Flow Cytometric Determination of the Frequency and Heterogeneity of Expression of Human Melanoma-associated Antigens

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ABSTRACT

We used flow cytometry to measure the expression of human melanoma antigens on cell suspensions dissociated from metastatic masses. The objective was to study the heterogeneity between tumor samples from different patients and between different tumors excised from a single patient. Fifty-three metastases excised from 34 melanoma patients were analyzed with a panel of nine murine monoclonal antibodies (MOABs). Melanoma cells were stained by an indirect fluorescent method and analyzed on a Coulter EPICS C flow cytometer after gating to exclude tumor-infiltrating leukocytes and dead cells. The most consistently and most strongly expressed antigen was the high-molecular-weight proteoglycan (detected by the MOAB 9.227), which was expressed on 95% of the melanoma specimens and by a high proportion of cells within each specimen (mean ± SE, 79.2 ± 5.5%). However, strong expression of this antigen was limited to melanoma cells that had been dissociated mechanically and was markedly diminished by exposure to collagenses. Cultures of collagenase-dissociated tumor cells for 24 to 48 h resulted in reexpression of the antigen. The expression of other melanoma-associated antigens was not affected by collagenase treatment, but for these antigens there was more variability between cells from an individual tumor and between tumors from different patients. The percentage of enzyme-dissociated tumors considered positive for MOAB binding (defined as at least 10% of cells positive) and the mean ± SE of the percentage of positive cells within a tumor were as follows: MOAB ME-9-61 (antigen, p97) = 84% ± (41.2 ± 5.4%); MOAB ME-20.4 (antigen, nerve growth factor receptor) = 40% ± (18.7 ± 5.1%); MOAB ME-24 (antigen, ganglioside G0) = 84% ± (50.8 ± 4.8%); MOAB ME-311 (antigen, ganglioside 9-O-acetyl-G0) = 76% ± (42.5 ± 5.1%); MOAB ME-361 (antigen, mainly ganglioside G2a) = 3% ± (1.9 ± 0.8%); MOAB 3F8 (antigen, ganglioside G2) = 36% (10.5 ± 3.8%); MOAB 14G2a (antigen, ganglioside G0) = 86% ± (46.0 ± 6.7%); MOAB L243 (antigen, HLA-DR) = 56% ± (22.5 ± 5.5%).

INTRODUCTION

A number of murine MOABs have been produced that recognize antigens strongly associated with human melanoma cells (1–4). For many of these antibodies, the antigens have been characterized and include such diverse chemical entities as high-molecular-weight proteoglycan (5), gangliosides (3), and transferrin-like proteins (6).

The degree to which melanoma cells within a tumor express these antigens is not only of considerable theoretical interest, but also of practical importance. Heterogeneous expression of melanoma-associated antigens could be a limiting factor in the usefulness of the antibodies as diagnostic or therapeutic agents. Moreover, if those antigens could be made immunogenic, consistency of their expression would be crucial for their inclusion in vaccines. Finally, even if this is not possible, the heterogeneity of their expression might be indicative of similar heterogeneity of other melanoma-associated antigens that are capable of inducing an antitumor immune response.

To date, much of the work on this subject has utilized melanoma cell lines (4) or tumor tissue sections that have been fixed and stained by immunoperoxidase methods (7). Expression of antigens by established cell lines may not reflect that of the tumor cells from which they originated. Immunoperoxidase studies are compromised by difficulty in quantitation and by fixation artifacts. In this study, we sought to avoid these pitfalls by analyzing melanoma cells dissociated from metastatic masses and by using a quantitative assay method, flow cytometry. We show that binding of a panel of well-characterized MOABs is quite heterogeneous between tumors from different patients, but that the heterogeneity does not appear to extend to different tumors from the same patient.

MATERIALS AND METHODS

Source of Tumors. Metastatic masses were excised from 34 patients with melanoma. For 19 patients, a second mass was excised 2 to 25 mo later, so that the total number of tumors examined was 53. All of the patients were treated with active immunotherapy, consisting of an autologous melanoma vaccine with cyclophosphamide pretreatment (8, 9). The sites of the original tumor specimens were as follows: lymph node, 18; subcutaneous, 11; lung, 2; liver, 1; and bowel, 2.

Preparation of Tumor Cells. We used a modification of the method of Peters et al. (10). Freshly excised tumor masses were trimmed of skin, fat, and necrotic tissue and minced in cold Hanks' balanced salt solution supplemented with 1% human albumin and 0.1% EDTA. Cells that were released into the medium by mechanical dissociation were put aside and stored separately. The minced tumor pieces were placed in an enzyme solution, consisting of collagenase (140 mg) and DNase (30 mg) in 100 ml of the same solution. The collagenase was type I (Sigma Chemical Co., St. Louis, MO) from Clostridium histolyticum; the DNase was type I (Sigma) extracted from bovine pancreas. The dissociation process was carried out in baffled, tropsinizing flasks in a 37°C water bath with constant stirring using magnetic stir bars and an immersible stirrer. After 30 min of dissociation, the enzyme solution containing the cell suspension was removed, and fresh enzyme solution was added. The dissociation process was continued until no visible tumor tissue remained. The total time that a tumor sample was exposed to collagenase/DNase varied from 60 to 90 min. The tumor cells were washed twice in the Hanks' solution, resuspended in freezing medium (RPMI 1640 plus 1% human albumin plus 10% dimethyl sulfoxide), and frozen in a controlled rate freezer (Union Carbide, Indianapolis, IN) at 1°C/min. They were stored in the liquid phase of liquid nitrogen until needed.

Monoclonal Antibodies. The source and specificity of the murine MOABs used are shown in Table 1.
Table 1 Sources and specificities of monoclonal antibodies

<table>
<thead>
<tr>
<th>MOAB</th>
<th>Isotype</th>
<th>Antigen Description</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ME-9-61</td>
<td>IgG2b</td>
<td>p97 (melanotransferin)</td>
<td>Wistar</td>
<td>Herlyn et al. (29)</td>
</tr>
<tr>
<td>ME-20.4</td>
<td>IgG1</td>
<td>Nerve growth factor receptor</td>
<td>Wistar</td>
<td>Ross et al. (13)</td>
</tr>
<tr>
<td>9.2.27</td>
<td>IgG2a</td>
<td>High-molecular-weight proteoglycan</td>
<td>NCI</td>
<td>Bumol and Reisfeld (2)</td>
</tr>
<tr>
<td>ME-24</td>
<td>IgM</td>
<td>Ganglioside GD3*</td>
<td>Wistar</td>
<td>Thurin et al. (30)</td>
</tr>
<tr>
<td>ME-311</td>
<td>IgG3</td>
<td>Ganglioside 9-O-acetyl-GD3</td>
<td>Wistar</td>
<td>Thurin et al. (24)</td>
</tr>
<tr>
<td>ME-361</td>
<td>IgG2a</td>
<td>Ganglioside GD3*</td>
<td>Wistar</td>
<td>Thurin et al. (21)</td>
</tr>
<tr>
<td>14G2a</td>
<td>IgG2a</td>
<td>Ganglioside GD2*</td>
<td>Scripps</td>
<td>Mujo et al. (22)</td>
</tr>
<tr>
<td>3F8</td>
<td>IgG3</td>
<td>Ganglioside GD1*</td>
<td>Sloan-Kettering</td>
<td>Cheung et al. (20)</td>
</tr>
<tr>
<td>L243</td>
<td>IgG2a</td>
<td>HLA-DR</td>
<td>Becton Dickinson</td>
<td></td>
</tr>
</tbody>
</table>

* M. Herlyn, Wistar Institute of Anatomy and Biology, Philadelphia, PA.
† J. Green, Biological Response Modifiers Program, National Cancer Institute.
‡ Minor cross-reactivity with ganglioside GD2.
§ IgG3 variant also tested and gave identical results.
¶ Minor cross-reactivity with ganglioside GD3.
‖ R. Reisfeld, Scripps Clinic and Research Foundation, LaJolla, CA.
* N-K. V. Cheung, Memorial-Sloan Kettering Cancer Center, New York, NY.
# Becton-Dickinson Immunocytometry Systems, Mountainview, CA.

**Fig. 1. Binding of MOABs to enzyme-dissociated melanoma cells. Each “+” represents a tumor from a different patient. The dotted (- - - -) line connects the mean values for each antibody.**

FACS Analysis. The melanoma samples were tested with the panel of anti-melanoma MOABs by a two-step procedure. Tumor cell suspensions were incubated with MOABs on ice for 30 min, washed twice, and resuspended in FITC-conjugated Fab fragment of affinity-purified goat anti-mouse immunoglobulin (Cappel-Organon Teknika, West Chester, PA) for 30 min. Then, they were washed again and analyzed with a Coulter EPICS C flow cytometer (Coulter Electronics, Hialeah, FL), equipped with a 488-nm argon laser (used at 500 mW). One aliquot of cells was incubated with a MOAB that binds to all human leukocytes (hybridoma HB12; American Type Culture Collection) to allow identification of leukocytes. Ethidium bromide (10 µg/ml) was added to all samples just before analysis to allow gating of dead cells.

Flow cytometry analysis was performed by a modification of the method of Schroff et al. (11). Gates were set on the forward light scatter histogram to include only cells in the size range of melanoma cells and to exclude debris, erythrocytes, and clumps. A second gate was set on the red fluorescence histogram to exclude dead cells that had taken up ethidium bromide. Next, the tumor cell sample stained with anti-human leukocyte antibody was analyzed with a two-dimensional histogram of forward light scatter versus log of green fluorescence; the lower limit of the forward light scatter window was adjusted upward until it contained fewer than 5% leukocytes. Finally, the samples stained with anti-melanoma antibodies were analyzed on the green fluorescence channel using logarithmic amplification. In the few cases in which the leukocytes could not be completely excluded from the melanoma cell light scatter gate, we monitored a two dimensional plot of log green fluorescence versus forward light scatter to be certain that any green positive cells exhibited sufficiently high forward light scatter to preclude their being leukocytes.

The percentage of cells “positive” for an antibody was determined by the Immuno program developed by Coulter Electronics. The negative histogram (tumor cells stained with FITC-goat anti-mouse only) is subtracted channel by channel from the positive histogram (FITC-goat anti-mouse plus MOAB) after the setting of a match range for the two histograms. This method allows for the counting of dimly stained cells, which could be disregarded by arbitrarily setting a dividing line between positive and negative populations. A tumor was considered “positive” for binding of a MOAB if at least 10% of the cells were “positive” by the Immuno program.

**RESULTS**

Enzymatically versus Mechanically Dissociated Melanoma Cells. Melanoma cell samples obtained by enzymatic dissociation of metastatic masses from 34 patients were analyzed for binding of the panel of 9 MOABs. Fig. 1 shows the percentage of cells within each tumor that bound each antibody. A high proportion of cells within most of the tumors expressed the antigens identified by MOABs ME-9-61 (p97), ME-24 (ganglioside GD3), ME-311 (ganglioside 9-O-acetyl-GD3), and 14G2a (ganglioside GD2). On the other hand, very few tumors were positive for the antigens identified by 9.2.27 (high-molecular-weight proteoglycan), ME-361 (ganglioside GD2), and 3F8 (ganglioside GD3). Intermediate levels of expression were observed for MOABs against nerve growth factor receptor (ME-20.4) and HLA-DR, consistent with other studies (12, 13).

Since Schroff et al. (11) have shown that the high-molecular-weight proteoglycan is sensitive to collagenase, we considered the possibility that the enzymatic dissociation process may have caused a loss of other antigens as well. For 22 of the melanoma samples, we were able to obtain sufficient quantities of cells dissociated mechanically without exposure to collagenase/DNase, and the results of analyses of these cells are shown in Fig. 2. As expected, the high-molecular-weight proteoglycan antigen identified by MOAB 9.2.27 was strongly expressed by mechanically dissociated cells, but the expression of the other antigens was similar to that observed with enzymatically dissociated cells. Thus, the minimal binding of MOABs ME-361 and 3F8 was unlikely to have been an artifact of the dissociation process. Fig. 3 is a summary graph showing the proportion of enzymatically versus mechanically dissociated tumors considered “positive” for each MOAB, i.e., at least 10% of the cells binding the MOAB as defined by the Immuno analysis.

As previously shown by Schroff et al. (11), incubation of enzymatically dissociated melanoma cells for 24 to 48 h in tissue culture medium resulted in strong reexpression of the proteoglycan antigen, but not of the GD2-associated antigens identified by MOABs ME-361 and 3F8 (data not shown).
HETEROGENEITY OF MELANOMA ANTIGENS

Heterogeneity of Expression of Melanoma-associated Antigens. For 19 patients we were able to obtain and analyze a second tumor specimen (Tumor 2) which arose 2 to 25 mo (median 4 mo) after excision of the original tumor (Tumor 1). In 17 cases, Tumor 2 developed during or shortly after a time when the patients were receiving immunotherapy with an autologous melanoma vaccine that had been prepared with cells from Tumor 1 (8, 9). Thus, differences in expression of MOAB-identified antigens between Tumor 1 and Tumor 2 could have been due to "natural" heterogeneity or to variations induced by successful immunization.

Surprisingly, we observed little variation between paired tumors from the same patient. Analysis by a t test for nonindependent samples showed no significant differences for any of the MOABs tested ($P > 0.10$ for all). Linear regression analyses were performed for each antibody by the following model

$$\% (+)\ of\ cells\ in\ Tumor\ 2 = K + C \times [\% (+)\ of\ cells\ in\ Tumor\ 1]$$

where $C$ is the regression coefficient, representing the slope of the regression curve, and $K$ is a constant representing the $Y$ axis intercept.

As indicated above, the initial tumors were uniformly positive for the antiproteoglycan MOAB 9.2.27 and uniformly negative for anti-GD2 MOABs, ME-361 and 3F8. This pattern was identical for the second tumors, and therefore a close correspondence was obvious without regression analysis. The linear regression data for the other six MOABs are shown in Table 2, and the regressions curve for four of the MOABs are shown in Figs. 2 and 3.

![Fig. 2. Binding of MOABs to mechanically dissociated melanoma cells. Each "+" represents a tumor from a different patient. The dotted (-----) line connects the mean values for each antibody.](image)

![Fig. 3. Comparison of MOAB binding to melanoma cells dissociated enzymatically (TCE) versus mechanically (TCM). Tumors were considered "positive" if at least 10% of the cells were positive for the antibody, as defined by Immunoanalysis.](image)

Table 2  Linear regression analysis data for binding of MOABs by cells from paired tumors from the same patient

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antigen</th>
<th>Regression coefficient (slope)</th>
<th>Constant (Y-intercept)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ME-9-61</td>
<td>p97</td>
<td>$0.667 \pm 0.171^{b}$*</td>
<td>$13.959 \pm 8.934^{d}$</td>
</tr>
<tr>
<td>ME-20.4</td>
<td>Nerve growth factor</td>
<td>$0.877 \pm 0.156^{b}$*</td>
<td>$4.387 \pm 5.309^{d}$</td>
</tr>
<tr>
<td>ME-24</td>
<td>GD3</td>
<td>$0.639 \pm 0.286^{d}$</td>
<td>$16.001 \pm 15.779^{d}$</td>
</tr>
<tr>
<td>ME-311</td>
<td>O-Acetyl-GD3</td>
<td>$0.785 \pm 0.188^{d}$</td>
<td>$2.936 \pm 9.700^{d}$</td>
</tr>
<tr>
<td>14G2a</td>
<td>GD2</td>
<td>$0.831 \pm 0.192^{d}$</td>
<td>$3.177 \pm 11.802^{d}$</td>
</tr>
<tr>
<td>L243</td>
<td>HLA-DR</td>
<td>$0.744 \pm 0.059^{d}$</td>
<td>$-0.446 \pm 1.456^{d}$</td>
</tr>
</tbody>
</table>

* $P < 0.001$.
* * $P < 0.001$.
* For all, $P > 0.20$.
* $P < 0.05$.
* $P < 0.01$

For all MOABs, the percentage of "+" cells in Tumor 1 was plotted against the percentage of "+" cells in Tumor 2 (see text).

![Fig. 4. For all of the MOABs there was a statistically significant linear relationship between the percentage of cells binding the MOAB in Tumor 2 versus Tumor 1. Moreover, in no case was the slope significantly different from 1 or the constant significantly different from 0. Thus, for all 9 MOABs the percentage of cells binding Tumor 2 could have been accurately predicted to be close to the percentage that was bound by Tumor 1.](image)

DISCUSSION

Flow cytometric analysis proved to be an efficient and reliable method of measuring the binding of a panel of well-characterized MOABs to melanoma cells dissociated from metastatic masses. The results give a somewhat different perspective of the expression of these melanoma-associated antigens than provided by previously published studies.

It is apparent that the expression of these antigens is more heterogeneous than indicated by assays of melanoma cell lines. For example, although the p97- and GD3-related antigens were each detected in 84% of tumors by MOABs ME-9-61 and ME-24, respectively, the percentage of positive cells within an individual tumor varied widely, from 0 to 91.9% for p97 and from 0 to 96.9% for GD3. The more uniform expression of these cell surface antigens in tissue culture (14) could be related to selection of cells that can adhere well to plastic. In fact, Cheresh et al. (15) have shown that membrane ganglioside expression is necessary for attachment of cells in vitro and that anti-ganglioside antibodies inhibit such attachment. Another major difference between melanoma cells in vivo and in vitro is the much higher proliferative rate of the latter. Although the antigen recognized by MOAB 9.2.27 is not cell cycle dependent (16), this may not be the case for other melanoma-associated antigens (12, 17).

The one antigen for which variability among patients' specimens was minimal was the high-molecular-weight proteoglycan antigen recognized by MOAB 9.2.27. All but one tumor was positive, and the percentage of positive cells within a tumor was consistently high (75.6 ± 5.6%). A similar result was reported by Morgan et al. (1), who also used flow cytometry to measure melanoma antigen expression. Those authors found that, in 26 of 30 melanoma specimens, more than 90% of the cells were positive for antibody 9.2.27. Lindmo et al. (18) and Schroff et al. (11) have shown that the high-molecular-weight proteoglycan antigen may be reversibly removed from the cell surface by trypsin or collagenase and that the antigen is regenerated after 48 h, an observation confirmed by us in this paper. It is possible that the sensitivity to collagenase was due to contamination of the relatively crude collagenase preparation.
with other proteolytic enzymes. However, this finding is important, because collagenase is commonly used in the preparation of tumor cell vaccines for use as immunotherapy (8, 9, 19). It is possible that other, as yet undefined, tumor-associated antigens are equally susceptible to collagenase and that more gentle treatments would result in more immunogenic vaccines.

We found minimal binding of the antibodies, ME-361 and 3F8, to our melanoma cell suspensions. The latter reagent has been shown to be specific for ganglioside G_{D3} (20), and the former binds predominantly to G_{D2} and to a much lesser extent to G_{D3} (21). These data are consistent with published work indicating that the G_{D2} is a major melanoma ganglioside only in cells adapted to tissue culture. For example, Tsuchida et al. (14) chemically assayed the ganglioside content of freshly excised surgical specimens and of tissue culture lines derived from those specimens. Although the cells lines were rich in G_{D2}, the freshly excised tissues contain minimal amounts; in contrast, G_{D3} levels were high in both surgical specimens and cell lines. The relatively strong binding of MOAB ME-361 to melanoma tissue observed by Thurin et al. (21) may have been due to the use of acetone fixation which allowed detection of small amounts of non-surface G_{D2}.

In light of these data, our observation that melanoma cells strongly and consistently bound the MOAB 14G2a is puzzling, since this reagent also seems to bind to G_{D2} (22). Furthermore, we observed strong and consistent binding of MOAB ME-311 which recognizes an alkali-labile ganglioside which is thought to represent an 9-O-acetylated form of G_{D3} (23, 24). Chemical assay of melanoma tissue by Tsuchida et al. (25) showed that amounts of G_{D2} and alkali-labile ganglioside were similarly low (G_{D2} = 2.0 µg/g, alkali-labile ganglioside = 3.7 µg/g; cf. G_{D3} = 47.7 µg/g). Thus, the high reactivity of these two MOABs is unexplained, but could be due to a concentration of 9-O-acetyl-G_{D3} on the cell surface or to previously undetected, low-level cross-reactivity to the predominant ganglioside, G_{D3}.

Although the expression of most of the melanoma-associated antigens exhibited considerable variability when specimens from different patients were compared, we were surprised to find that the melanoma phenotype of an individual patient was quite stable over time. Regression analysis indicated that the degree of binding of each MOAB by a "new" tumor was similar to that of a tumor excised 2 to 25 mo previously. This observation is remarkable in view of: (a) the relatively small sample size; (b) the technical variability inherent in surface marker analysis; and (c) the intervening administration of a melanoma cell vaccine.

The subject of phenotypic variability of melanoma-associated antigens among tumors removed from the same patient has not been extensively addressed in the literature. Albino et al. (26) reported that cell lines derived from three metastatic masses from the same patient varied in growth rate and reactivity with a battery of MOABs. However, it is not possible to determine whether these differences reflected distinct populations present in vivo or were due to variations related to adaptation to tissue culture. Natali et al. (27) studied freshly harvested metastatic masses from seven melanoma patients by immunofluorescent staining of cryostat tissue sections. Although these investigators interpreted their findings as indicating heterogeneity in the expression of the high-molecular-weight proteoglycan antigen and of HLA-DR, their assay was semiquantitative and thus did not allow for statistical analysis. The only published study comparable to ours was that of Morgan et al. (28) who analyzed by flow cytometry 30 tumor specimens from 10 patients; these authors reported that multiple tumors from the same patient exhibited comparable, i.e., high, levels of MOAB 9.2.27 binding, similar to what we have observed.

In summary, the result of previously published studies do not contradict our finding that the melanoma phenotype exhibits little variability among the metastatic lesions of a given patient, at least in regard to the antigens recognized by the limited panel of MOABs that we studied. The critical question of whether this finding applies to melanoma antigens relevant to immunologically mediated tumor regression remains to be answered.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the assistance of the following individuals: Carmella Clark and Carlotta Green, who processed and

Fig. 4. Comparison of paired tumors from the same patient, linear regression analysis. Each panel shows a scattergram plot of the percentage of MOAB-positive cells in the original tumor (Tumor 1) versus that of a tumor (Tumor 2) excised 2 to 25 mo (median, 4 mo) later. For all four MOABs (ME-9-61, ME-24, ME-311, and 14G2a) the calculated regression curves were statistically significant (see Table 2).
analyzed the tumor specimens; Marsha Golden, who performed the flow cytometry analysis; and Ellen Hart, RN, who monitored the patients and arranged for timely specimen delivery.

REFERENCES


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