Increased Labeling of Human Melanoma Cells in Vitro Using Combinations of Monoclonal Antibodies Recognizing Separate Cell Surface Antigenic Determinants

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ABSTRACT

A panel of mouse anti-melanoma monoclonal antibodies (MoAb) were analyzed for reactivity with human melanoma cells singly and in combination. Five MoAb, ZME-018, 96.5, P94, 4.2, and 5.1, reactive with individual cell surface melanoma-associated antigens were tested with seven melanoma cell lines and seven fresh tumor biopsies. Cells were incubated with the MoAb, indirectly stained with fluorescein-conjugated goat anti-mouse immunoglobulin, and analyzed by flow cytometry. Percentage of labeled cells and relative fluorescence intensity (FI) with individual MoAb varied with different cell lines and biopsy samples. The most reactive MoAb, ZME-018, 96.5, and P94, labeled 29–93% of the cells from cell lines with relative FI of 2–59 units, thereby demonstrating phenotypic diversity of these cells. Similar results were obtained with cells derived from tumor biopsies, where 1–73% of cells were labeled and relative FI ranged from 0–27. These variations were reduced by using a “cocktail” of MoAb which recognized different melanoma-associated antigens. In cell lines both the percentage of labeled cells (range, 82–95%) and relative FI (range, 36–115) increased substantially (P < 0.025 and P < 0.005, respectively) when a “cocktail” prepared from all five MoAb rather than individual MoAb was used. A cocktail of MoAb increased the percentage of labeled tumor biopsy cells (range, 53–78%; P < 0.01) and relative FI (range, 11–68; P < 0.025). The mean FI obtained by incubating cells with a cocktail of suboptimal concentrations of three MoAb (ZME-018, 96.5, P94) was 48 ± 12 (SD), which was significantly increased compared to the mean FI seen with suboptimal concentrations of MoAb alone (ZME-018, 7 ± 10; 96.5, 8 ± 7; P94, 2 ± 2; P < 0.005). These findings were confirmed by radioimmunocassay using a combination of two MoAb, ZME-018 and 96.5. The data suggest that cocktails of MoAb were more effective than single MoAb alone for melanoma tumor cell labeling in vitro and might be more effective for tumor imaging and therapy.

INTRODUCTION

The development of hybridoma methodology (1) has greatly increased our ability to detect and characterize tumor-associated antigens. Currently more than 30 MoAb2 have been developed which recognize individual MAA on human malignant melanomas (2). Several of these MoAb have been evaluated in humans either alone (3, 4) or coupled to radioisotopes for use in diagnostic imaging (5–7).

There are several problems which need to be solved before MoAb can be used effectively for imaging or therapy. One problem is the heterogeneity of surface antigen expression on fresh tumor biopsies (8, 9) and cell lines (10–13). This heterogeneity has also been demonstrated on individual clones of tumor cell lines (10). Variations in antigen expression and density on individual tumor cells within primary and metastatic tumors (14–16) could account for the difficulties in detecting all known lesions in vivo using a single radiolabeled MoAb. One approach to this problem might be to use a combination of MoAb which recognize several different or distinct surface antigens and/or antigenic epitopes covering the range of heterogeneous antigen expression.

In this report we compared the binding of a panel of anti-melanoma MoAb reactive with different MAA singly and in combination (cocktail) to cells from melanoma cell lines and fresh tumor biopsies. Our results indicate that both the percentage of tumor cells labeled and the relative amount of MoAb bound per cell were significantly augmented by using MoAb cocktails compared to individual MoAb alone.

MATERIALS AND METHODS

Cell Lines. Melanoma cell lines BMCL, G, M-40, and RON were obtained from Dr. James Bowen, Section of Virology, M. D. Anderson Hospital and Tumor Institute, University of Texas. All of these lines were established in culture from melanoma biopsy specimens; lines G and M-40 produced melanin. Melanoma cell lines C-81-46 and C81-61 were obtained from Dr. Frank Meyaskis, University of Arizona Cancer Center. Melanoma cell line 294T is commercially available and was described by Creasey et al. (17). Adult human fibroblasts, used as a negative control, were obtained from Dr. Christopher Reading, Department of Tumor Biology, M. D. Anderson Hospital and Tumor Institute. Daudi, a Burkitt’s lymphoma cell line established by Klein et al. (18), also used as a negative control, was cultured in sterile 500-ml plastic culture flasks (Falcon Plastics, Oxnard, CA) in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum, 0.5% gentamicin, and 1% (v/v) L-glutamine. Melanoma cells and fibroblasts were initially cultured at a density of 2 x 10³ cells/ml in Leibowitz L-15 medium (Gibco) supplemented with 10% fetal calf serum in a humidified incubator at 37°C with 5% CO₂ in air. Cells were routinely split at confluence every 6–7 days and maintained in long term culture. Prior to labeling with MoAb cells were removed from culture flasks by gentle scraping or aspiration (Daudi cells), washed in PBS, and counted. Viability as assessed by trypan blue was greater than 70% in all cell lines tested.

Preparation of Melanoma Tumor Cell Suspension. Fresh unfixed surgical specimens were cut and minced into 1-mm³ fragments in RPMI media supplemented with 10% fetal calf serum and passed through a
0.38-µm sieve to obtain a single cell suspension. The cell suspension was enriched for viable tumor cells by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density gradient centrifugation, washed with PBS, and counted. Viability of cells as assessed by trypan blue dye exclusion ranged from 60–95%. Nucleated bone marrow cells obtained from normal donors were purified by Ficoll-Hypaque density gradient centrifugation as above and used as a negative control with the MoAb labeling procedure.

Monoclonal Antibodies. Murine anti-human melanoma MoAb were obtained from Hybridtech, Inc., San Diego, CA. All antibodies were produced and purified by Hybridtech using conventional hybridoma techniques. MoAb ZME-018 is a murine IgG2a antibody specific for a high molecular weight cell surface MAA (19). A second murine MoAb, subclass IgG2b reacting with a single 94,000-M, glycoprotein, was also used (20). For purposes of simplicity the MoAb is designated P94 in this study. MoAb 96.5 is murine IgG2a which reacts specifically with P97 antigen epitope (21). MoAb 5.1 is specific for MAA P210 and is of the IgG1 subclass (22). MoAb 4.2 is a murine IgM MoAb specific for a ganglioside antigen, expressed on the majority of human melanomas (23). As an irrelevant control we used a MoAb with a structure and subclass identical to that of MoAb 96.5 but nonreactive with P97 and any other known MAA antigen (obtained from Hybridtech, Inc.).

Immunofluorescent Techniques. Fresh tumor cells and cell lines suspended at a concentration of 1–3 x 10^6 cells in 50 µl of PBS were mixed with 50 µl of individual MoAb at concentrations ranging from 1 to 50 µg/ml and incubated for 30 min at 4°C. The final concentration of MoAb ranged from 0.5 to 25 µg/ml. Cells were washed twice with PBS and incubated for an additional 30 min at 4°C with 75 µl of a 1:50 dilution of fluorescein-conjugated F(ab')2 goat anti-mouse immunoglobulin (Kallestad Laboratories, Austin, TX). The cells were then washed twice with PBS, and the number of cells labeled with MoAb or MoAb combinations as well as the relative fluorescence intensity of labeling (FI) were determined by flow cytometry. The flow cytometer (Spectrum III-Ortho Diagnostic Systems, Westwood, MA) was standardized daily with Fluorotrol-GF (Ortho). Tumor cell populations were determined by narrow forward and right angle light scatter characteristics. Careful gating procedures were used to ensure exclusion of dead cells and lymphocytes (24). Values for FI were controlled by determination of autofluorescence of unstained melanoma cells. Relative FI is reported as units of fluorescence on a scale from 0 to 250.

Radioimmunoassay Procedures. Cells from the melanoma cell line 294T along with Daudi cells were suspended in 15 x 100-mm glass tubes (Fischer Scientific, Dallas, TX) at a concentration of 1–2 x 10^6 cells/tube in 50 µl of PBS. One mg each of two MoAb, 96.5 and ZME-018, were labeled with 1 mCi ¹¹¹In using a modification of the technique of Krezcarek and Tucker (25). A more detailed report of the labeling procedure has been published previously (26). One hundred ng of each MoAb ¹¹¹In conjugate (i.e., equivalent of 100 nCi ¹¹¹In or approximately 150,000 cpm) and a cocktail of MoAb In conjugates (i.e., 50 ng each of labeled 96.5 and ZME-018) were incubated with melanoma and Daudi cells in a total volume of 0.1 ml PBS for 30 min at 4°C. Cells were washed twice in PBS and radioactivity was determined using a gamma counter (Packard Instruments, Des Plaines, IL). Blocking studies were also performed by incubating cells with unlabeled MoAb for 30 min, washing twice, and then incubating with the respective ¹¹¹In labeled MoAb. The actual ng quantities of MoAb bound were calculated using the formula:

\[ \text{ng MoAb bound} = \frac{\text{cpm bound to cells}}{\text{cpm added} \times \text{ng MoAb added}} \]

RESULTS

Reactivity of Different MoAb Cocktails with Melanoma Cell Lines. In initial experiments optimal binding of MoAb to most cell lines occurred when cells were collected between 6 and 7 days of growth after being subcultured. MoAb reactivity with MAA was less if cells were analyzed prior to 5 days or after 7 days in culture (data not shown). Hence all experiments were performed at a time when maximal MAA expression occurred, to compensate for variations in antigen expression which might be related to cell cycle or culture conditions.

In preliminary experiments we tested the reactivity of two of the five available MoAb (96.5 and ZME-018) against the melanoma cell line 294T at final MoAb concentrations ranging from 0.5 to 25 µg/ml. Reactivity was detected at individual MoAb concentrations ranging from 5 to 25 µg/ml; above 25 µg/ml no further increase in either the percentage of cells labeled with MoAb or the relative FI was observed (data not shown). Hence a 25-µg/ml concentration of each MoAb was used in experiments described below.

Immunoreactivity of five anti-melanoma MoAb with seven different human melanoma cell lines is shown in Table 1. Overall the most reactive individual MoAb were ZME-018, 96.5, and P94. However, there was considerable heterogeneity in MAA expression on the different cell lines with variability in the percentage of labeled cells and FI. For example MoAb ZME-018 showed high binding with all cell lines except RON. Similarly MoAb 96.5 showed high binding with BMCL cells but low reactivity with C81-46 and RON. Of all the MoAb used, only P94 reacted well with the RON cell line. In contrast a cocktail prepared from all five MoAb reacted very well with all cell lines. The percentage of cells labeled by the cocktail were in the range of 82–95% (median, 89%) compared to 29–93% (median, 71%) with ZME-018 alone (25 µg/ml) (P < 0.025). The percentage of cells reacting with 96.5 alone (25 µg/ml) ranged from 22–81% (median, 62%), and the range for P94 alone was 31–83% (median, 61%).

The relative FI was also greater with cocktails compared to single MoAb. When cell lines were examined for FI they showed the highest intensity with ZME-018 (range, 9–96 units; median, 49), followed by 96.5 (range, 2–49 units; median, 23) and P94 (range, 4–31 units; median, 16). FI for other MoAb were in the range of 0–38 units. A MoAb cocktail prepared from all five MoAb demonstrated significantly higher FI (range, 36–115; median, 93; P < 0.005). A representative example of the percentage of labeled cells and FI using a single MoAb and a cocktail of MoAb is shown in Chart 1.

Improved Labeling of Cell Lines with Suboptimal Concentrations of MoAb in Combination. In the previous experiments all MoAb were used at optimal concentrations (i.e., 50 µl of a 50-µg/ml concentration per 1–3 x 10^6 cells in a total of 0.1 ml of PBS or an equivalent of MoAb of 25 µg/ml). To determine whether increases in the percentage of labeled cells and FI could occur if suboptimal concentrations of MoAb were added, 10 µl of 50-µg/ml dilutions of each of the three most reactive MoAb (ZME-018, 96.5, and P94) were added individually (total, 5 µg/ml) and in combination (total, 15 µg/ml) to the 294T melanoma cell line (Table 2). The percentage of cells labeling with the suboptimal concentration (i.e., 5 µg/ml) of each MoAb was equivalent to those labeling at the 15-µg/ml concentration; however, the relative FI was markedly diminished. The FI increased with a cocktail of three MoAb (total MoAb concentration, 15 µg/ml). The mean FI was significantly greater [48 ± 12 (SD); P < 0.002] than the sum of the FI obtained for each MoAb [8 + 7 + 2] added singly.
Table 1
Reactivity of MoAb with melanoma cell lines

<table>
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<tr>
<th>Melanoma cell lines</th>
<th>Individual MoAb</th>
<th>Cocktail</th>
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<tr>
<td></td>
<td>NR</td>
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<td>15</td>
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<tr>
<td>C 81-51</td>
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Control cell lines
- Fibroblasts
- Daudi cells
- Bone marrow

Concentration of MoAb, 25 μg/ml/1-3 x 10^5 cells.
Mixture of five MoAb at a total concentration of 25 μg/ml/1-3 x 10^5 cells.
Nonimmunoreactive MoAb. This MoAb was similar to 96.5 in subclass IgG2a.
Represents mean of duplicate or, in the case of M 40 cell line, triplicate experiments.
Differences between single MoAb and cocktail are statistically significant for labeling (P < 0.025) and FI (P < 0.005). Paired t test was used for statistical evaluation.

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at individual concentrations of 15 μg/ml. Higher individual concentrations of MoAb in the cocktail (i.e., 15 μg each) did not improve either the mean percentage of cells labeled (82 ± 6) or the mean FI (49 ± 6) over a MoAb cocktail made up of 5 μg/ml concentrations of each MoAb.

Similar results were obtained using ng amounts of 111In-labeled MoAb in a radioimmunoassay (see "Materials and Methods"). As shown in Table 3, 500-ng/ml concentrations of a combination of two MoAb (ZME-018 and 96.5) bound to 294T to a greater extent (12.97 ng) than did 1000-ng/ml amounts of each MoAb alone (4.75 and 5.16 ng, respectively; P < 0.0001). Binding to cells was effectively blocked if cold antibody was first added followed by the respective labeled antibody. Of further interest was the finding that addition of cold unlabeled 96.5 followed by 111In-labeled ZME-018 increased binding of ZME-018 (11.75 ± 5.54 ng) over that which occurred for 111In-labeled ZME-018 alone (4.75 ± 1.70 ng).

Reactivity of Different MoAb and MoAb Cocktails with Single Cell Suspensions of Melanoma Tumors Derived from Human Biopsy Materials. The pattern of reactivity of different
fresh tumor biopsies and MoAb is demonstrated in Table 4. Mixtures of two, three, or five MoAb were compared to each MoAb added alone. A total of seven individual tumor cell suspensions were evaluated. Several distinct patterns of reactivity emerged. In sample 1 the majority of labeled cells expressed the antigens P97 and high molecular weight MAA and hence demonstrated good labeling with either 96.5 or ZME-018. Therefore a mixture of other antibodies along with 96.5 and ZME-018 increased only the relative FI and did not increase the percentage of labeled cells. In sample 2 cells reacted predominantly with MoAb 4.2. Similar to the above example the addition of the four other MoAb in a cocktail increased only the relative FI and not the percentage of labeled cells. A heterogeneous population of tumor cells was found in samples 3, 4, and 7, and low expression of all five MAA as recognized by MoAb was present. In contrast to the previous samples a mixture of MoAb recognizing all five MAA increased both the percentage of labeled cells and the relative FI. In sample 6 there were two populations of cells, one predominantly labeling with P94 and the other with ZME-018. A combination of these MoAb increased the percentage of labeled cells to the same level as a combination of all five MoAb. In all samples tested the binding of the MoAb cocktails was higher than that of single MoAb alone. A cocktail prepared from two MoAb, ZME-018 and 96.5, showed less reactivity than a cocktail prepared from three MoAb. In general MoAb cocktails prepared from five MoAb showed higher reactivity than cocktails prepared from three MoAb.

**DISCUSSION**

A variety of MoAb reactive with MAA have been described (2, 9, 19–23). In this report we analyzed the ability of a panel of MoAb to react singly and in combination with a panel of melanoma cell lines and fresh tumor samples. Most of the melanoma cell lines used had a high expression (i.e., from 60–80%) of three previously defined antigens, high molecular weight MAA, P97, and P94. Other antigens, 5.1 and 4.2, were expressed to a lesser extent. Considerable heterogeneity in MAA expression existed among the cell lines and fresh tumor samples.

Phenotypic diversity of cancer cells is a well described phenomenon (15, 16). Burchiel et al. (11, 12) analyzed binding of MoAb recognizing HLA-AB antigens, la-likes antigens, and two MAA (M, 280,000 and 94,000) in human melanoma cell lines by flow cytometry. Considerable heterogeneity with regard to MAA antigen expression was found which correlated to a limited extent with cell size and cell cycle. Both the M, 280,000 and 94,000 MAA were differentially expressed during the cell cycle with maximal expression occurring during G2 + M phases. In another study (10) melanoma cell lines were cloned in methylcellulose and expanded in liquid culture; the antigenic profile of these metastatic tumor cell clones was analyzed using a panel of eight MoAb to MAA. Both a quantitative and a qualitative heterogeneity of antigen expression was observed among and within the different clones in spite of the cloning procedure. Heterogeneity of antigen expression was also described by Houghton et al. (27) using bulk cultures of melanoma cells. The authors attributed changes in antigen expression as arising along a melanocyte differentiation pathway. Similar results have been published for other tumor cell lines, particularly oat cell and squamous cell lung cancer (28).

The most important finding of this study was that variations in antigen expression for a single MoAb could be significantly reduced by using a "cocktail" of MoAb which recognized a series of different MAA. Both the percentage of cells which bound MoAb and the actual quantity of MoAb bound, as assessed by relative FI on flow cytometer, increased significantly in both cell lines and fresh tumor cells. In this instance the relative FI histogram is a rough measure of antigen density; however, it cannot discriminate individual "brightly staining" cells from an overall increase in antigen density per cell. A second important observation was that the percentage of labeled cells and more significantly the relative FI was increased substantially if suboptimal concentrations of three MoAb with relatively high affinity (ZME-018, 96.5, P94) were added to cells rather than saturating concentrations of each MoAb alone or combined. In this case the mean FI with suboptimal amounts of MoAb in the cocktail was actually higher than the sum total FI expected for each MoAb added individually in optimal concentrations suggesting a synergistic effect (Table 2).

This represents one of the first studies to examine the efficacy
of using cocktails of MoAb reacting with distinct MAA to improve labeling of melanoma cell lines and fresh tumor cells in vitro. Dantas et al. (29) were able to detect melanoma cells in bone marrow using a combination of three MoAb which recognized separate epitopes on P97: 96.5, 8.2, and 11B.1. Preliminary work by Colnaghi et al. (3) demonstrated that a panel of MoAb reactive against breast (30) and ovarian carcinoma (31) significantly augmented the sensitivity of detecting both circulating antigens and metastatic tumor cells in bone marrow over individual MoAb used alone. Ceriani et al. (4) recently demonstrated that a cocktail of anti-breast cancer MoAb appeared to be more effective in eradicating tumor cells in nude mice than was single MoAb alone.

Binding of ng quantities of a combination of two MoAb, ZME-018 and 96.5, to T294 cells was significantly greater (P < 0.00001) than each MoAb alone as well as the sum of both antibodies (P < 0.025) as determined by a sensitive radioimmunoassay technique (Table 3). Of additional interest was the observation that quantitative binding of 111In-labeled ZME-018 MoAb was increased substantially if unlabeled 96.5 was first added followed by 111In-labeled ZME-018. Although the explanation for this phenomenon is unclear it suggests that one MoAb, depending on its structure or other characteristics, may actually augment the binding of a second MoAb, perhaps by exposing cryptic antigen sites through cross-linking, etc. Further studies are needed in an attempt to understand the mechanisms involved.

Similar findings were also observed using fresh tumor cell suspensions (Table 4). In contrast to data using cell lines P94 bound much more avidly to fresh tumor cells. The reason for this difference is uncertain although it is possible that more fresh tumor cells were in G1-G2 phase transition than cell lines (11). In general the degree of binding of MoAb cocktails to fresh tumor suspensions was reflected by the affinity of each MoAb for its respective MAA. For example if both ZME-018 and 96.5 bound individually to over 80% of cells, the percentage of cells labeled did not increase substantially with a cocktail of both MoAb. On the other hand if MoAb bound to a lesser extent individually, the percentage of cells and more importantly the FI increased significantly if a cocktail of the respective antibodies was used.

In conclusion the findings reported above could have considerable importance in the clinical setting. In view of the heterogeneity within tumors it is likely that a cocktail of MoAb could be more efficacious for tumor imaging and/or therapy. However, one should be cautious in overinterpreting our results to imply that "more is better." It is likely that either optimal or suboptimal combinations of MoAb exist depending on their respective affinities and interactions when used in combination; hence certain MoAb might either inhibit or augment binding of another MoAb depending on their individual characteristics. Also pharmacokinetic considerations and nonspecific uptake may influence the binding of single MoAb versus cocktails in vivo.

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REFERENCES


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