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

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


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 alloimmunizations. 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's 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 cell lines 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 x 10⁷ cells/ml for 30 min at 4° 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 x g for 5 min and stored at -20°.

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 x 10⁵ 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 radioimmunoassay was performed in micropipette 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 li 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 polyvinyl chloride 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
(pH 7.0) with 5% calf serum were added. Following incubation at room temperature for the indicated time, wells were washed 3 times with 0.05% Tween 20-0.9% NaCl solution. Then 125I-labeled antibodies (10^5 cpm) were added to each well. Plates were incubated for the indicated time at room temperature and then washed 5 times with 0.05% Tween 20-0.9% NaCl solution. Wells were then cut and counted in a γ-counter. Nonspecific binding was assessed by incubating the antigen source and the radiolabeled antibody in wells coated with an irrelevant MoAb (i.e., directed to an antigen different from the HMW-MAA). Specifically bound radioactivity was determined by subtracting the nonspecifically bound cpm from the total bound cpm. Results are expressed as ng of 125I-labeled bound MoAb/1 × 10^5 cells.

RESULTS

Significant amounts of 125I-MoAb 225.28S bound to microtiter plates which had been coated with the MoAb 763.74T and added with an NP40 extract of cultured melanoma cells Colo 38. A series of experiments were then performed to analyze the specificity, sensitivity, and reproducibility of the DDIA to measure the HMW-MAA.

Specificity. The following lines of evidence indicate the specificity of the DDIA: (a) there is a progressive increase in the amount of the 125I-MoAb 225.28S bound to MoAb 763.74T-coated plates preincubated with increasing volumes of sources of HMW-MAA (Chart 1); (b) less than 0.01 ng of the 125I-MoAb 225.28S is bound to MoAb 763.74T-coated plates preincubated with extracts from cells lacking the HMW-MAA (i.e., cultured lymphoid cells and cultured carcinoma cells) and their spent media (Chart 2); and (c) less than 0.1 ng of the 125I-MoAb 225.28S is bound to plates coated with the MoAb 465.12S to a cytoplasmic MAA and preincubated with an NP40 extract of cultured melanoma cells Colo 38 is incubated in wells coated with the MoAbs 225.28S and 763.74T, respectively.

Sensitivity. The sensitivity of the DDIA is influenced by the combinations of the antibodies tested and by their use either as a radiolabeled tracer or in insolubilized form (Chart 1). The combinations MoAb 763.74T-[125I]-MoAb 225.28S and MoAb 225.28S-[125I]-MoAb 763.74T detected the highest (6 ng of 125I-labeled MoAb bound) and lowest (0.5 ng of 125I-labeled MoAb bound) amount of HMW-MAA, respectively; combinations of the latter 2 antibodies with the MoAb 149.53 detected intermediate levels of the HMW-MAA (Chart 3). These differences are not likely to reflect a change in conformation of the molecule which is induced by reaction with one MoAb for the following reasons. Coating of melanoma cells Colo 38 with one MoAb does not affect the binding of the other 2 MoAbs. Prior incubation of [125I]-labeled NP40 extracts of cultured melanoma cells Colo 38 (3 × 10^6 cpm) with up to 10 μl of ascorbic fluid of one MoAb does not affect the amount of radioactivity bound by the 2 Sepharose 4B-bound MoAbs.

The incubation time of the antigen source with the antibody bound to the plate as well as with the radiolabeled antibody significantly affects the sensitivity of the assay. In both steps, prolonging the incubation time to 2 hr markedly increases the extent of cpm bound without affecting the background level; longer incubation periods cause only a slight increase in the amount of 125I-MoAb 225.28S bound by the MoAb 763.74T-coated wells after incubation with a 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 used in the test. The use of radiolabeled antibody in the amount of 1 × 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 added to MoAb 763.74T-coated wells causes a proportional increase in the amount of 125I-MoAb 225.28S bound.

Reproducibility. Testing of an NP40 extract of cultured melanoma cells Colo 38 on 5 occasions using the same batch of polyvinyl chloride microtiter plates yielded a mean value of 0.826 ng of 125I-labeled MoAb bound, with S.D. values lower than 0.06 ng within each determination and range values of 0.752 and 0.982 ng.
were 2370 samples; the variation within each duplicate has been less than 10%.

The DDIA has been performed in the following way. On the basis of the data derived from the above-mentioned experiments, the DDIA has been performed in the following way.

The DDIA was performed as follows:

1. Microtiter plates were coated with antibodies by adding to each well 100 µl of a MoAb 763.74T solution (100 µg/ml) in 0.1 M NaHCO₃ buffer (pH 9.5). Following an overnight incubation at room temperature, wells were washed 3 times. The ¹²⁵I-labeled MoAb 225.28S (1 x 10⁵ cpm) was added to each well, incubated for 2 hr at room temperature, and then washed 5 times before being counted in a γ-counter. The sensitivity of the assay performed in this way allows for the detection of 1.6 fmol of HMW-MAA in 200 µl of sample (1000 cpm are bound with a background of 200 cpm); this corresponds to the amount of HMW-MAA solubilized from 6 x 10⁶ cultured melanoma cells Colo 38.

**Use of the DDIA to Quantitate the HMW-MAA in Extracts 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 parts of the same patient.

**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.

Chart 2. Level of HMW-MAA in NP40 extracts and in spent media of human melanoma cell lines SK-MEL 93 (C), Colo 38 (D), M21 (E), SK-MEL 37 (F), and BP 906 (G), of carcinoma cell lines A549 (lung carcinoma), H494 and Du145 (prostate carcinoma), Mano and T24 (bladder carcinomas), of a human fibroblast cell strain, of the T-lymphoid cell line Mott 4, of B-lymphoid cell lines Lo-2, Raji, Victor, and Wi-L2, and of the erythroid cell line K562.

Chart 3. Effect of the combination of MoAbs on the sensitivity of the DDIA. The MoAbs 149.53, 225.28S, and 763.74T were used either to coat microtiter plates or as radiolabeled tracers. The antigen source was a cell extract prepared by solubilizing 2 x 10⁷ cells with 1 ml of a 1% NP40 solution in phosphate-buffered saline with the addition of 10 mM phenylmethylsulfonyl fluoride. Binding of ¹²⁵I-MoAb 225.28S to antigen incubated in MoAb 465.12S-coated plates.

Chart 4. Effect of incubation time and addition sequence of antigen source and radiolabeled MoAb on the sensitivity of the DDIA. Top, results obtained by incubating the MoAb 763.74T-coated plates with the antigen source for the indicated time. Following 3 washings, plates were added with ¹²⁵I-MoAb 225.28S and incubated for 2 hr. Middle, results obtained by incubating the MoAb 763.74T-coated plates with the antigen source for 2 hr. Following 3 washings, plates were added with ¹²⁵I-MoAb 225.28S and incubated for the indicated time. Bottom, results obtained by adding consecutively (without washings) the antigen source and the ¹²⁵I-MoAb 225.28S to MoAb 763.74T-coated plates and incubating the mixture for the indicated time. The antigen source was 25 (A), 50 (B), and 100 µl (C) of a NP40 extract of cultured melanoma cells Colo 38. The latter was prepared by solubilizing 2 x 10⁷ Colo 38 cells with 1 ml of a 1% NP40 solution in phosphate-buffered saline with the addition of 10 µM phenylmethylsulfonyl fluoride. ---, binding of ¹²⁵I-MoAb 225.28S to antigen incubated in MoAb 465.125-coated plates.

0.935 ng. The variation between values was about 25% when the same antigen source was tested with 2 batches of microtiter plates coated with the same antibody preparation. This variability is likely to reflect differences in protein binding capacity of different batches of plastic plates. The variation between values was about 17% when an antigen source was tested with the same MoAb 763.74T-coated plate but preparations of MoAb 225.28S radiolabeled on different occasions. This variability is likely to reflect differences in the specific activity of the MoAb 763.74T used in the above-mentioned experiments. The DDIA has been performed in the following way.

**Chart 3.** Effect of the combination of MoAbs on the sensitivity of the DDIA. The MoAbs 149.53, 225.28S, and 763.74T were used either to coat microtiter plates or as radiolabeled tracers. The antigen source was a cell extract prepared by solubilizing 2 x 10⁷ cells with 1 ml of a 1% NP40 solution in phosphate-buffered saline with the addition of 10 mM phenylmethylsulfonyl fluoride. Binding of ¹²⁵I-MoAb 225.28S to antigen incubated in MoAb 465.12S-coated plates.

**Chart 4.** Effect of incubation time and addition sequence of antigen source and radiolabeled MoAb on the sensitivity of the DDIA. Top, results obtained by incubating the MoAb 763.74T-coated plates with the antigen source for the indicated time. Following 3 washings, plates were added with ¹²⁵I-MoAb 225.28S and incubated for 2 hr. Middle, results obtained by incubating the MoAb 763.74T-coated plates with the antigen source for 2 hr. Following 3 washings, plates were added with ¹²⁵I-MoAb 225.28S and incubated for the indicated time. Bottom, results obtained by adding consecutively (without washings) the antigen source and the ¹²⁵I-MoAb 225.28S to MoAb 763.74T-coated plates and incubating the mixture for the indicated time. The antigen source was 25 (A), 50 (B), and 100 µl (C) of a NP40 extract of cultured melanoma cells Colo 38. The latter was prepared by solubilizing 2 x 10⁷ Colo 38 cells with 1 ml of a 1% NP40 solution in phosphate-buffered saline with the addition of 10 µM phenylmethylsulfonyl fluoride. ---, binding of ¹²⁵I-MoAb 225.28S to antigen incubated in MoAb 465.125-coated plates.
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 10^5 cells/ml) was added with human leukocyte interferon (5 x 10^3 units/ml) or exposed to 42°C for 2 hr. Then, cultures were incubated in a 5% CO₂ humidified atmosphere at 37°C 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 showed 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 1**

<table>
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<th>Patient</th>
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<th>IIF</th>
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</tr>
<tr>
<td>C. G.</td>
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<td>&lt;0.05</td>
<td>-</td>
</tr>
<tr>
<td>F. F.</td>
<td>Normal skin</td>
<td>&lt;0.05</td>
<td>-</td>
</tr>
<tr>
<td>C. F.</td>
<td>Blue nevus</td>
<td>&lt;0.05</td>
<td>-</td>
</tr>
<tr>
<td>D. T.</td>
<td>Blue nevus</td>
<td>&lt;0.05</td>
<td>-</td>
</tr>
<tr>
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<tr>
<td>C. C.</td>
<td>Melanoma metastasis 1</td>
<td>&lt;0.05</td>
<td>-</td>
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</table>

* (ng of ^125I-MoAb 225.28S bound/1 x 10⁶ cells extracted.)

**Chart 5.** Effect of interferon treatment on the shedding of the HMW-MAA. A culture of melanoma cells Colo 38 was added with human leukocyte interferon (5 x 10³ units/ml) and then grown at 37°C in a 5% CO₂ humidified atmosphere. The concentration of alive cells (I) and dead cells (II) in the culture with interferon added was compared with that in a control culture (II, III) (top). The amount of HMW-MAA shed by treated (A) and control (B) melanoma cells was measured by testing 200 µl of culture medium in the DDIA (middle). Bottom, amount of shed HMW-MAA, taking into account the number of cells in the cultures.

**Chart 6.** Effect of hyperthermia on the shedding of the HMW-MAA. A culture of melanoma cells Colo 38 was exposed to 42°C for 2 hr in a 5% CO₂ humidified atmosphere. The concentration of viable (III) and dead cells (II) in the treated culture was compared with that in a control culture (I, II) (top). The amount of HMW-MAA shed by treated (A) and control (C) melanoma cells was measured by testing 200 µl of culture medium in the DDIA (middle). Bottom, amount of shed HMW-MAA, taking into account the number of cells in the cultures.

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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 lα antigens changes their conformation and their reactivity with other MoAbs (15) suggested to us a similar mechanism for our finding. 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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