Protein Antigens of Normal and Malignant Human Cells Identified by Immunoprecipitation with Monoclonal Antibodies*

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Joseph P. Brown, Peter W. Wright, Charles E. Hart, Richard G. Woodbury, Karl Erik Hellstrom, and Ingegerd Hellstrom

From the Division of Tumor Immunology, Fred Hutchinson Cancer Research Center, Seattle, Washington 98104

Spleen cells from a mouse immunized with human melanoma cells were fused with mouse myeloma cells, and somatic cell hybrids were grown in selective medium. Eight hybrids, which secreted antibodies to protein antigens of the melanoma cell line, were identified by immunoprecipitation of a 125I-labeled melanoma cell lysate followed by sodium dodecyl sulfate-gel electrophoresis of the immunoprecipitates and autoradiography. Seven of the eight melanoma proteins identified in this way were present at the cell surface. Two of the cell surface proteins, p80 and p97, were not detected in autologous fibroblasts.

The identification and characterization of structures at the surface of normal and neoplastic cells have become the subjects of intensive research. Antibodies are among the most powerful tools available for such studies but, until recently, were difficult to obtain. However, monoclonal antibodies of defined specificity can now be produced by somatic cell hybridization (1, 2), and systematic serological analysis of the surface of normal and neoplastic cells has become feasible. Biochemical analysis of antigens identified by monoclonal antibodies will be necessary if newly defined antigens are to be related to known cell surface molecules. Protein antigens, for example, may be characterized with respect to their molecular weights by immunoprecipitation from radiolabeled cell lysates followed by SDS-polyacrylamide gel electrophoresis (3, 4).

In this paper, we report the use of an immunoprecipitation technique to select eight hybridomas secreting antibodies to protein antigens of a human melanoma cell line; all but one of these proteins were located at the melanoma cell surface. Five of the proteins, p23, p33, p40, p260, and p270, were present at the surface of autologous skin fibroblasts. Two of them, p80 and p97, were absent from the fibroblasts and are thus possible markers of differentiation or neoplasia.

METHODS

Human and mouse cells were cultured as described previously (5). P3-NS1/Ag 4-1 (NS-1) (6) is an azaguanine-resistant BALB/c myeloma, which was kindly provided by C. Milstein (Medical Research Council Laboratory of Molecular Biology, Cambridge, England). Antibody-secreting hybridomas were generated by mixing 2.5 × 10^6 spleen cells from an immunized mouse with 2.5 × 10^6 NS-1 myeloma cells and centrifuging them for 5 min at 250 × g. The pellet was suspended in 1 ml of 50% polyethylene glycol 1500 (Merck-Schuchardt, Höhenbrunn bei München) in serum-free RPMI 1640 medium and stored gently for 2 min. The polyethylene glycol was gradually diluted with 10 ml of culture medium, and the cells were spun down and resuspended in 100 ml of HAT medium (5) containing 4 × 10^-6 thymocytes (7). Aliquots (200 μl) of the cell suspension were dispersed into five 96-well microtiter plates and placed in a humid 37°C incubator with 7% CO_2 in air. Seven days later, microscopic examination revealed an average of three colonies of hybrid cells per well, a fusion frequency of 20 hybrids per 10^6 spleen cells, and the hybrids were fed by replacing half of the medium in each well with fresh HAT medium.

Monoclonal mouse IgG was purified from the ascites fluid of BALB/c mice that had been inoculated with hybridoma 3.2 (6) by affinity chromatography on a column of Staphylococcus aureus protein A coupled to Sepharose (8). IgG concentrations were determined spectrophotometrically, assuming an ε_280 of 14.0. Seventeen milligrams of IgG was obtained from 4 ml of ascites fluid. Monoclonal antibody W6/32, specific for HLA-A, B, and C heavy chains (9) was obtained from Accurate Scientific and Chemical Corp. (Hicksville, N.Y.).

The purified IgG was labeled with 125I by the method of McConahey and Dixon (10). Forty micrograms of IgG was incubated with 1 mCi of Na^221 (Amersham, Arlington Heights, Ill.) and 10 μg of chloramine-T in 400 μl of phosphate-buffered saline (0.9% NaCl solution), pH 7.2, for 10 min at 0°C. The reaction was stopped by adding 10 μg of sodium metabisulfite, and the 125I-labeled antibody was separated from reagents by gel filtration on a 10-ml column of Sephadex G-25 superfine equilibrated with TNE buffer (20 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, pH 8.0). The 125I-labeled IgG (specific radioactivity, 4 × 10^6 cpm/μg) was stored at -70°C.

Spent medium from hybridoma cultures was tested for IgG by a competition assay. Samples (20 μl) were dispensed into V-bottomed microtiter wells (Dynatech Laboratories, Inc., Alexandria, Va.), followed by 100 μl of TNE buffer containing 10^6 cpm of 125I-labeled IgG, 10^5 cpm of Na^35CrO_4 (New England Nuclear, Boston, Mass.), and 20 μl of TNE buffer containing 20 μg of heat-inactivated, formalin-fixed S. aureus (New England Enzyme Center, Boston, Mass.). The contents of the wells were mixed by gently agitation the microtiter plate. After 30 min at 20°C, the microtiter plate was centrifuged for 3 min at 1000 × g. Samples (40 μl) of the supernatant were counted for 0.5 min in a Packard Auto-Gamma scintillation counter. From the 125I/^35Cr ratio, the amount of 125I-labeled IgG bound to the bacteria was calculated, as described previously (11). Unlabeled IgG was used to standardize the assay.

Cell lysates were prepared by incubating a confluent 150 cm^2 monolayer of cells (approximately 10^6 cells) with 10 ml of TNE buffer for 5 min at 0°C. The lysate was centrifuged for 2 min at 2000 × g to remove nuclei, and then for 10 min at 300,000 × g. The supernatant was gel-filtered on a 5-ml column of Sephadex G-25 superfine equilibrated with TNE buffer and stored at -70°C. Membrane lysates were prepared by incubating a confluent 150 cm^2 monolayer of cells with 10 ml of 1 mM NaHCO_3 buffer containing 1 mM phenylmethylsulfonyl fluoride for 5 min at 0°C. The hypotonically swollen cells were scraped from the culture flask and broken with five strokes of the pestle in a Dounce homogenizer. The nuclei were removed by centrifugation for 2 min at 2000 × g. The membranes were pelleted by centrifugation for 1 min at 50,000 × g, suspended in 0.5 ml of TNE buffer and spun for 10 min at 300,000 × g. The
supernatant was gel-filtered on a 5-ml column of G25 superfine equilibrated with TEN buffer and stored at -70°C. The protein concentrations of the lysates, determined as described previously (12), were 100 to 500 μg/ml.

Cell membrane lysates were labeled with 125I. Protein (4 to 20 μg) in 40 μl of TEN buffer was incubated with 1.5 mCi of Na125I and 10 μg of chloramine-T in 400 μl of phosphate-buffered saline for 10 min at 0°C. The reaction was stopped by adding 10 μg of sodium metabisulfite, and the labeled proteins were gel-filtered on a 10-ml Sephadex G25 superfine column equilibrated with immunoprecipitation buffer (TEN buffer containing 2% bovine serum albumin, 0.5% sodium deoxycholate, and 10 mM NaI). The 125I-labeled lysate, containing 1.5 × 10^6 cpm, was stored at -70°C.

For immunoprecipitation tests, 20 μl of hybridoma culture medium containing 0.1 to 0.4 μg of IgG2 was incubated for 1 h at 0°C with 100 to 200 μl of 125I-labeled cell or membrane lysate (3 to 10 × 10^6 cpm), to which SDS had been added to a final concentration of 0.1%. One milligram of formalin-fixed, heat-inactivated S. aureus was added in 1 ml of immunoprecipitation buffer containing 0.1% SDS. After 10 min at 0°C, the bacteria were pelleted by centrifugation for 5 min at 2000 × g, and washed twice with 1 ml of immunoprecipitation buffer containing 0.1% SDS and twice with TEN buffer diluted 1/10 with water. Bound antigens were eluted by incubation for 10 min at 100°C in 50 μl of sample buffer (13), the bacteria were spun down, and 40 μl of the supernatant were analyzed by electrophoresis on a 10% SDS-polyacrylamide slab gel. The molecular weight markers were rabbit muscle myosin (200,000), Escherichia coli β-galactosidase (116,248), rabbit muscle phosphorylase b (97,422), bovine serum albumin (66,269), chicken ovalbumin (45,000), bovine catalase (29,980), and soybean trypsin inhibitor (20,095); they were obtained from Bio-Rad Laboratories (Richmond, Calif.). The gel was fixed with 25% trichloroacetic acid, stained with 0.1% Coomassie Brilliant Blue in 25% trichloroacetic acid, destained in 7% acetic acid, dried, and autoradiographed. By superimposing the autoradiograph on the dried gel, the apparent molecular weight of each labeled protein was determined by comparison with the unlabeled markers in the same tracks using a semilog plot.

125I-labeled protein A assays for antibody binding to surface antigens of viable cells were performed as described previously (15). Membrane immunofluorescence tests were done as follows: A viable monolayer of melanoma cells on a glass slide was incubated for 1 h at 37°C first with culture medium containing 5 to 20 μg/ml of monoclonal antibody and then with a 1/10 dilution in culture medium of fluorescently labeled goat antibody to mouse IgG (Miles Biochemicals, Elkhart, Ind.). The cells were washed with phosphate-buffered saline, fixed with 10% buffered formalin, and examined under a Leitz Orthoplan fluorescence microscope.

**RESULTS AND DISCUSSION**

A melanoma cell line was established from a retroperitoneal lymph node metastasis from a female patient (G. D.) with malignant melanoma. The origin of the cells was confirmed by electron microscopy, which revealed the presence of premelanosomes. We immunized a 3-month-old BALB/c mouse with 2 intraperitoneal inoculations of 10^7 G. D. melanoma cells 1 month apart. Three days after the second immunization, spleen cells from the immunized mouse were fused with NS-1 myeloma cells and hybrid cells grown in selective medium. Ten days after the fusion, when the fastest growing hybrids had reached confluence, samples of the culture medium were removed for testing.

The medium samples were first tested for IgG with high affinity for S. aureus protein A, in the mouse, the IgG2 and IgG1 isotypes bind protein A with high affinity, but the IgG2 isotype is much more common (16). We used a competition assay in which IgG2 in the test sample competes with 125I-labeled IgG2 for binding to a limiting amount of protein A-bearing S. aureus. Unlabeled IgG2 was used to standardize the assay, which can be used to measure IgG2 concentrations as low as 1 μg/ml. Of 480 medium samples from the five plates of hybrids, 132 contained more than 4 μg/ml of IgG2, and these were then tested by immunoprecipitation.

The number of immunoprecipitation tests required for the screening was minimized by testing pooled samples. The 132 IgG2-containing samples were arranged in a two-dimensional array of 11 rows of 12 microtest wells. The samples were then pooled along rows, 11 tubes each receiving 50 μl of medium from each well in one row, and also pooled along columns. The 23 row and column pools were tested in immunoprecipitation. In order to keep the final incubation volume at a minimum, the IgG2 antibodies in the pooled samples were first adsorbed to S. aureus. One milligram of S. aureus in 1 ml of immunoprecipitation buffer was added to each tube. After 10 min at 22°C the bacteria, along with adsorbed IgG, were spun down, incubated with a 125I-labeled detergent lysate of melanoma cells for 1 h at 0°C, and washed extensively. In screening subsequent fusions, we have pooled 10-μl samples from each well and used the immunoprecipitation procedure described under "Methods." The bound antigens were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.

The autoradiographs (Fig. 1) revealed at least eight proteins...
that were precipitated by certain of the pooled hybridoma medium samples but were not seen when control culture medium was tested. Most of these proteins were precipitated by more than one row and column pool, showing that antibodies precipitating them were present in multiple wells. Individual wells containing antibody precipitating each of the eight proteins were identified by retesting 19 individual medium samples. For example, a 33-kilodalton protein, p33, was precipitated by row pools C and J and by column pools 8 and 11. Wells C8, C11, J8, and J11 were retested; C11 and J8 were found to contain the precipitating antibody.

The hybrids were cloned by dispensing five hybridoma cells into each well of a 96-well microtiter plate in 200 μl of HAT medium with 4 × 10^{-2} thymocytes as a feeder layer. Wells containing single colonies were identified by microscopic examination, and medium samples were tested for IgG production and by immunoprecipitation. Antibody-producing clones, designated 96.1, 96.2, etc. (see Table I), were obtained from all eight hybrids. Spent medium from dense cultures (2 to 8 × 10^6 cells/ml) of the cloned hybridomas contained 5 to 20 μg/ml of IgG and was stored at -70°C.

Immunoprecipitation tests with the monoclonal antibodies confirmed the results obtained during the initial screening. The molecular weights of the precipitated proteins were determined by co-electrophoresis with markers. The proteins are identified by their apparent molecular weights (Table I). Several of the antibodies precipitated more than one polypeptide. Antibody 96.6, for example, precipitated polypeptides of 27, 80, and 110 kilodaltons. Whether these polypeptides are distinct proteins with common antigenic determinants, or are the result of specific or nonspecific aggregation of several proteins, one of which binds to the monoclonal antibody, or proteolytic degradation products of a high molecular weight polypeptide, is unknown.

The cellular localization of the antigens identified by the eight monoclonal antibodies was investigated. Immunoprecipitation assays with viable monolayers of melanoma cells showed that seven of the eight antibodies recognized membrane-associated polypeptides. However, since the membrane lysates tested were not derived from purified plasma membranes but from a microsome fraction, this did not establish that the proteins were located at the cell surface. Antibody 96.7 did not precipitate a protein from the membrane lysates, though it did precipitate a 60-kilodalton protein from cell lysates. Antibody 96.1 precipitated 33- and 50-kilodalton polypeptides from the membrane lysate. Only small amounts of the 50-kilodalton polypeptide were precipitated from the whole cell lysate, but the reason for this discrepancy remains unexplained. Membrane immunofluorescence and ^125I-protein A assays with viable monolayers of melanoma cells showed that all seven of the antibodies that precipitated proteins from the membrane lysate bound to the outer surface of the plasma membrane, demonstrating that they recognized cell surface antigens. Antibody 96.7 did not bind to the melanoma cells, indicating that p60, the antigen recognized by this antibody, is intracellular. These results are summarized in Table I. The observation that seven of eight hybridomas, derived from spleen cells of a mouse immunized with intact melanoma cells and selected by immunoprecipitation of a whole cell lysate, recognized cell surface proteins indicates that, under the immunization conditions used, cell surface antigens are more immunogenic than intracellular antigens.

Autologous skin fibroblasts were tested. Immunoprecipitation tests (Fig. 2) and ^125I-labeled protein A assays showed that the antigens recognized by antibodies 96.1, 96.2, 96.3, and 96.4 were present at the surface of the fibroblasts. Only small amounts of antibody 96.10 bound to the fibroblasts and the immunoprecipitation tests detected only low levels of p40. Thus, we have obtained six hybridomas that defined cell-surface proteins common to both normal and neoplastic cells. Even though our major objective is to identify cell-surface

| Antibody | Protein precipitated | Presence at cell surface |
|----------|---------------------|-------------------------|
| 96.1     | p33, p50            | +                       |
| 96.2     | p23                 | +                       |
| 96.3     | p270                | +                       |
| 96.4     | p200                | +                       |
| 96.5     | p97                 | +                       |
| 96.6     | p80, p27, p110      | +                       |
| 96.7     | p60                 | –                       |
| 96.10    | p40                 | +                       |
proteins associated with neoplasia, we feel that the identification and characterization of proteins on the surface of normal cells is worthwhile, if only to use them as controls for the study of tumor-associated proteins. Antibodies 96.5 and 96.6, however, were negative when tested on fibroblasts both in immunoprecipitation and in binding assays, indicating the absence of p80 and p97. It is thus possible that p80 and p97 are markers of differentiation or neoplasia. Recent work in our laboratory using another hybridoma antibody indicates that p97 may indeed be a tumor-associated antigen (17). Whether p80 or p97 is related to other melanoma cell surface antigens identified by melanoma patients’ sera (18) or monoclonal antibodies (19) is unknown.

These results demonstrate that the immunoprecipitation technique can be used as a rapid and informative screening method for identifying hybridomas secreting antibodies to protein antigens of cultured human cells. Preliminary experiments (data not shown) indicate that the method can also be applied to tissues and tumors that have not been cultured. Since the immunoprecipitation technique allows protein antigens to be identified by their polypeptide molecular weights, the two-dimensional screening method can be used, greatly reducing the number of tests required to screen several hundred hybridomas. Wells containing several different antibodies can also be analyzed. Well C9, for example, contained antibodies to both p23 and p80, but we were able to isolate a clone (hybridoma 96.6) secreting antibody to p80. More importantly, the ability to characterize protein antigens recognized by hybridoma antibodies with respect to their molecular weights will enable one to search systematically for antibodies to proteins of known molecular weight and also to compare newly identified antigens with previously characterized proteins.

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