Double Determinant Immunoassay to Measure a Human High-molecular-weight Melanoma-associated Antigen¹

Patrizio Giacomini,² Ah Kau Ng, Raphe R. S. Kantor,³ Pier Giorgio Natali, and Soldano Ferrone⁴


ABSTRACT

Using monoclonal antibodies to distinct determinants of a human high-molecular weight melanoma-associated antigen (HMW-MAA), a double determinant immunoassay has been developed. The assay is specific and reproducible. Its sensitivity is influenced by the incubation time of antibodies with antigen sources and the combination of antibodies, as well as by the pH of the buffer and the incubation time used to coat plates with antibodies. Testing with the double determinant immunoassay of Nonidet P-40 extracts of human cell lines and of surgically removed normal and malignant tissues has confirmed the restricted tissue distribution of the HMW-MAA. In addition, significant differences have been found in the level of HMW-MAA in melanoma cell lines, as well as in melanoma lesions removed from different patients and from different sites of a given patient. The amount of HMW-MAA shed by various melanoma cell lines does not correlate with their cell surface expression and with their level in Nonidet P-40 extracts. Interferon and hyperthermia increase the shedding of the HMW-MAA by melanoma cells.

INTRODUCTION

Studies with sera from patients with melanoma and from animals immunized with melanoma cells suggested that malignant transformation of human melanocytes is associated with the appearance of MAA (for review, see Ref. 4). Conclusive evidence about the expression of MAA by melanoma cells was provided by Carey et al. (3), who used autologous systems in their studies, thus avoiding the interference of antibodies elicited by allogemizations. Recently, the hybridoma technology has been applied by several investigators, and MoAbs have been generated to MAA with distinct tissue distribution and molecular profile (for review, see Ref. 12). We have developed MoAbs to distinct determinants of a HMW-MAA which has been detected thus far on melanoma, nevi, and some skin carcinoma cells (10).

Since DDIA are more specific and sensitive than are other serological assays (1, 2), in this study, we have utilized these MoAbs to apply the DDIA methodology to measure the HMW-MAA in various antigen sources.

MATERIALS AND METHODS

Human Cells. The carcinoma, erythroblastoid, lymphoid, and melanoma cell lines and the fibroblast strain used in this study were perpetuated in Roswell Park Memorial Institute Medium 1640 with 10% calf serum added. The antigenic profiles and the growth characteristics of these cells have been described previously (7, 10, 11, 17). Cells were surface labeled with ¹²⁵I (Amersham/Searle Corp., Arlington Heights, Ill.) by the iodogen method (13). Cell extracts were prepared by incubating packed cells at a concentration of 2 × 10⁷ cells/ml for 30 min at 4°C in a 1% solution of NP40 detergent in phosphate-buffered saline, containing 10 μM phenylmethylsulfonyl fluoride (Sigma Chemical Company, St. Louis, Mo.). The extract was cleared by centrifugation at 7000 × g for 5 min and stored at −20°C.

Human Tissues. Tissues were from patients who underwent surgery. A cell suspension was prepared by mincing the tissue with scissors, tearing it with forceps, and filtering through a fine-mesh metal net. Following 5 washings with 0.01 M phosphate-buffered saline (0.15 M, pH 7.2), cells were solubilized with NP40 as described for cells in long-term culture.

Interferon. Partially purified human leukocyte interferon (6 × 10⁶ units per ml; 1.5 mg of protein per ml) was a generous gift from Dr. K. Cantell (Central Public Health Laboratory, Helsinki, Finland).

MoAbs. The MoAb 149.53, an IgG1, the MoAb 225.28S, an IgG2a, and the MoAb 763.74T, an IgG1, to distinct determinants of the HMW-MAA, and the MoAb 465.12S, to a cytoplasmic MAA, have been prepared and characterized as described elsewhere (7, 10, 17).

The MoAbs were purified from ascitic fluid by sequential ammonium sulfate precipitation, ion-exchange chromatography on DEAE, and gel filtration on Sephadex G-200. The purified antibodies were radiolabeled with ¹²⁵I using the chloramine-T method (6).

Protein concentration of the ¹²⁵I-labeled MoAb solution was determined by the method of Lowry et al. (9). MoAbs were covalently coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia, Uppsala, Sweden).

Serological Assay. The ¹²⁵I radioimmunometric assay was performed in microtiter plates as described elsewhere (7). The IIF assay was performed as described elsewhere (10), using 4-μm thick acetone-fixed cryostat sections and MoAb solution (5 to 25 μg/ml) in Roswell Park Memorial Institute Medium 1640 containing 10% fetal calf serum. Fluorescent patterns were observed in a Leitz Ortholux II microscope equipped for phase-contrast microscopy and fluorescent epillumination. Incubation of tissue sections with supernatant from the murine myeloma cell line P3-X63 was used as a negative control. The immunofluorescence staining of lesions was scored as follows: negative, when no stain was detected even with oil-immersion objectives and with reactions performed with MoAb to distinct determinants of the HMW-MAA; bare trace, when the stain was detected only with oil immersion objectives; trace, when the stain was homogeneous and weak; 1+, when at least 50% of the lesion had bright stain; and 2+, when the stain was homogeneously bright.

Unless otherwise stated, the DDIA was performed according to the following procedure. Wells of polystyrene microtiter plates (Dynatech, Alexandria, Va.) were coated with antibody and washed 3 times with 0.9% NaCl solution containing 0.05% Tween 20. Then, the antigen source or its dilutions in 0.05% Tween 20 in phosphate-buffered saline.
RESULTS

Significant amounts of 

Coating the plates with solutions of antibody at concentrations lower than 100 μg/ml reduces the antibody binding capacity of each well. Use of antibody solutions at higher concentrations results in binding of amounts of antibody much in excess of that required to measure the level of antigen usually measured in the test. The use of radiolabeled antibody in the amount of 1 x 10^6 cpm is in excess of the amount of antigen usually measured, since a progressive increase (80-fold) of a Colo 38 melanoma cell extract, but they also increase the background level (Chart 4). Simultaneous addition and a single incubation of the antigen source and of the 125I-MoAb 225.28S in MoAb 763.74T-coated wells reduces the sensitivity of the assay by about 30% after 2 hr and reaches a plateau at 4 hr of incubation (Chart 4).

Coating of plates with solutions of antibody at concentrations lower than 100 μg/ml reduces the antigen binding capacity of each well. Use of antibody solutions at higher concentrations results in binding of amounts of antibody much in excess of that required to measure the level of antigen usually measured in the test. The use of radiolabeled antibody in the amount of 1 x 10^6 cpm is in excess of the amount of antigen usually measured, since a progressive increase (80-fold) of a Colo 38 melanoma cell extract, but they also increase the background level (Chart 4). Simultaneous addition and a single incubation of the antigen source and of the 125I-MoAb 225.28S in MoAb 763.74T-coated wells reduces the sensitivity of the assay by about 30% after 2 hr and reaches a plateau at 4 hr of incubation (Chart 4).

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Optimal Conditions under Which to Perform the DDIA. On the basis of the data derived from the above-mentioned experiments, the DDIA has been performed in the following way.

Use of the DDIA to Quantitate the HMW-MAA in Externals and Spent Media of Human Cell Lines. NP40 extracts and spent media of a variety of human cell lines were tested for their HMW-MAA content using the DDIA. Representative results are summarized in Chart 2. The following findings are noteworthy: (a) the results of the DDIA are in agreement with those of other serological assays of the restricted distribution of the HMW-MAA (10); (b) there are significant differences in the level of the HMW-MAA found in extracts and spent media of the melanoma cell lines tested; and (c) there is no relationship between the level of HMW-MAA in the spent media of the melanoma cell lines analyzed and either the cell surface expression of the HMW-MAA or its content in cell extracts.

Use of the DDIA to Quantitate the HMW-MAA in Surgically Removed Tissues. Testing of extracts of 8 surgically removed melanoma tissues detected the HMW-MAA in 4 lesions. Marked differences were found in the level of HMW-MAA in lesions removed from different patients and in lesions removed from different melanoma cell lines.
different sites from a given patient (Table 1). Furthermore, the DDIA did not detect the HMW-MAA in extracts of normal skin from 3 donors, of blue nevi from 2 patients, of one congenital nevus, of brain cortex, kidney, liver, lung, and skeletal muscle from several donors. Representative results are shown in Table 1. It is of interest that the results obtained with the DDIA agreed with those of the IIF staining of frozen sections in all but 3 cases.

Use of the DDIA to Measure the HMW-MAA Shed by Cultured Melanoma Cells Colo 38 Treated with Human Leukocyte Interferon or Hyperthermia. The usefulness of the DDIA to measure the HMW-MAA shed by melanoma cells under various experimental conditions was evaluated by testing the effect of interferon and hyperthermia on the shedding of the HMW-MAA. A culture of melanoma cells Colo 38 (2 x 10⁵ cells/ml) was added with human leukocyte interferon (5 x 10⁵ units/ml) or exposed to 42°C for 2 hr. Then, cultures were incubated in a 5% CO₂ humidified atmosphere at 37°C for up to 120 hr. The number of viable cells in the interferon-treated culture increased less than in a control culture (Chart 5). Melanoma cells harvested after 12 and 24 hr from interferon-treated cultures did not differ from controls in their binding capacity for the MoAb 225.28S and 763.74T. Cells harvested after at least 48 hr of incubation displayed about 10% higher antibody-binding activity. The level of the HMW-MAA increased progressively in the medium harvested daily from the control cultures. Medium harvested during the first 3 days from interferon-treated cultures contained amounts of HMW-MAA similar to those of controls, while medium harvested on the fourth and fifth days contained lower amounts of HMW-MAA. However, if the cell concentration in the culture is taken into account, then interferon appears to increase the shedding of HMW-MAA per melanoma cell.

In hyperthermia-treated cultures, the number of viable cells did not increase over a 4-day incubation period, while the number of dead cells, as measured by trypan blue uptake, progressively increased and was higher than 50% following 48 hr of incubation at 37°C (Chart 6). Melanoma cells exposed to hyperthermia did not change significantly in their ability to bind MoAb 225.28S and

<table>
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<th>IIF</th>
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<tr>
<td>C. F.</td>
<td>Blue nevus</td>
<td>&lt;0.05</td>
<td>—</td>
</tr>
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<td>—</td>
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<td>—</td>
</tr>
<tr>
<td>C. C.</td>
<td>Melanoma metastasis 1</td>
<td>&lt;0.05</td>
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*a (ng of ¹²⁵I-MoAb 225.28S bound/1 x 10⁶ cells extracted.)
*b The criteria used to score the staining of the lesions have been indicated in "Materials and Methods."
763.74T. The medium harvested for up to 24 hr from the culture exposed to 42° did not differ from the control in the content of HMW-MAA; medium harvested after 48 hr of culture contained lower amounts of HMW-MAA than did controls, and the difference became more marked in media harvested at later times. When the data are expressed by taking into account the cell concentration of the cultures, then hyperthermia appears to increase the shedding of the HMW-MAA per melanoma cell.

**DISCUSSION**

DDIAs have been used previously to quantitate human tumor-associated antigens, such as a p97 MAA, α-fetoprotein, and a colon carcinoma monoganglioside (1, 2, 14, 16). The present study, in agreement with the data in the literature, shows that the DDIA we have applied is a simple, sensitive, and reproducible assay to measure a HMW-MAA in various antigen sources.

The sensitivity of the assay is significantly affected by the combination of the antibodies used. It is not known whether this applies also to DDIAs used for other types of human tumor-associated antigens (1, 2, 14, 16), since no mention is made by the other investigators of the criteria to select the antibodies used. Several mechanisms may be envisioned for our results. The recent report that binding of a MoAb to soluble human la antigens changes their conformation and their reactivity with other MoAbs (15) suggested to us a similar mechanism for our results. However, this possibility appears to be unlikely, since incubation of a melanoma cell extract with one MoAb did not affect the amount of radiolabeled antigen immunoprecipitated by the other 2 MoAbs tested. On the other hand, a role can be played by the heterogeneous distribution of the determinants recognized by the 3 MoAbs used on the pool of HMW-MAA-bearing molecules synthesized by a given cell line (8). Furthermore, the exquisite sensitivity of the HMW-MAA to proteolysis and the distribution of the determinants recognized by the MoAbs 149.53, 225.28S, and 763.74T on different fragments (17) suggest the nonrandom generation of antigenically distinct fragments as another mechanism for the effect of combinations of MoAbs on the sensitivity of the DDIA.

The sensitivity of the DDIA we have used appears to be similar to that of the assays utilized to detect other types of tumor-associated antigens (1, 2, 16). On the other hand, the DDIA in microtiter plates we have used is about 5 times more sensitive than is the DDIA we have developed previously in tubes with antibodies covalently bound to Sepharose 4B (5). In addition, the use of microtiter plates instead of tubes facilitates the handling of a large number of samples.

Analysis of surgically removed malignant and benign tissues with the DDIA has confirmed the results of the IIF testing of the restricted tissue distribution of the HMW-MAA and the heterogeneity in its expression among different patients and among lesions from different sites from a patient. These results together suggest that the HMW-MAA may be a useful marker for radioimaging and immunotherapy and that combinations of MoAbs to various types of MAA may be preferable to single antibody preparations to overcome the antigenic heterogeneity. From a technical viewpoint, the disparities in the results of IIF and of DDIA may reflect at least 2 mechanisms. The section tested with IIF may not be representative of the sample tested with the DDIA. Furthermore, the expression on the same molecule of HMW-MAA of the 2 determinants recognized by the MoAbs being used is necessary for a positive result in the DDIA but is not a requirement for a positive reaction in IIF.

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