Binding Parameters and Idiotype Profile of the Whole Immunoglobulin and Fab' Fragments of Murine Monoclonal Antibody to Distinct Determinants of the Human High Molecular Weight-Melanoma Associated Antigen

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ABSTRACT

The nonspecific accumulation of radioactivity in bone marrow, liver, and spleen in patients with melanoma injected with radiolabeled whole IgG of anti-high molecular weight-melanoma associated antigen (HMWMMAA) monoclonal antibody (mAb) hampers the application of immunoscintigraphy to visualize melanoma lesions. This nonspecific background can be reduced by utilizing fragments which do not contain the Fc portion of anti-HMW-MAA mAb. Since the in vivo targeting to melanoma lesions of radiolabeled F(\(ab\)\(^\prime\)) and Fab' fragments of anti-HMW-MAA mAb is critically dependent on their binding parameters, we have analyzed the effect of fragmentation on the binding characteristics of the anti-HMW-MAA mAbs 225.28, 763.74, and TP41.2. The three mAbs recognize distinct and spatially distant determinants with a heterogeneous distribution on the pool of HMW-MAA molecules synthesized by melanoma cells. The determinant recognized by mAb TP41.2 is detectable on a markedly smaller population of HMW-MAA molecules than those recognized by mAbs 225.28 and 763.74. \(^{125}\)I-labeled F(\(ab\)\(^\prime\)) fragments of the three mAbs displayed an immunoreactive fraction similar to that of the whole IgG, while Fab' fragments displayed a lower one. Fragmentation of mAbs 225.28 and TP41.2 to F(ab')\(^\prime\) produced a 2- and 1.5-fold reduction in their association constants but did not cause a significant change in that of mAb 763.74. Cleavage of F(\(ab\)\(^\prime\)) fragments to Fab' fragments produced 2-, 40-, and 7-fold reductions in the association constants of mAbs 225.28, 763.74, and TP41.2, respectively. These changes qualitatively fit the predictions of theory for univalent and bivalent mAb binding, since mAbs 763.74 and TP41.2 appear to show bivalent binding to melanoma cells and mAb 225.28 to show univalent binding. The affinity constant of IgG, F(\(ab\)\(^\prime\)), and Fab' fragments of mAbs 225.28, 763.74, and TP41.2 displays an inverse relationship with the extent of their time-dependent release from the membrane of melanoma cells. Since no endocytosis of mAb could be detected, the latter results suggest that radioactivity remains bound to melanoma cells in vivo for a longer time following injection of F(\(ab\)\(^\prime\)) fragments than following that of Fab' fragments of each of the anti-HMW-MAA mAb tested. Radiolabeled Fab' fragments of mAbs 763.74 and TP41.2 displayed a marked reduction in their reactivity with some of the antidiidiotypic mAb tested. The loss of some idiotopes is likely to be caused by changes in the conformation of the molecules associated with the fragmentation of IgG and by damage during the iodination procedure.

INTRODUCTION

The well-defined specificity of mAbs' recognizing human melanoma associated antigens has reignited interest in the use of immunoscintigraphy to visualize malignant lesions in patients with melanoma. It has been a general experience that the use of the whole immunoglobulin of radiolabeled anti-MAA mAb in imaging is hampered by the nonspecific accumulation of radioactivity in bone marrow, liver, and spleen (for review, see Ref. 1). This nonspecific background can be reduced by utilizing fragments of anti-MAA mAb that do not contain the Fc portion of immunoglobulin (2). The available information (3-5) suggests that the effective in vivo localization of F(\(ab\)\(^\prime\)) and Fab' fragments of anti-MAA mAb is critically dependent on their affinity and on the density of the corresponding determinants. Therefore, characterization of the quantitative binding parameters of F(\(ab\)\(^\prime\))\(^2\) and Fab' fragments of anti-MAA mAb provides an important background to optimize immunoscintigraphy in patients with melanoma. Nevertheless, to the best of our knowledge, no study has analyzed the effect of fragmentation of anti-MAA mAb on their equilibrium binding characteristics. Furthermore, injections of murine anti-MAA mAb have been reported to induce the formation of antibodies not only to the Fc portion but also to idiotopes of the injected mAb (6-8). Although antidiidiotypic antibodies can interfere with the localization of the injected mAb in melanoma lesions, no study has compared the idiotype profile of whole immunoglobulin with that of Fab' fragments of anti-MAA mAb.

In the present study, we have compared the binding to melanoma cells and the idiotype profile of whole immunoglobulin, F(\(ab\)\(^\prime\))\(^2\) fragments, and Fab' fragments of mAbs 225.28, 763.74, and TP41.2, which recognize distinct and spatially distant determinants of human HMW-MAA. The latter has been selected for these studies, since results of in vitro and in vivo investigations have shown that it represents a useful marker for immunoscintigraphy. Specifically, immunohistochemical staining with mAbs of surgically removed melanoma lesions and normal tissues has shown that HMW-MAA is expressed in at least 85% of melanoma lesions, displays a limited heterogeneity among melanoma lesions removed from different anatomic sites and among melanoma cells in a given lesion, and has a restricted distribution in normal tissues (for review, see Ref. 9).

The results of these in vitro studies have been corroborated by the selective localization of radioactivity in melanoma lesions in patients given injections of radiolabeled anti-HMW-MAA mAb (10-12).

MATERIALS AND METHODS

Human Cell Lines. Cultured melanoma cells (Celo 38) and cultured B-lymphoid cells (LG-2) were grown in RPMI 1640 supplemented with 10% fetal calf serum and 2 mm L-glutamine. Cell viability, as determined by trypan blue exclusion, was higher than 95%.

mAb and Conventional Antiserum. mAbs 225.28, an IgG2a, and 763.74, an IgG1, reacting with distinct determinants of HMW-MAA; anti-intercellular adhesion molecule 1 mAb CL203.4; syngeneic antiidiotype mAbs MF11-30, MF11-97, TK1-F2, TK3-121, and TK3-125 elicited with mAb 225.28; syngeneic antiidiotype mAbs MK2-23 and MK2-120 elicited with mAb 763.74; and syngeneic antiidiotype mAbs...
TK6-74, TK6-81, TK6-107, TK6-123, and TK6-316 elicited with mAb TP41.2 were developed and characterized as described previously (13–15). The antiidiotype mAbs TK3-125 and TK6-316 recognize idiotopes outside the antigen combining site of the corresponding immunizing anti-HMW-MAA mAb. The remaining antiidiotype mAb recognize idiotopes in the antigen combining site of the immunizing anti-HMW-MAA mAb. The anti-HMW-MAA mAb TP41.2, an IgGl, was generated from a BALB/c mouse immunized at 10-day intervals with three injections of 5 ⋅ 10^5 cultured human melanoma cells (Colo 38) which had been incubated with recombinant γ-interferon (250 units/ml) for 72 h. mAb VF20VT26 to a M, 120,000 glycoprotein expressed on melanoma cells was generated from a BALB/c mouse immunized at weekly intervals with three injections of 1 ⋅ 10^7 recombinant γ-interferon (1000 units/ml for 24 h) treated melanoma cells (Colo 38). Splenocytes from immunized mice were hybridized with murine myeloma P3-X63-Ag8.653 cells. Hybridization, subcloning, and growth of hybridomas in tissue culture and in the peritoneal cavity of BALB/c mice were performed according to standard procedures (16). Affinity-purified rabbit anti-mouse IgG antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (Avondale, PA).

Indirect Immunoprecipitation and SDS-PAGE. Cells were surface labeled with ^125I using the lactoperoxidase method (17). Then, ^125I-labeled cells were washed 3 times with HBBS and solubilized by incubation (1 ⋅ 10^5 cells/ml) for 30 min at 4°C in lysis buffer containing 0.5% NP40, 10 mM Tris-HCl (pH 8.2), 0.5 mM NaCl, 1 mM EDTA, and 0.1 mg/ml BSA. Following centrifugation at 500 × g for 5 min and at 100,000 × g for 1 h, an aliquot of the radiolabeled extract (5 ⋅ 10^6 cpm) was incubated for 2 h at 4°C with 10 μl of Staphylococcus aureus protein A-Sepharose 4B beads (Pharmacia Fine Chemicals AB, Uppsala, Sweden) precoated with 5 μg of mAb. Immunocomplexes were washed 5 times with lysis buffer. Antibodies were eluted by heating at 100°C for 4 min in 0.06 M Tris-HCl buffer (pH 6.8) containing 2% SDS and 2% 2-mercaptoethanol and electrophoresed in a 3-15% linear gradient polyacrylamide gel (18). Slab gels were dried and autoradiographed using a Kodak XAR-5 film (Eastman Kodak Company, Rochester, NY). Immunodepletion was performed by serial 2-h incubations at 4°C of a radiolabeled cell extract (3 ⋅ 10^6 cpm) with 100 μg of mAbs 225.28, 763.74, or TP41.2 bound to Affi-Gel 10 (Bio-Rad Laboratories, Richmond, CA) (2 mg of mAb/ml of gel) to completely remove the corresponding antigen. The depleted solution was divided into three portions which were immunoprecipitated with each of the anti-HMW-MAA mAbs. After elution, the antigens were analyzed by SDS-PAGE and autoradiographed as described above.

Purification of mAb and Preparation of Fab' Fragments. Ascitic fluids were clarified by centrifugation, diluted, and precipitated with 50% saturated ammonium sulfate. The precipitated proteins were dialyzed against 0.05 M Tris-HCl buffer, pH 7.5 (5 mM for mAbs 225.28 and 763.74 and 20 mM for mAb TP41.2) and chromatographed on DEAE-Sephadex (Pharmacia) in the same buffers using gradients of 0–100 mM NaCl. Purity of fractions was determined by SDS-PAGE in a 7.5% gel under nondenaturing conditions; fractions without detectable contaminants were combined and concentrated to 5–10 mg/ml on an Amicon PM-30 membrane. This product was used directly for preparation of F(ab'); fragments. Samples to be radiiodinated were purified further by high performance liquid chromatography on a TSK 3000 column in 0.15 M sodium phosphate buffer, pH 7.5.

F(ab'); fragments were prepared by digesting mAb (1 mg/ml) with pepsin (0.02 mg/ml) in 0.10 M sodium citrate-0.02% NaN₃, for 18 h at 37°C. The pH of the reaction mixture was 4.1 for IgGl and 4.4 for IgG2a mAbs. Products were chromatographed on carboxymethylcellulose (Whatman CM-52) using a gradient of 0–0.25 M NaCl in 0.10 M sodium acetate buffer, pH 5.0. Fractions were examined by SDS-PAGE in a 10% gel under nondenaturing conditions and processed as above.

Fab' fragments were prepared by mixing F(ab'); fragments (2 mg) with 20 mM sodium phosphate (pH 8.0), 5 mM EDTA, and 2-mercaptoethanol (1 mM for mAb 225.28 and 2 mM for mAbs 763.74 and TP41.2) in a total volume of 1 ml. After 1 h incubation at 37°C, 50 μl of 2,2'-dithiodipyridine (0.1 mM in acetonitrile) were added and incubation was continued at 37°C for 10 min. Each reaction mixture was concentrated to about 150 μl with a Centricon-30 microfiltration apparatus (Amicon) and purified by high performance liquid chromatography as described above. SDS-PAGE was performed with a 12.5% gel under nondenaturing conditions (18).

Radiola beling of mAbs. mAbs were labeled with ^125I by the Iodo-Gen method with minor modifications (19). Five μl of NaI^125I (100 μCi/ml) (Amersham, Arlington Heights, IL) together with 50 μl of a mAb preparation (1 mg/ml in 0.15 M sodium phosphate buffer, pH 7.5) were added to a glass reaction tube (coated with 5 μg of Iodo-Gen) (Pierce Chemical Co., Rockford, IL). The reaction was allowed to proceed for 10 min at 0°C, mixing every 30 s. Unreacted ^125I was removed using a 100-μl Dowex AG1-X8 chloride column (Bio-Rad) equilibrated in ultrapure H₂O. The reaction tube and the column were washed twice with 100 μl of 0.15 M sodium phosphate buffer (pH 7.5). Intact IgG, F(ab'); fragments, and Fab' fragments of mAb were labeled with ^125I at specific activities of 6–8, 9–12, and 18–22 μCi/μg, respectively.

Serological Assays. These were performed in 96 V-bottomed well microtiter plates (Dynatech Laboratories, Alexandria, VA). The immunoreactive fraction of radiola beled IgG and fragments of mAb was measured by linear extrapolation to infinite antigen excess according to the method of Lindmo et al. (20). Briefly, ^125I-labeled IgG or fragments of mAb (2 ⋅ 10⁵ cpm) were incubated with five 2-fold dilutions of Colo 38 cells (starting from 4 ⋅ 10⁴ cells/well) in a total volume of 100 μl HBBS-BSA. After a 2-h incubation at 4°C on a rotator, cells were pelleted by centrifugation, supernatants were carefully aspirated by suction with a No. 22 needle, and cell-bound radioactivity was measured in a gamma counter. Nonspecific binding was measured utilizing the B-lymphoblastoid LG-2 cells which do not express the HMW-MAA.

The inhibition binding assay with radiola beled mAb to map the corresponding determinants was performed by incubating target cells (1 ⋅ 10⁵) with an excess of nonradiolabeled mAb for 1 h at 4°C. Then ^125I-labeled mAb (1 ⋅ 10⁶ cpm/well) was added. After an additional 1-h incubation at 4°C on a rotator, cell-bound radioactivity was measured as above. Results are expressed as percentage of reduction of the binding of radiola beled mAb to target cells compared to the binding in the presence of an unrelated mAb.

Affinity constants of IgG and fragments of mAb were measured by mixing constant numbers of Colo 38 cells (0.5–1 ⋅ 10⁵) with twelve 1:1.4 serial dilutions of ^125I-labeled IgG or fragments of mAb (initial concentration, 0.5–1 ⋅ 10⁸ m) in a 100-μl total volume of HBBS-BSA. At the end of a 90-min incubation at 4°C on a rotator, cell-bound radioactivity was measured as above. Results were plotted in a linear form of the law of mass equilibrium according to the method of Scatchard (21), taking into account the immunoreactive fraction of each immunoglobulin species. Slopes and intercepts were calculated by linear regression. Nonspecific binding was determined by the specific binding with an excess of nonradiolabeled homologous mAb.

The binding of ^125I-labeled mAb to antiidiotype mAb-coated plates was performed as described elsewhere (22). Briefly, microtiter plates were coated with antiidiotype mAb by a 16-h incubation of 10 μg of antiidiotype mAb/100 μl of 0.1 M bicarbonate buffer (pH 9.6) in each well. Following three washings with phosphate-buffered saline (pH 7.4), plates were blocked with HBBS-BSA for 1 h at room temperature and ^125I-labeled mAb (1 ⋅ 10⁶ cpm/100 μl HBBS-BSA/well) was added. At the end of a 4-h incubation at room temperature on a rotary shaker, supernatants were aspirated with a No. 22 needle. Bound radioactivity was measured in a gamma counter. Nonspecific binding was determined by inhibiting the specific binding with an excess of nonradiolabeled homologous mAb. Specific binding was determined by subtracting nonspecific binding from total bound radioactivity.

RESULTS

mAbs 225.28, 763.74, and TP41.2 precipitate molecules with the characteristic electrophoretic profile of HMW-MAA from cultured melanoma cells (Fig. 1). Three mAbs recognize distinct and spatially separate determinants of the HMW-MAA, since coating of Colo 38 cells with each of the three mAbs does not
WHOLE IgG AND Fab' FRAGMENTS OF ANTI-HMW-MAA mAb

Fig. 1. Structural relationship among molecules recognized by anti-HMW-MAA mAbs 225.28, 763.74, and TP41.2 in an extract of cultured human melanoma cells Colo 38. A NP40 extract of 125I-labeled cultured melanoma cells Colo 38 (3 x 10^6 cpmp) was immunoextracted with insolubilized mAb 225.28, 763.74, or TP41.2. The depleted extract was divided into three aliquots which were immunoprecipitated with mAb 225.28 (Lane A), 763.74, or TP41.2 (Lane B). The anti-intercellular adhesion molecule 1 mAb CL203.4 was used as a specificity control.

Table 1 Mapping of the determinants defined by mAbs 225.28, 763.74, and TP41.2 on cultured human melanoma Colo 38 cells

<table>
<thead>
<tr>
<th>mAb</th>
<th>225.28</th>
<th>763.74</th>
<th>TP41.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>whole IgG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonradiolabeled mAb</td>
<td>93.9</td>
<td>4.5</td>
<td>4.3</td>
</tr>
<tr>
<td>225.28</td>
<td>2.8</td>
<td>96.2</td>
<td>6.0</td>
</tr>
<tr>
<td>763.74</td>
<td>2.2</td>
<td>7.2</td>
<td>96.8</td>
</tr>
</tbody>
</table>

* Results are expressed as percentage of inhibition of the binding of radiolabeled mAb to target cells in the presence of the unrelated mAb VF20VT26 which recognizes a M, 120,000 glycoprotein.

Fig. 2. SDS-PAGE analysis of whole IgG (Lane A), F(ab')2 fragments (Lane B) and Fab' fragments (Lane C) of anti-HMW-MAA mAb 225.28, 763.74, and TP41.2. Proteins were electrophoresed under nonreducing conditions in a 3–15% gradient SDS-PAGE. Slab gels were stained with Coomassie Brilliant Blue R-250 and destained in 1% acetic acid and 25% methanol (v/v) in distilled water.

Affect the binding of the other two (Table 1). F(ab')2 fragments were prepared from each mAb by pepsin digestion; Fab' fragments were prepared by subsequent reduction of disulfide bonds. Analysis by SDS-PAGE indicated the essential purity of each preparation (Fig. 2). Radiolabeled whole IgG and F(ab')2 fragments of the three mAbs had an immunoreactive fraction of at least 83%. Radiolabeled Fab' fragments of the three mAbs had an immunoreactive fraction of at least 61% (Table 2).

The apparent association constant of the whole IgG of mAb TP41.2 with Colo 38 cells is higher than that of mAb 763.74, which in turn is higher than that of mAb 225.28 (Fig. 3; Table 2). Fragmentation of IgG to F(ab')2, resulted in a reduction in the association constant of mAbs 225.28 and TP41.2 by factors of 2 and 1.5, respectively, but did not cause a significant change in that of mAb 763.74. Cleavage of F(ab')2 to Fab' fragments produced 2-, 40-, and 7-fold reductions in the association constants of mAbs 225.28, 763.74, and TP41.2, respectively. The association constant of the Fab' fragments of mAb TP41.2 was significantly higher than that of the Fab' fragments of the other two mAbs; it was comparable to the association constant of the intact IgG of mAb 225.28. Representative Scatchard plots and calculated association constants are shown in Fig. 3 and Table 2, respectively. It should be noted that, for practical reasons, the binding of the whole IgG, F(ab')2 fragments, and Fab' fragments of the three mAbs to melanoma cells could not be measured in the same experiment. The same batch of Colo 38 melanoma cells was used with the whole IgG, F(ab')2 fragments, and Fab' fragments of each mAb; different batches of melanoma cells were used to analyze the three mAbs. The variation in the affinity constants and in the number of antigenic sites recognized by each mAb on different batches of Colo 38 melanoma cells are less than 2-fold. This variation does not affect the comparison of affinity constants of the mAb determined with different batches of cells but does affect that of the number of antigenic sites recognized by the IgG and fragments of the three mAbs. Therefore, we have restricted the latter comparisons to results obtained with the same batch of melanoma cells.

The number of whole IgG and F(ab')2 fragments of mAb 225.28 bound per melanoma cell at antibody saturation is similar to that of the Fab' fragments, suggesting that the binding of whole IgG and of F(ab')2 fragments of mAb 225.28 to melanoma cells is univalent. On the other hand, the number of whole IgG and F(ab')2 fragments of mAbs 763.74 and TP41.2 bound per melanoma cell at antibody saturation is about one-half of that of the corresponding Fab' fragments, suggesting that the binding of whole IgG and of F(ab')2 fragments of these two mAbs is bivalent. Repploting of the data by the method of Klotz (23) gave results consistent with the estimates of the numbers of binding sites described here.

These conclusions are also supported by the results of an experiment in which the binding parameters of mAbs 225.28, 763.74, and TP41.2 were compared using the same batch of melanoma cells (Fig. 4); mAb 763.74 bound approximately one-half the number of antigenic sites bound by mAb 225.28. Surprisingly, mAb TP41.2 bound a lower number of antigenic determinants than mAb 763.74. To clarify the molecular basis of the latter finding, sequential immunodepletion experiments

Table 2 Binding analysis of whole IgG, F(ab')2 fragments, and Fab' fragments of anti-HMW-MAA mAbs 225.28, 763.74, and TP41.2 to cultured human melanoma Colo 38 cells

<table>
<thead>
<tr>
<th>mAb</th>
<th>IgG</th>
<th>Immunoactive fraction (%)</th>
<th>Affinity constant (X10^9 M)</th>
<th>mAb molecules/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>225.28</td>
<td>Whole</td>
<td>98</td>
<td>1.2</td>
<td>1.3</td>
</tr>
<tr>
<td>F(ab')2</td>
<td>83</td>
<td>0.6</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Fab'</td>
<td>67</td>
<td>0.3</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>763.74</td>
<td>Whole</td>
<td>94</td>
<td>7.5</td>
<td>1.4</td>
</tr>
<tr>
<td>F(ab')2</td>
<td>94</td>
<td>8.8</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>Fab'</td>
<td>61</td>
<td>0.2</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>TP41.2</td>
<td>Whole</td>
<td>96</td>
<td>14.1</td>
<td>0.5</td>
</tr>
<tr>
<td>F(ab')2</td>
<td>86</td>
<td>9.5</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Fab'</td>
<td>66</td>
<td>1.4</td>
<td>1.0</td>
<td></td>
</tr>
</tbody>
</table>

* Approximately equal molar concentrations of whole IgG, F(ab')2 fragments, and Fab' fragments, 1 x 10^5 cpmp of each, were incubated with varying concentrations of cells.

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were performed. Incubation of a NP40 extract of radiolabeled Colo 38 cells with either mAb 225.28 or mAb 763.74 immunoprecipitated all the molecules recognized by mAb TP41.2 and almost all those recognized by mAbs 763.74 and 225.28, respectively (Fig. 1).

On the other hand, incubation of the NP40 extract with mAb TP41.2 immunoprecipitated only a fraction of the molecules immunoprecipitated by mAb 225.28 or 763.74. These results indicate that the determinant defined by mAb TP41.2 is expressed by only a fraction of the HMW-MAA molecules which carry the determinants recognized by mAbs 225.28 and 763.74. A large proportion of the latter two epitopes are expressed on the same population of HMW-MAA molecules; only a minor portion of the two determinants display a differential distribution on the population of HMW-MAA molecules. Molecules that express the mAb TP41.2-defined determinant but lack either of the other two determinants were not detected.

In some antigenic systems, coating of target cells with mAb to specific determinants of an antigenic structure has been reported to change the reactivity of other determinants with their corresponding mAb (24). Therefore, the effect of coating Colo 38 cells with F(ab')2 fragments of two of the anti-HMW-MAA mAbs on the association constant of F(ab')2 fragments of the third one was investigated. No significant change was detected. On the other hand, the association constant of the three mAbs showed a 3-fold increase when the incubation temperature was raised from 4°C to 37°C (data not shown).

To compare the fate of the intact IgG, F(ab')2 fragments, and Fab' fragments of mAbs 225.28, 763.74, and TP41.2 bound to melanoma cells, Colo 38 cells were coated with the 125I-labeled probes, washed, and incubated in antibody-free medium for up to 72 h. At the indicated times, supernatants were aspirated and cell-bound radioactivity was measured in a gamma counter. As shown in Fig. 5, the loss of radiolabeled whole IgG of mAbs 763.74 and TP41.2 occurs gradually and is about 50% after 48 h of incubation in antibody-free medium. On the other hand, the loss of 125I-labeled mAb 225.28 occurs sharply during the first hours of incubation and is already more than 50% following 8 h of incubation; then the loss is gradual and is about 60% following 48 h of incubation. The kinetics of the loss of F(ab')2 and Fab' fragments of the three mAbs has a pattern similar to that of the whole IgG. The extent of the release appeared to be inversely related to the affinity constant of the antibody species. Specifically, the extent of the release of the whole IgG of mAb 225.28 was less marked at each incubation time tested than that of its F(ab')2 fragments and Fab' fragments; the latter were released more rapidly than the former. F(ab')2 fragments of mAbs 763.74 and TP41.2 were released at each incubation time tested to an extent similar to that of the corresponding whole IgG, while Fab' fragments were released to a greater extent. It is noteworthy that Fab' fragments of mAb TP41.2 and whole IgG of mAb 225.28, which display similar affinity constants, have similar release kinetics.

To determine whether the isotope bound to melanoma cells reflected the presence of mAb on the cell membrane, the experiments with radiolabeled mAb were repeated with the three unlabeled anti-HMW-MAA mAbs. After 12, 24, and 36 h of incubation, the level of mAb on the cell membrane was measured by determining the binding of 125I-labeled anti-mouse IgG xenoantibodies. The decrease in the reactivity of anti-mouse immunoglobulin xenoantibodies was parallel to the loss of isotope bound to melanoma cells that had been coated with 125I-labeled anti-HMW-MAA mAb. These results suggest that anti-HMW-MAA mAbs are not endocytosed but remain on the membrane of melanoma cells until when they are released in the medium.

To compare the idiotypic profile of the whole IgG, F(ab')2 fragments, and Fab' fragments of mAbs 225.28, 763.74, and TP41.2, the three molecular species were tested in a binding assay with syngeneic antiidiotypic mAb. F(ab')2 fragments of mAb 225.28 reacted with the antiidiotype mAbs MF11-30, MF11-97, TK1-F2, TK3-121, and TK3-125 to the same extent as the whole IgG, while the Fab' fragments displayed a lower reactivity. The whole IgG, F(ab')2 fragments, and Fab' fragments of mAb 763.74 reacted to a similar extent with the
antiidiotypic mAb MK2-23. On the other hand, F(ab')2 fragments displayed a lower reactivity with antiidiotypic mAb MK2-120 than whole IgG but a higher reactivity than that of Fab' fragments. The whole IgG, F(ab')2 fragments, and Fab' fragments of mAb TP41.2 displayed a similar reactivity with the antiidiotypic mAbs TK6-74 and TK6-107. The reactivity of F(ab')2 fragments with antiidiotypic mAbs TK6-81, TK6-123, and TK6-316 was similar to that of the whole IgG but higher than that of Fab' fragments (Fig. 6). The decrease in the reactivity of Fab' fragments of mAbs 763.74 and TP41.2 with some of the antiidiotypic mAbs tested is likely to reflect a destruction of the corresponding idiotopes during the fragmentation as well as in the radiolabeling procedure, since cold Fab' fragments displayed a reduced reactivity with radiolabeled antiidiotypic mAb. Furthermore, exposure of Fab' fragments of mAb to the oxidizing conditions of the radiolabeling procedure in presence of nonradioactive iodine caused an additional marked reduction in the extent of their reactivity with radiolabeled antiidiotypic mAb.

**DISCUSSION**

The present study has compared the binding characteristics of whole IgG, F(ab')2 fragments, and Fab' fragments of the mAbs 225.28, 763.74, and TP41.2, which recognize three distinct and spatially distant determinants of HMW-MAA. Following labeling with 125I, Fab' fragments of the three mAbs display a lower immunoreactive fraction than the corresponding whole IgG and F(ab')2 fragments. This finding may reflect nonspecific reduction of disulfide bonds during cleavage of F(ab')2 fragments with 2-mercaptoethanol. We attempted to minimize reduction of intra- and inter-H'-L chain disulfides by using the lowest feasible concentration of reducing agent and by stopping the reaction with excess 2,2'-dithiodipyridine. This reagent forms reactive mixed disulfides between 2-mercaptoethanol or protein sulfhydryl groups and pyridine-2-thiol (25). We expected this to cause specific reoxidation of reduced disulfides in environments where the sulfhydryl groups are held in close proximity by the three-dimensional structure of the protein. Reduced groups in the "hinge" region would not be reoxidized to F(ab')2 because of dissociation of the two Fab' moieties. In our hands, this was superior to stopping the reduction reaction and blocking free sulfhydryl groups with N-ethylmaleimide or by carrying out the reduction with a high concentration of dithiothreitol at low pH (26). After purification of Fab' fragments, no F(ab')2 fragment or other higher molecular weight form was ever detected by SDS-PAGE carried out under non-reducing conditions.

Purified Fab fragments produced by digestion of IgG with papain are probably more homogeneous than the Fab' frag-
ments used in the present investigation, since partially denatured molecules are digested further by the protease. In the present investigation, we have analyzed Fab' fragments, since we plan to use them as carriers of groups more complex than iodine in future studies. Fab' fragments can have one or more free sulphydryl groups near the carboxyl terminus of the H' chain; these groups are particularly useful for attaching complex molecules that may have diagnostic or therapeutic properties. Since the free sulphydryl groups are far from the antigen-combining site of the antibodies, substitution of even large groups at this position is expected not to affect the affinity for antigen.

The changes in the affinity constants of mAbs 225.28, 763.74, and TP41.2 associated with the fragmentation of whole IgG to F(ab')2, and Fab' fit the predictions of theory for univalent and bivalent antibodies. An antibody that simultaneously binds two cell surface antigen molecules (bivalent binding) would be expected to show little change in affinity when converted to a F(ab')2 fragment but should display a marked reduction in its affinity when converted to a Fab' fragment. This behavior is exhibited by mAbs 763.74 and TP41.2. Both show bivalent binding to melanoma cells, since the number of Fab' fragments binding to the cell is roughly twice that of the corresponding whole IgG and F(ab')2 fragments. The intact IgG and the F(ab')2 fragments of an antibody which binds univalently to the corresponding cell surface antigen are expected to display only a 2-fold larger affinity than the univalent Fab' fragments. This behavior is shown by mAb 225.28. It binds univalently to melanoma cells, since the number of Fab' fragments bound to melanoma cells is roughly equal to that of whole IgG and F(ab')2 fragments. The differences between binding of mAb 225.28 and of mAbs 763.74 and TP41.2 may reflect steric constraint imposed on antigen-antibody complexes by the location of the determinants on the antigenic structure. The epitope recognized by mAb 225.28 may be oriented in such a way that a single bivalent antibody molecule cannot span the distance between two binding sites, even allowing for rotational and translational mobility of the antigen molecule in the membrane. The epitopes recognized by mAbs 763.74 and TP41.2 may be located on more accessible regions of the antigen where bivalent binding is possible.

An interesting result of our studies has been the markedly lower level of HMW-MAA molecules immunoprecipitated from cultured melanoma cells by mAb TP41.2 than by mAbs 225.28 and 763.74. This result cannot be attributed to the differential affinity of the mAb, since mAb TP41.2 has a higher affinity than mAb 225.28 or 763.74; it is likely to reflect the distribution of the epitope binding to mAb TP41.2 on a narrower population of HMW-MAA bearing molecules than those defined by mAb 225.28 or 763.74. Heterogeneity in distribution of determinants of HMW-MAA has already been detected with a number of mAbs by Morgan et al. (27) and by ourselves (28, 29); however, in no case has the difference been so marked. Furthermore, the affinity constants of the mAbs used were never determined; therefore, one could not establish whether the differential reactivity of mAb with the pool of HMW-MAA molecules synthesized by a melanoma cell line reflected heterogeneity in the expression of the corresponding determinants or differences in the affinity constants. It should also be noted that the reactivity pattern of mAb TP41.2 with a panel of melanoma cell lines was not different from that of mAbs 225.28 and 763.74. These results suggest that the subpopulations of HMW-MAA with different antigenic profiles do not have a clonal distribution in a melanoma cell population but are expressed uniformly on different cells.

Radiolabeled Fab' fragments of anti-HMW-MAA mAb were found to display a marked reduction in their reactivity with some of the antiidiotypic mAbs tested. These findings, which parallel similar results with polyclonal antibodies (30), reflect not only destruction caused by the iodination procedure but also changes in conformation of the molecules caused by reduction of intra- and interchain disulfide bonds during the fragmentation process, since the idiotopes analyzed are conformational (15). An implication of these results is that immunoscintigraphy with radiolabeled Fab' fragments of anti-HMW-MAA mAb in patients with melanoma may reduce the interference of antiidiotypic antibodies which may be found following injections of murine mAb (6-8). This advantage may offset the limitations imposed by the reduced immunoreactivity of radiolabeled fragments, especially since methods are now available to purify immunoreactive antibodies following radiolabeling (31).

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REFERENCES

WHOLE IgG AND Fab' FRAGMENTS OF ANTI-HMW-MAA mAb


Binding Parameters and Idiotypic Profile of the Whole Immunoglobulin and Fab' Fragments of Murine Monoclonal Antibody to Distinct Determinants of the Human High Molecular Weight-Melanoma Associated Antigen

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