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Immunology

Cell surface antigens of human melanoma identified by monoclonal antibody

(tumor antigen/hybridoma/serology)

MING-YANG YEH[ab], INGEGERD HELLSTRÖM[ab], JOSEPH P. BROWN[ac], GLENN A. WARNER[d], JOHN A. HANSEN[efg], AND KARL ERIK HELLSTRÖM[ac]

Divisions of [a]Tumor Immunology and [c]Oncology, Fred Hutchinson Cancer Research Center, Seattle, Washington 98104; Departments of [b]Microbiology/Immunology, [c]Pathology, and [f]Medicine, University of Washington, Seattle, Washington 98195; [d]Swedish Hospital Tumor Institute and Medical Center, Seattle, Washington 98104; and [g]Histocompatibility Laboratory, Puget Sound Blood Center, Seattle, Washington 98104

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ABSTRACT **Mouse NS-1 myeloma cells were fused with spleen cells from mice that had been immunized with cells from a human melanoma, M1804. Hybrid cells were grown in selective medium and tested for production of antibody to surface antigens of M1804 cells. Three hybrids that produced antibodies that bound to the melanoma cells but not to autologous skin fibroblasts were cloned. Antibodies produced by two of the clones were cytotoxic to M1804 cells in the presence of rabbit complement. Extensive specificity tests showed that the antibodies produced by the clones bound strongly only to M1804 cells; significant, although weaker, binding occurred with 2 of 11 allogeneic melanomas. Apart from weak binding of the antibody produced by one of the clones to a breast carcinoma, binding assays of five carcinomas, one sarcoma, and fibroblasts from 17 individuals were negative, as were cytotoxic tests of 10 lymphoblastoid cell lines and peripheral blood lymphocytes from 68 normal donors and 12 chronic lymphocytic leukemia patients. This suggests that we have identified one or more determinants of a melanoma-associated antigen(s), whose expression is limited to a small proportion of melanomas.**

Human melanomas express cell surface antigens to which melanoma patients can mount an immune response (1). Sera from such patients sometimes contain antibodies to cell surface antigens of their melanomas. In some cases the antigens have been detected only on the patient's own melanoma (2, 3), and in other cases, also on allogeneic melanomas (3–5). There is also evidence for cell-mediated immunity, both to antigens whose expression is limited to a small number of melanomas (6) and to antigens that are shared by most, or possibly all, melanomas (7–9).

The introduction by Köhler and Milstein (10) (see also ref. 11) of a method for producing large amounts of monoclonal antibodies of defined specificity promises to revolutionize serological analysis of tumor antigens. Koprowski *et al.* (12) have used this approach and obtained a monoclonal mouse antibody that identifies what may be a melanoma-specific antigen.

This paper reports the results of a fusion of mouse NS-1 myeloma cells with spleen cells from mice that had been immunized with a short-term explant of a human melanoma (M1804). Three of the cell hybrids produced antibodies that bound to the immunizing melanoma, but not to autologous skin fibroblasts. These hybrids have been cloned and tested extensively by means of a ^{125}I-labeled protein A (^{125}I-protein A) binding assay and complement-dependent cytotoxicity assays. Results have shown that the antibodies produced by the clones define antigenic determinants expressed strongly on M1804 melanoma cells and weakly on some allogeneic melanomas, but not appreciably on other human cell types.

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MATERIALS AND METHODS

Cells. M1804 is a short-term explant of a melanoma metastasis that was removed from a lymph node of a 51-yr old white male 6 months after excision of the primary tumor (superficial spreading L III). Skin fibroblasts from the same patient (N1804) have also been cultured. Melanomas M1462, M1697, M1698, M1720, M1799, M1801, and M1885; renal cell carcinoma C1391; liposarcoma S1079; skin fibroblasts N1799 and N1859; lung fibroblasts N1879 and N1881; B-lymphoblastoid cell lines PA-3, SB, CAH, T5-1, HA-1, Daudi, and 3001-4; and T-lymphoblastoid cell lines CCRF-CEM, Molt 4, and RPMI-8402 have been cultured at the Fred Hutchinson Cancer Research Center. Melanoma lines M1480 (G.S.), M1481 (M.S.), M1482 (MeWo), and M1477 (L.E.), bladder carcinoma C1483 (T24), lung carcinoma 1893 (SKMES), and skin fibroblast cultures N1896–N1907 were obtained from M. Bean (Virginia Mason Research Center, Seattle, WA). Breast carcinoma C0925 and lung carcinoma C0928 were obtained from R. Herberman (Bionetics, Kensington, MD). Human cells were grown in 5% CO_2 in air in Waymouth's culture medium buffered with $NaHCO_3$ and supplemented with fetal calf serum (300 ml/liter), potassium penicillin G (100 units/ml), streptomycin (100 mg/liter), nonessential amino acids (10 ml/liter), sodium pyruvate (1 mM), and L-glutamine (290 mg/liter). Serum and other supplements were obtained from GIBCO, except sodium pyruvate (Microbiological Associates, Bethesda, MD).

P3-NS1/1-Ag4-1 (NS-1) is an azaguanine-resistant BALB/c myeloma, which was kindly provided by C. Milstein (Medical Research Council Laboratory of Molecular Biology, Cambridge, England). NS-1 cells were grown at a density of 0.5–4 $\times 10^5$ cells per ml in 7% CO_2 in air in RPMI 1640 culture medium (GIBCO no. 430-1800) buffered with $NaHCO_3$ and supplemented with heat-inactivated fetal calf serum (150 ml/liter), penicillin (100 units/ml), streptomycin (100 mg/liter), sodium pyruvate (1 mM), and L-glutamine (290 mg/liter), which is referred to as NS-1 culture medium.

Immunization and Cell Fusion. Four 3-month-old BALB/c mice were immunized with two intraperitoneal inoculations of 10^7 viable M1804 melanoma cells 1 week apart and were killed 4 days later. Their spleens were removed and the cells were suspended in serum-free NS-1 culture medium. Erythrocytes were lysed by hypotonic shock. The spleen cells (1.5×10^8) were mixed with NS-1 cells (3.8×10^7) and pelleted by centrifugation at $200 \times g$ for 5 min. The cells were placed in a 37°C water bath, resuspended over a period of 1 min in 1 ml of serum-free NS-1 culture medium containing 50% (wt/wt) polyethylene glycol 1540 (Baker, cat. no. 9-U220, lot no. 730355), and stirred gently for 1 min. Two milliliters of NS-1 culture medium was then added over a period of 2 min while the cells were resuspended by gentle stirring. This was followed

by addition of an additional 8 ml of culture medium. The cells were pelleted by centrifugation at 200 × *g* for 5 min and resuspended in 21 ml of NS-1 culture medium. One hundred microliters of the cell suspension was dispensed into each of 192 Falcon 3040 microtest wells (Falcon). The plates were placed in a humidified 37°C incubator with 7% CO_2 in air.

Growth of Antibody-Producing Cell Hybrids. One day after the fusion, 100 μl of NS-1 culture medium containing hypoxanthine (13.6 mg/liter), aminopterin (0.176 mg/liter), and thymidine (3.88 mg/liter) (HAT medium) was added to each well. For 2 weeks, the cultures were fed at 2- to 3-day intervals by replacing the medium with 100 μl of fresh selective medium. For the third week, NS-1 culture medium containing hypoxanthine and thymidine but not aminopterin was used, and, subsequently, NS-1 culture medium with none of these additives was used.

Hybrids selected for further study were plated at 10 cells per well in Falcon 3040 microtest plates with 6 × 10^5 BALB/c thymocytes per well as a feeder layer and tested for antibody production 1 week later. Cells from selected wells were cloned formally by plating the cells at low density in microtest wells with a thymocyte feeder layer. One week later the wells were examined microscopically for single clones, which were retested for antibody production. Antibody-producing clones were expanded to about 10^7 cells, frozen in RPMI containing heat-inactivated fetal calf serum (300 ml/liter) and dimethyl sulfoxide (100 ml/liter), and stored in liquid nitrogen. Spent medium from the cultures was stored frozen for additional specificity tests.

Antisera. Rabbit antiserum to mouse Ig (IgG, IgA, IgM), cat. no. 21-574-901, lot no. 8802D, was obtained from Behring Diagnostics (Somerville, NJ). Rabbit antisera to mouse IgA, IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, and IgM (cat. nos. 8403-01, -03, -04, -06, -08, and -09, with respective lot nos. AI100, AI099, AH096, 231-69-1, AJ106, and AJ105) were obtained from Bionetics.

Assays for Antibodies to Cell Surface Antigens. An autoradiographic modification of the ^{125}I-protein A assay (13) was used for rapid screening. Two thousand target cells in 10 μl of culture medium were added to wells of Falcon 3034 microtest plates. After overnight incubation the cells, which formed an adherent monolayer, were incubated for 45 min with 10 μl of spent hybridoma culture medium (or NS-1 culture medium as negative control). The plate was washed twice with 5 ml of RPMI. The cells were then incubated with rabbit antiserum to mouse Ig diluted 1:100 in RPMI containing heat-inactivated fetal calf serum (330 ml/liter) and washed with RPMI containing bovine serum albumin (20 g/liter). Finally, the cells were incubated with ^{125}I-protein A at 10^6 cpm/ml in RPMI containing bovine serum albumin and washed. The plate was dried at 70°C and autoradiographed overnight at −70°C with preflashed Kodak XR-2 film and a Rarex B Mid Speed intensifying screen (GAF Corporation, New York) (14).

For more quantitative assays 10,000–20,000 target cells were used, either as adherent monolayers in wells of Falcon 3040 microtest plates or in suspension in wells of V-bottomed microtest plates (cat. no. 1-220-25-A, Cooke, Alexandria, VA). Incubation volumes were 50 μl, whereas wash volumes were 200 μl. Rabbit anti-mouse Ig serum was used at a dilution of 1:1000, and ^{125}I-protein A was used at 2 × 10^6 cpm/ml. At the end of the assay, the cells were transferred to test tubes (adherent cells were first dissolved in 200 μl of 2 M NaOH) and counted in a Packard Auto-Gamma scintillation counter.

Adherent cells were also tested in a complement-dependent cytotoxicity assay (15). Briefly, 200 target cells, suspended in 5 μl of culture medium that contained normal rabbit serum at a dilution of 1:20 as complement source, were added to serial dilutions of spent hybridoma culture medium (5 μl) in wells of the microtest plates and incubated for 20 hr. The plates were rinsed with phosphate-buffered saline, and the remaining cells were stained and counted by an electronic image analyzer.

Lymphocytes and lymphoblastoid cell lines were tested in a standard National Institutes of Health two-stage complement-dependent cytotoxicity assay (16). Two thousand peripheral blood lymphocytes were incubated in Terasaki plates with hybridoma culture medium (final dilutions of 1:2, 1:10, and 1:50) for 30 min at 22°C in 2 μl. Five microliters of pretested, pooled normal rabbit serum was added as a source of complement. After 1 hr at 22°C, 3 μl of eosin (50 ml/liter) was added to each well, followed 10 min later by addition of 3 μl of buffered formalin. Cell viability was then determined by phase microscopy. For tests of lymphoblastoid lines, the rabbit serum was diluted 1:4 in RPMI containing fetal calf serum (150 ml/liter). For tests of CCRF-CEM cells, the rabbit serum was first absorbed with CCRF-CEM cells (1 ml of cells per ml of serum for 30 min at 4°C) and then diluted 1:5.

RESULTS

Growth and Selection of Hybridomas. Spleen cells from mice immunized with M1804 melanoma cells were fused with NS-1 myeloma cells and seeded into microtest wells. Hybrids, which were selected by growth in medium containing hypoxanthine, thymidine, and aminopterin, grew in about half the wells and reached confluence in 2–3 weeks. Supernatant from

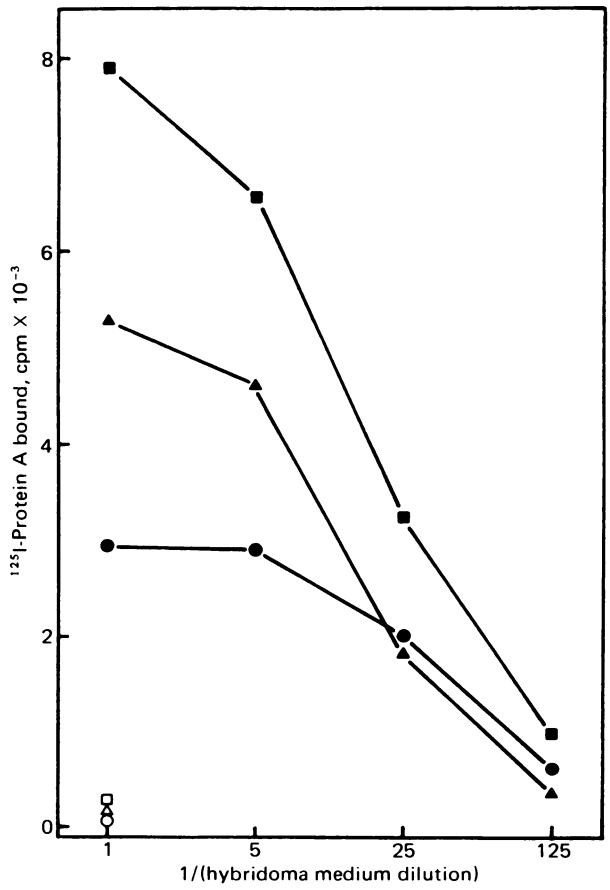

FIG. 1. ^{125}I-protein A assays of hybridoma culture media. Serial dilutions of spent culture media from hybridomas 3.1 (■), 3.2 (▲), and 3.3 (●) were tested in duplicate on M1804 melanoma cells (in suspension). Results are given as ^{125}I-protein A bound per 10^4 cells. The media were also tested on skin fibroblasts from the melanoma patient (open symbols).

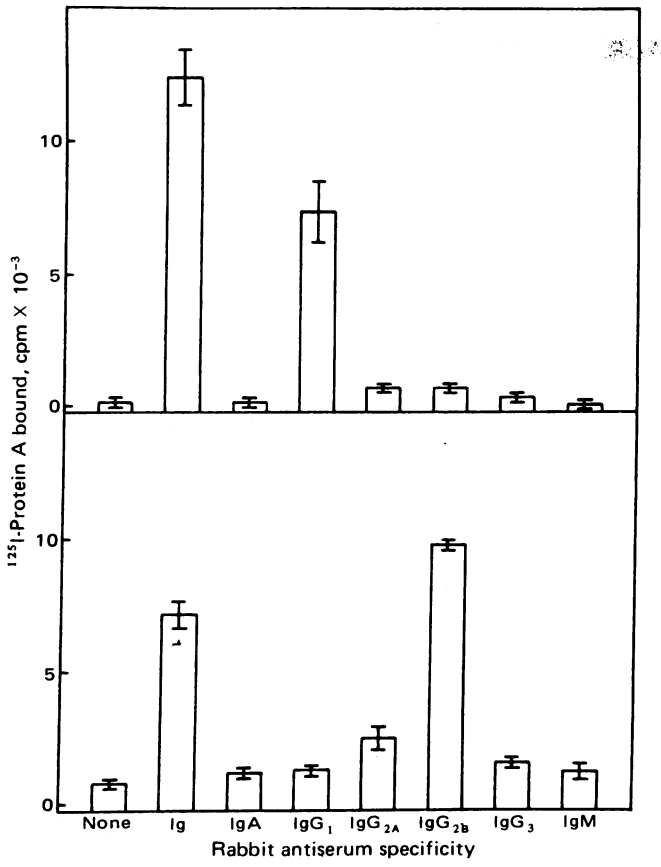

FIG. 2. Determination of Ig class of hybridoma antibodies. Spent culture media from hybridomas 3.1 (*Upper*) and 3.3 (*Lower*) at a dilution of 1:25 were tested on M1804 melanoma cells (in suspension). The ^{125}I-protein A assay was used as described in *Materials and Methods* except that rabbit antisera specific for different mouse Ig classes and subclasses were used in the second incubation. Results are given as ^{125}I-protein A bound per 10^4 cells.

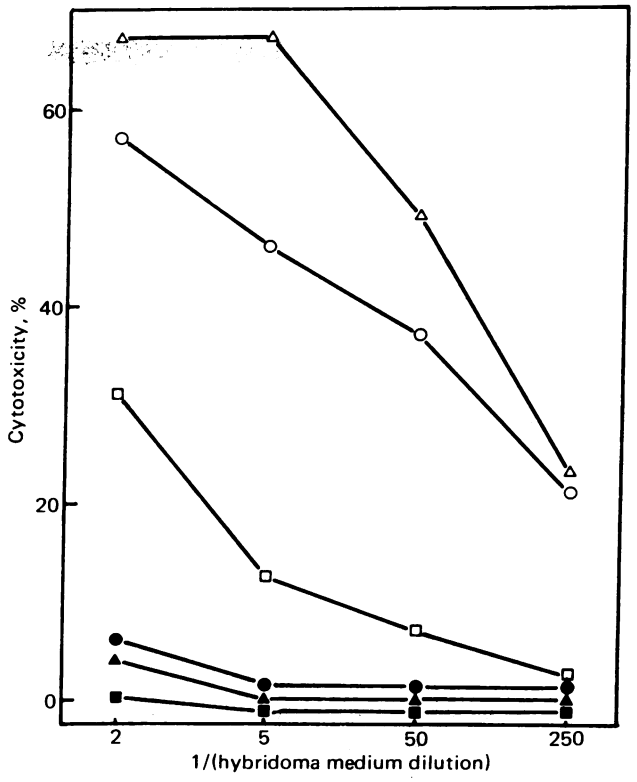

FIG. 3. Complement-dependent cytotoxicity assays of hybridoma culture media. Serial dilutions of spent culture media from hybridomas 3.1 (□), 3.2 (△), and 3.3 (○) were tested in duplicate on M1804 melanoma cells. Results are given as percentage cytotoxicity, measured as a reduction in plating efficiency; all values greater than 10% were statistically significant ($P < 0.05$). Cytotoxicity in the presence of heat-inactivated complement is also shown (closed symbols).

each well was tested in the autoradiographic ^{125}I-protein A assay for antibody against several target cell types, including the immunizing M1804 melanoma and fibroblasts from the same patient. Three wells were found to contain antibody that bound strongly to M1804 melanoma cells but not to autologous skin fibroblasts or cells from other tumors. The hybrids in these three wells were cloned and are referred to as hybridomas 3.1, 3.2, and 3.3. Supernatants from cultures of hybridomas containing 4–8 × 10^5 cells per ml were used for the remainder of the tests, as outlined in the following sections.

Titration of Hybridoma Supernatants on M1804 Melanoma Cells. Serial 1:5 dilutions of supernatants from cultures of hybridomas 3.1, 3.2, and 3.3 were assayed for binding to M1804 melanoma cells by the ^{125}I-protein A assay. As shown in Fig. 1, supernatants from all 3 hybridomas gave significant binding to M1804 cells up to a dilution of 1:125. No binding was seen when the same supernatants were tested against autologous skin fibroblasts.

Characterization of the Hybridoma Antibodies. We examined the three hybridoma antibodies with respect to (*i*) ability to bind protein A directly, (*ii*) heavy chain class, and (*iii*) ability to lyse M1804 melanoma cells in the presence of complement.

By replacing with culture medium the rabbit anti-mouse Ig serum used in the second incubation of ^{125}I-protein A assay, we were able to determine whether the antibodies were able to bind protein A directly. The amounts of ^{125}I-protein A bound when the rabbit anti-mouse Ig serum was omitted were 0.5, 98.3, and 10.7% of levels obtained when the rabbit antiserum was included, for hybridomas 3.1, 3.2, and 3.3, respectively.

The low level of direct protein A binding by antibodies from hybridomas 3.1 and 3.3 allowed us to determine their Ig class. We did this by using class-specific rabbit anti-mouse Ig sera in the second step of the ^{125}I-protein A assay. Fig. 2 shows that the antibodies produced by hybridomas 3.1 and 3.3 are IgG_1 and IgG_{2b}, respectively.

Next we determined whether the hybridoma antibodies were cytotoxic to M1804 cells in the presence of complement. Fig. 3 shows that antibodies produced by hybridomas 3.2 and 3.3 gave significant killing of M1804 cells up to a dilution of 1:250, whereas the antibody produced by hybridoma 3.1 gave significant killing only at a dilution of 1:2.

Specificity of the Three Hybridoma Antibodies. We used the ^{125}I-protein A assay to test the three hybridoma antibodies against various cells, including melanomas, carcinomas, a sarcoma, and fibroblasts. Fig. 4 summarizes the results of these tests. It is apparent that the three different hybridoma antibodies bound much more strongly to the immunizing M1804 melanoma cells than to any of the other cells tested, including 11 melanomas. Fig. 4 also shows that we detected weak, but significant, binding to some other cell types. Most strikingly, the antibodies from all three hybridomas bound to M1801 melanoma cells. In addition, antibody from hybridoma 3.1 bound significantly to melanoma M1477 and to breast carcinoma C0925. Antibodies from hybridomas 3.2 and 3.3 did not bind to any cell types other than M1804 and M1801 when evaluated by the criteria outlined in the legends to Fig. 4.

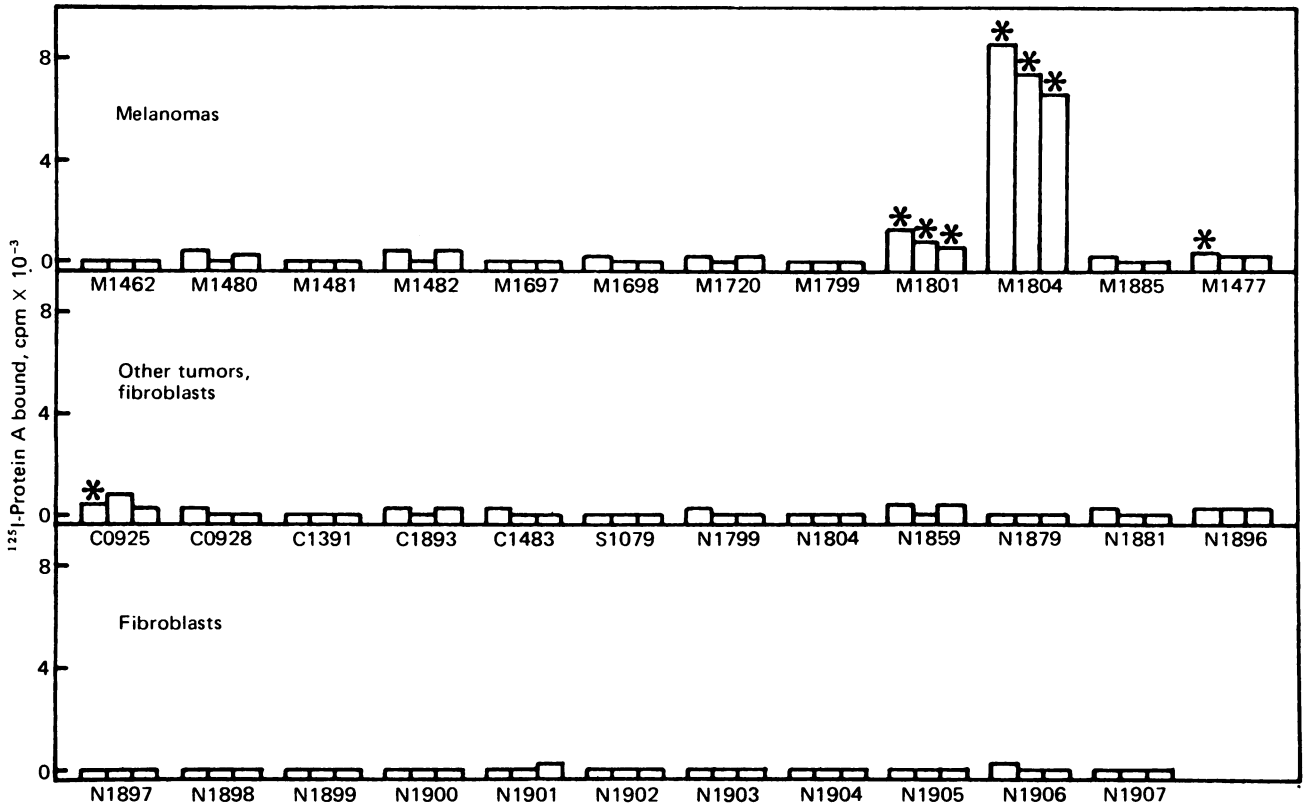

FIG. 4. ^{125}I-Protein A assay of hybridoma culture media. Spent hybridoma culture media at a dilution of 1:5 were tested in duplicate on 35 cell types. For each cell type three bars are shown, representing, from left to right, hybridomas 3.1, 3.2, and 3.3, respectively. Results are given as ^{125}I-protein A bound per 10^4 cells. Reactions in which the amount of ^{125}I-protein A bound was more than twice control values and statistically significant ($P < 0.05$) in both of two separate experiments are indicated (*).

Binding assays of human A, B, and O erythrocytes were negative.

We also tested the antibodies produced by hybridomas 3.2 and 3.3, which were cytotoxic to melanoma M1804 in the presence of complement (Fig. 3), for cytotoxicity to other cell types. The antibody produced by hybridoma 3.2 was cytotoxic to melanoma M1801 up to a dilution of 1:250. No cytotoxicity was seen either to autologous skin fibroblasts (N1804), to melanomas M1462, M1698, or M1720, or to carcinoma C1391. Hybridoma 3.3 antibody was tested on the same panel of cells, but was cytotoxic only to M1804.

Finally, serial 1:5 dilutions of the three hybridoma antibodies were tested in the complement-dependent cytotoxicity assay against normal and cultured lymphocytes for evidence of reactivity to lymphocyte antigens, particularly HLA antigens. We tested peripheral blood lymphocytes from 68 donors, malignant B cells from 12 patients with chronic lymphocytic leukemia, seven B-lymphoblastoid cell lines (PA-3, SB, CAH, T5-1, HA-1, Daudi, and 3001-4), and three T-cell lines (CCRF-CEM, Molt 4, and RPMI-8402). All tests were negative, with less than 10% cytotoxicity at all dilutions of the hybridoma media tested.

DISCUSSION

Spleen cells from mice immunized against a human melanoma (M1804) were fused with NS-1 mouse myeloma cells, and the cell hybrids were grown in selective medium. Three hybrids (hybridomas) that produced antibodies binding to M1804 cells but not to autologous skin fibroblasts were selected and cloned.

Spent culture medium from each of the hybridomas contained antibody to melanoma M1804 cells detectable in the ^{125}I-protein A assay at dilutions up to 1:125 (Fig. 1). Hybridoma 3.1 produces an IgG_1 antibody that does not bind protein A and is poorly cytotoxic in the presence of complement. Hybridoma 3.2 antibody binds protein A well and is cytotoxic; we believe it to be IgG_2. Antibody from hybridoma 3.3 is IgG_{2b}, binds protein A, though not as well as hybridoma 3.2 antibody, and is cytotoxic.

In order to determine the specificity of the three hybridoma antibodies, we tested a total of 126 cell types. In one series of experiments, peripheral blood lymphocytes from 68 donors, 12 patients with chronic lymphocytic leukemia, and seven B- and three T-lymphoblastoid cell lines were tested in a microcytotoxicity assay used routinely for HLA typing. All were negative, strongly suggesting that the hybridoma antibodies do not recognize HLA-A, -B, -C, or, -DR (Ia) antigens, or, indeed, any other lymphocyte cell surface antigens.

For tests of adherent cells, the ^{125}I-protein A assay was used. Skin fibroblasts from 15 individuals, including the patient from whom the M1804 melanoma was obtained, and lung fibroblasts from two individuals were all negative. Tests of five carcinomas and a sarcoma were negative, except for weak binding of hybridoma 3.1 antibody to C0925, a breast carcinoma. Finally, 12 melanomas were tested. All three hybridoma antibodies, in addition to binding to M1804, bound significantly to M1801. Antibody from hybridoma 3.1 bound weakly to M1477. Neither melanoma bound more than 15% of the antibody bound by M1804. These results have recently been confirmed by indirect immunofluorescence tests. Strong membrane fluorescence was seen on all M1804 cells and weaker fluorescence was seen on about 40% of M1801 cells. The weak binding to M1477 and C0925 detected in the ^{125}I-protein A assay was confirmed and shown to be due to a few (less than 20%) positive cells.

Complement-dependent cytotoxicity tests of a more limited range of cell types showed that hybridoma 3.2 antibody killed melanomas M1804 and M1801, but not melanomas M1462, M1698, M1720, carcinoma C1391, or N1804 skin fibroblasts, in complete agreement with the binding assays. Hybridoma 3.3 antibody was tested against the same panel, but killed only M1804.

We conclude that the antibodies produced by hybridomas 3.2 and 3.3 recognize determinants of antigen(s) that are expressed strongly by the immunizing M1804 melanoma, less strongly by one allogeneic melanoma (M1801), and weakly or not at all by 57 other cell types (hybridoma 3.1 antibody bound to an additional allogeneic melanoma and to a breast carcinoma). Either these monoclonal antibodies identify determinants that are virtually unique to M1804 melanoma, or these results suggest that, as a class, melanoma-associated antigens are highly polymorphic. The antigen defined by Koprowski *et al.* (12), who also used monoclonal antibodies, appears to be of wider distribution among melanomas. Taken together, our data and those of Koprowski *et al.* (12) agree with studies of sera from melanoma patients (3): human melanomas express cell surface-localized tumor antigens, some of limited distribution and some shared by many different melanomas. We believe that the hybridoma technique will be useful both for defining the specificity of these antigens and for characterizing them structurally through the use of hybridoma antibodies as immunoadsorbents for antigen purification. It should then be possible to develop radioimmunoassays for the antigens to be employed for diagnosis and for monitoring melanoma patients. Ultimately, hybridoma antibodies or the antigens identified by them may also be useful for tumor therapy.

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1. Hellström, K. E. & Brown, J. P. (1979) in *The Antigens*, ed. Sela, M. (Academic, New York), Vol. 5, pp. 1–82.
2. Lewis, M. G., Ikonopisov, R. L., Nairn, R. C., Phillips, T. M., Hamilton-Fairly, G., Bodenham, D. C. & Alexander, P. (1969) *Br. Med. J.* **3**, 547–552.
3. Shiku, H., Takashashi, T., Oettgen, H. F. & Old, L. J. (1976) *J. Exp. Med.* **144**, 873–881.
4. Morton, D. L., Malmgren, R. A., Holmes, E. C. & Ketcham., A. S. (1968) *Surgery* **64**, 233–240.
5. Cornain, S., De Vries, J. E., Collard, J., Vennegoor, C., Wingerden, I. V. & Rümke, P. (1975) *Int. J. Cancer* **16**, 981–997.
6. Hellström, I. & Hellström, K. E. (1973) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **32**, 156–159.
7. Hellström, I., Hellström, K. E., Sjögren, H. O. & Warner, G. O. (1971) *Int. J. Cancer* **7**, 1–16.
8. McCoy, J. L., Jerome, L. F., Dean, J. H., Perlin, E., Oldham, R. K., Char, D. H., Cohen, M. H., Felix, E. L. & Herberman, R. B. (1975) *J. Natl. Cancer Inst.* **55**, 19–23.
9. Halliday, W. J., Maluish, A. E., Little, J. H. & Davis, N. C. (1975) *Int. J. Cancer* **16**, 645–658.
10. Köhler, G. & Milstein, C. (1975) *Nature (London)* **256**, 495–497.
11. Melchers, F., Potter, M. & Warner, N. L., eds. (1978) *Curr. Top. Microbiol. Immunol.* **81**.
12. Koprowski, H., Steplewski, Z. & Herlyn, D. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 3405–3409.
13. Brown, J. P., Klitzman, J. M. & Hellström, K. E. (1977) *J. Immunol. Methods* **15**, 57–66.
14. Swanstrom, R. & Shank, P. R. (1978) *Anal. Biochem.* **86**, 184–192.
15. Hellström, I., Brown, J. P., Klitzman, J. M. & Hellström, K. E. (1978) *J. Immunol. Methods* **22**, 369–381.
16. National Institute of Allergy and Infectious Diseases (1977) *NIAID Manual of Tissue Typing Techniques*, DHEW no. (NIH)76-545 (GPO, Washington, DC), pp. 22–24.