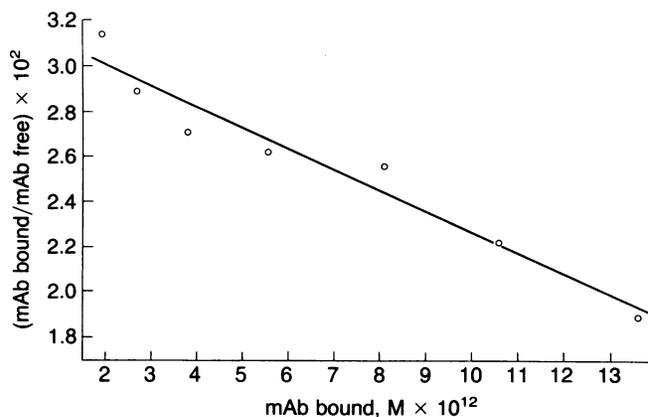


FIG. 2. Modified Scatchard plot of mAb M2A binding to HEY cells. HEY cells were incubated with various concentrations of mAb M2A, followed by <sup>125</sup>I-labeled (Fab')<sub>2</sub> fragment of sheep anti-mouse Ig antibody. Calculations are based on formulae published in ref. 13.

tumors was assayed using cryostat sections and an immunoperoxidase reaction (Table 1). mAb M2A reacted with germ cells in the seminiferous tubules of a fetal (24 weeks of gestation) testis (Fig. 3A) but not with germ cells or sustentacular cells of normal seminiferous tubules in adult testis. Tumor cells of 12 classical testicular seminomas stained positively, as did seminoma cells infiltrating

seminiferous tubules. In contrast, lymphocytes between the seminoma cells did not stain (Fig. 3 C-F). No other type of testicular tumor reacted with mAb M2A (Table 1). The mAb reacted with three primary ovarian dysgerminomas but not with normal adult ovary or the other ovarian tumors tested (Table 1). In addition to reactivity with testicular seminomas and ovarian dysgerminomas, there was less intense staining

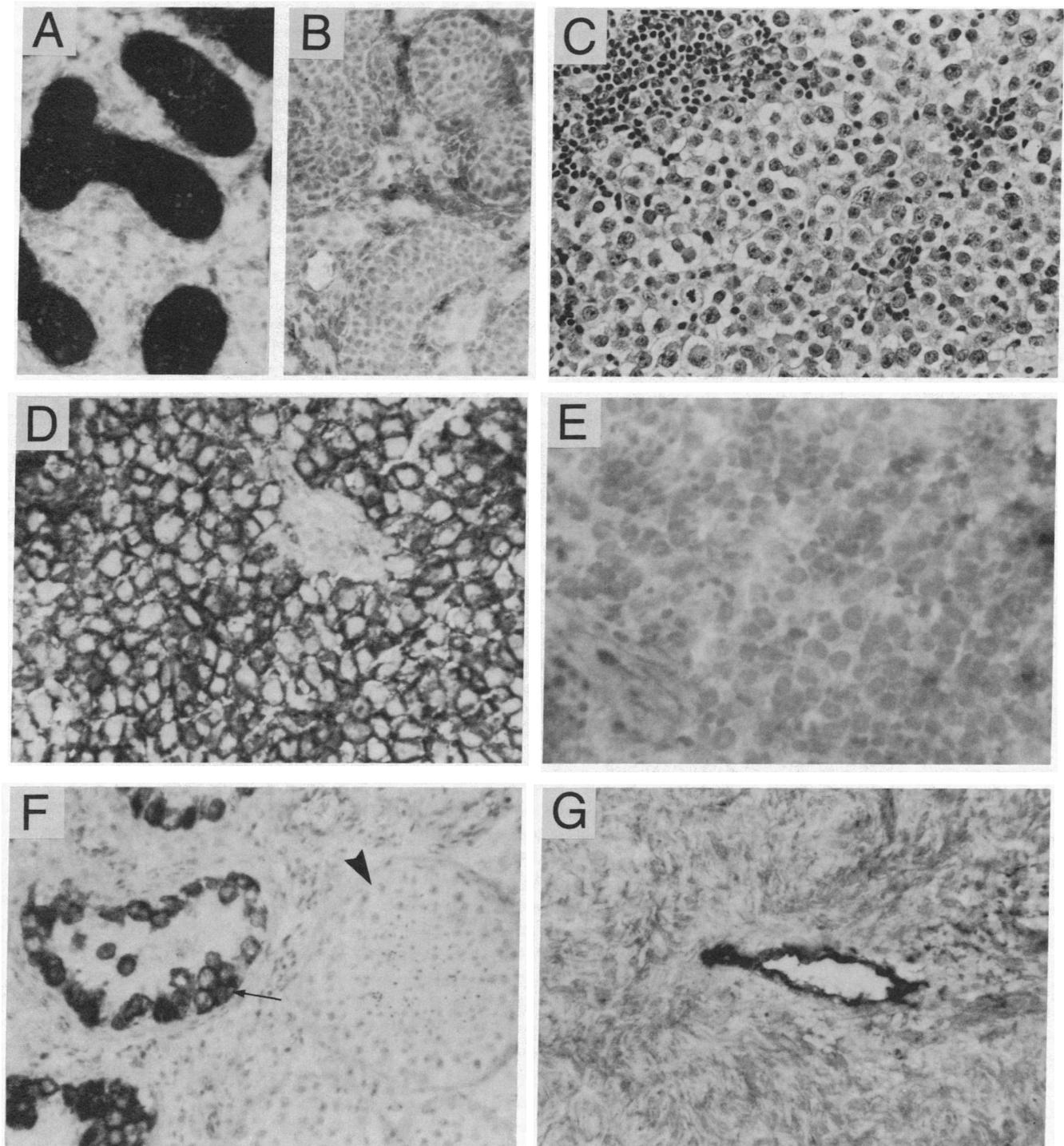


FIG. 3. (A and B) Fetal testis, showing positive staining of seminiferous tubules with mAb M2A (A) but absence of staining when mAb M2A was omitted (B). (Immunoperoxidase;  $\times 120$ .) (C) Classical seminoma, showing seminoma cells and lymphocytic infiltrate. (Hematoxylin/eosin;  $\times 150$ .) (D) Classical seminoma, showing positive immunoperoxidase staining of the seminoma cells with mAb M2A. (Immunoperoxidase;  $\times 150$ .) (E) Classical seminoma, showing no staining of the seminoma cells without mAb M2A. (Immunoperoxidase;  $\times 150$ .) (F) Classical seminoma, showing seminoma cells growing within a seminiferous tubule. The seminoma cells show positive immunoperoxidase staining with mAb M2A (arrow), whereas a tubule containing germ cells at various stages of maturation was not stained (arrowhead). (Immunoperoxidase;  $\times 100$ .) (G) Fibrous connective tissue, showing positive staining of capillary endothelium with mAb M2A. (Immunoperoxidase;  $\times 220$ .)

Table 1. Reactivity of mAb M2A with testicular and ovarian tumors, nongonadal tumors, and normal adult tissues

	No. positive/ no. tested
<b>Testicular tumors</b>	
Seminoma (classical)	12/12
Immature teratoma	0/1
Embryonal carcinoma	0/1
Mixed teratoma/embryonal carcinoma	0/2
Endodermal sinus tumor	0/1
Leydig cell tumor	0/2
<b>Ovarian tumors</b>	
Dysgerminoma	3/3
Thecoma	0/1
Granulosa cell tumor	0/1
Cystadenofibroma	0/2
<b>Epithelial</b>	
Serous	0/5
Mucinous	0/2
Endometrioid	0/3
Clear-cell	0/1
Undifferentiated	0/2
Nongonadal tumors*	0/34
Normal adult tissues†	0/14

Reactivity was assessed by immunoperoxidase staining (see *Materials and Methods*).

\*No reactivity was seen with any of the nongonadal tumors examined, which were as follows (no. of cases in brackets): adenocarcinoma of colon [7], carcinoma of breast [4], osteogenic sarcoma [3], Wilms tumor [2], rhabdomyosarcoma [2], carcinoma of esophagus [1], adenocarcinoma of lung [1], melanoma [1], hepatoblastoma [1], Ewing sarcoma [1], leiomyosarcoma [1], pheochromocytoma [1], lymphoma [4], thymoma [1], cystic hygroma [1], cavernous hemangioma [1], angiosarcoma [1], retroperitoneal anaplastic seminoma [1] (see text).

†None of the normal adult tissues tested reacted with mAb M2A. One sample of each of the following tissues was tested: testis, ovary, kidney, adrenal, thyroid, pancreas, liver, stomach, colon, spleen, thymus, lymph node, epidermis, and periosteum.

of mesothelium and the endothelium of capillaries (Fig. 3G). mAb M2A did not react with a large sample of normal adult human tissues or non-testicular or non-ovarian tumors. Included among the latter was a primary tumor of the retroperitoneum, which was diagnosed histologically as an anaplastic seminoma (Table 1).

**Identification of the Antigen Reacting with mAb M2A.** The mAb reacted with an antigen in HEY cell lysates having a molecular weight of approximately 40,000 when analyzed by the immunoblotting technique under both reducing (Fig. 4A) and nonreducing (data not shown) conditions. The antigen is a glycoprotein, since it was labeled metabolically by both [<sup>35</sup>S]methionine and [<sup>3</sup>H]glucosamine (Fig. 4B), and it was localized to the surface of HEY cells on the basis of its accessibility to enzymatic radioiodination by lactoperoxidase (Fig. 4C).

## DISCUSSION

mAb M2A was shown in the present study to react with an oncofetal antigen shared by the germ cells of seminiferous tubules of immature testis and the tumor cells of seminomas and dysgerminomas. Mesothelial cells and vascular endothelium also showed slight reactivity with the mAb (Fig. 3). This crossreactivity implies that mesothelial cells and vascular endothelium bear an epitope similar to one on the surface of the seminoma cells. The significance of this, including whether this epitope is part of the same macromolecule in different tissues, remains to be determined.

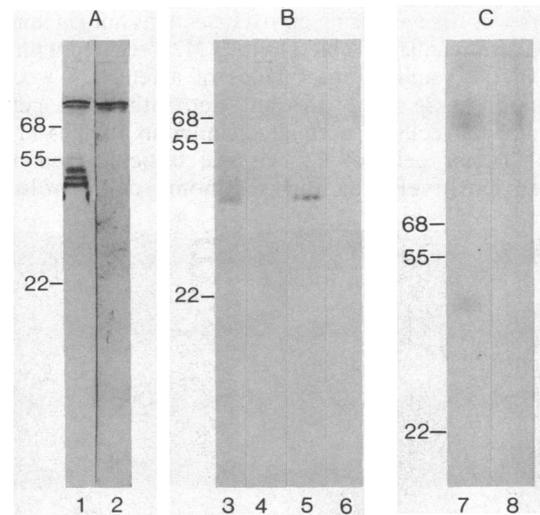


FIG. 4. Characterization of antigen recognized by mAb M2A. (A) Aliquots (150  $\mu$ g of protein) of HEY cell lysates were analyzed by immunoblotting after electrophoresis in 10% polyacrylamide slab gels under reducing conditions, using mAbs M2A (lane 1) and D10 (lane 2). (B) Aliquots of lysates of HEY cells labeled metabolically with [<sup>35</sup>S]methionine ( $30 \times 10^6$  cpm; lanes 3 and 4) or [<sup>3</sup>H]glucosamine ( $3 \times 10^6$  cpm; lanes 5 and 6) were immunoprecipitated with mAb M2A (lanes 3 and 5) or D10 (lanes 4 and 6) and analyzed by electrophoresis in 10% polyacrylamide slab gels under reducing conditions, followed by autoradiography. (C) HEY cells were radioiodinated, using <sup>125</sup>I and lactoperoxidase. Aliquots ( $10^6$  cpm) of cell lysates were immunoprecipitated with mAb M2A (lane 7) or D10 (lane 8) and analyzed by electrophoresis in 7.5% polyacrylamide slab gels under reducing conditions, followed by autoradiography. The positions of migration of molecular weight standards are indicated ( $M_r \times 10^{-3}$ ).

Although mAb M2A was raised against and reacted in an immunoperoxidase reaction with the epithelial ovarian adenocarcinoma cell line HEY, it did not react with other epithelial adenocarcinomas of the ovary or normal adult ovary (Table 1). In a previous study, mAb M2A was also shown not to react with the original HEY ovarian adenocarcinoma xenograft grown in thymectomized CBA/CJ mice, from which the HEY cell line was derived (unpublished data). This antigenic difference was assumed to be due to selection of a rare stem cell from the original xenograft population during derivation of the HEY cell line. The results presented in this work support this hypothesis, since we have shown that HEY cells display an antigen that is defined by mAb M2A and that likely is also present on a population of testicular and ovarian stem cells that can give rise to the seminoma and dysgerminoma types of tumor, respectively. The antigen was detectable in fetal but not adult testis, presumably because it was lost during normal differentiation of the stem cells. Expression of the antigen by seminomas suggests that these tumors arise by the malignant transformation of a rare stem cell remaining in the adult testis.

The antigen defined by mAb M2A is a monomeric glycoprotein migrating with a molecular weight of approximately 40,000 under both reducing and nonreducing conditions. The three bands visualized by immunoblotting probably represented species with different degrees of glycosylation (Fig. 4A and B). The antigen recognized by mAb M2A is present on the cell surface, since the mAb reacted with HEY cells in a cell-binding RIA and the antigen was accessible to enzymatic radioiodination by lactoperoxidase (Figs. 1 and 4C).

The potential diagnostic importance of mAb M2A is based on its absolute reactivity with seminomas and dysgerminomas and not with other testicular or ovarian tumors or non-testicular or non-ovarian tumors, including those with

which seminomas and dysgerminomas might be confused histologically, such as large-cell lymphomas, melanomas, and anaplastic carcinomas. Moreover, since mAb M2A did not stain normal testicular germ cells, it might be of diagnostic value in cases where there is only intratubular spread of seminoma. The histochemical techniques that are presently available for dealing with this problem cannot distinguish between intratubular seminoma cells and other types of normal or abnormal germ cells (16). It is not known whether all types of seminomas and dysgerminomas will react with mAb M2A, and additional tumors of these types must be examined. However, it is noteworthy that the only seminoma that did not react with mAb M2A in this study was one that was highly anaplastic, was located retroperitoneally, and was not associated with a primary testicular seminoma. Since this tumor was diagnosed solely on the basis of morphologic criteria, the possibility must be considered that it was not a seminoma.

If the antigen recognized by mAb M2A proves to be a specific marker for seminomas and dysgerminomas in a larger series of testicular and ovarian tumors, reactivity with mAb M2A could be a useful diagnostic criterion for primary seminomas and dysgerminomas and their metastases; for metastatic seminomas and dysgerminomas for which no primary can be found; and for extragonadal seminomas and dysgerminomas arising in the mediastinum, retroperitoneum, or central nervous system, especially in cases where diagnosis based on morphologic criteria alone is not conclusive.

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