Antibody-induced Movement of Common Melanoma Membrane Antigens on the Surface of Unfixed Human Melanoma Cells

Stanley P. L. Leong, Sidney R. Cooperband, Peter J. Deckers, Carl M. Sutherland, James F. Cesare, and Edward T. Kremetz

ABSTRACT

Antisera to common human melanoma antigens were obtained from melanoma patients receiving autologous immunization with their own irradiated cultured melanoma cells and Bacillus Calmette-Guérin. The antibody thus derived was used to detect common antigens on the plasma membrane of three different human melanoma cell lines by membrane immunofluorescence. The antigen-antibody complexes on the surface of melanoma cells would move to a pole (capping) and would subsequently be extruded into the extracellular milieu at room temperature. Approximately 25 to 30% of viable cells were positive by immunofluorescence. However, when the cells were fixed with methanol, 60 to 70% of cells demonstrated membrane binding. Capping was inhibited at 0°C or when the cells were pretreated with vincristine sulfate. It can be concluded that common tumor antigens exist on the surface of viable human melanoma cells and that the redistribution of antigen-antibody complexes is an active process. The extrusion of antigen-antibody complexes in vitro may represent a mechanism of antigenic modulation in vivo and could indicate a basic method of tumor survival since presumably the antigen-denuded cell is viable and capable of replication but not of recognition by subsequent effector immune events.

INTRODUCTION

The presence of common tumor-associated antigens within or on the surface of human malignant melanoma cells continues to be a controversial issue. By immunofluorescence, common membrane antigens have been observed on the plasma membrane (4, 12, 20), in the cytoplasm (13, 20, 21, 24, 26), and in the nucleolus (18). To complicate matters further, Lewis and Phillips (14) reported that melanoma membrane antigens were individually specific. The reason for these conflicting results are not clear, but they are probably related in part to sources of antisera and melanoma cells (2). Most previous studies have utilized sera from melanoma patients who have not been immunized with melanoma vaccines. These sera may or may not contain significant quantities of antibody to detect all antigens, or they may contain antibody against one antigen and not another.

In a recent immunofluorescence study (12), our group reported the development of antimelanoma antisera in melanoma patients undergoing autologous immunization. When these patients were serially immunized with their irradiated autologous melanoma cells in the presence of the adjuvant BCG,3 strong antimelanoma antisera developed in 2 to 6 months. These antisera were found to bind to the surface of a variety of human melanoma lines derived from different patients after these cells had been prepared by fixation with methanol (12). When viable melanoma cells were incubated with these antisera, fixed with methanol, and again incubated with fluorescent goat anti-human γ-globulin, sequential steps of full-surface fluorescence, capping, and extrusion of antigen-antibody complexes could be observed (11). Since methanol fixation was used in these 2 studies, there still remains a nagging doubt that the antibody is binding to surface antigenic determinants which are artificially produced by fixation. Therefore, we used unfixed viable melanoma cells throughout the entire immunofluorescence procedure in this study. The immunofluorescence and phase-contrast data presented in this report show the existence of common melanoma membrane antigens, which may cap and be extruded from viable melanoma cells.

MATERIALS AND METHODS

Immunization of Melanoma Patients. The immunization program used to produce the antimelanoma antisera has been described elsewhere (12). The postimmune plasma of one (R. L.) of the 5 patients undergoing autologous immunization was used in this study. Approximately 1 to 4 x 10⁶ irradiated (2 to 7 x 10⁶ rads) tumor cells suspended in 0.5 ml of Roswell Park Memorial Institute Tissue Culture Medium 1640 plus Galaxo BCG (1 to 3 x 10⁵ viable organisms) were injected intra-dermally at approximately 2-week intervals. IgM/IgG-fluorescent antibody titers were followed during the course of RL autologous immunization. The target cell in this immunofluorescent study was TU-M (Tulane University Melanoma)-PK which was not used in the immunization of R. L. The antibody titers are shown in Chart 1. The specificity of the antisera for melanoma antigens is shown in Table 1. Preimmune plasma (September 30, 1974) and postautoimmune plasma (October 13, 1975) from Patient R. L. were used. Absorption was carried out with a variety of normal and malignant non-melanoma viable human cells. There was no apparent loss of antibody when tested by immunofluorescence on melanoma cells. Similar results on the specificity of the antisera have already been published (12).

Two human malignant melanoma cell lines, CaCL 74-36 (16) and RPMI-8322 (15), were obtained from Dr. S. K. Liao, McMaster University, Hamilton, Ontario, Canada. CaCL 74-36

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The abbreviations used are: BCG, Bacillus Calmette-Guérin; PBS, phosphate-buffered saline (8.8 g NaCl, 1.135 g Na₂HPO₄, and 0.276 g NaH₂PO₄·H₂O in 1000 ml distilled water, pH 7.4).
was developed by Dr. Liao in Hamilton, and RPMI-8322 was initially developed by Dr. G. Moore in Denver. The third cell line, TU-M-BW (11, 12, 30), was developed at Tulane University. CaCL 74-36 passage 40-42, RPMI-8322 passage 45-47, and TU-M-BW passage 16-20 were used in this study.

Cells were grown in plastic bottles (Falcon Plastics; 75-sq cm flasks) with complete Earle’s minimal essential medium containing 10% fetal calf serum, antibiotics [100 units penicillin & 100 μg streptomycin per ml and 1% glutamine (200 mM)] at 37° under 5% CO₂. The culture medium was changed every 3 to 4 days. Passage of the cells was accomplished by trypsinization (0.25%) for 1 to 3 min.

Membrane Immunofluorescence Studies. Möller’s membrane immunofluorescence technique was used (19). Cultured melanoma cells were harvested by scraping with a rubber policeman. The cells were then washed 3 times in PBS. During the last wash, the cell suspension was divided equally into 10-ml centrifuge glass tubes, and each tube contained approximately 3 × 10⁷ cells. One drop of RL postimmune plasma (October 13, 1975; diluted 1:8) was applied onto the cell pellet with mixing and incubated at room temperature for 2, 5, 12, and 20, and 30 min. One set of cells were incubated in ice bath throughout the entire procedure to see the effect of low temperature on capping. Controls included RL preimmune plasma (September 30, 1974; diluted 1:4), 2 nonimmune melanoma sera, 2 normal human sera, and PBS. At the end of each incubation, 9 ml of ice-chilled PBS were poured into the tube to stop any further metabolic activity (i.e., capping), and the tubes were placed in an ice bath. At the end of the last incubation, the cell suspensions were centrifuged at 4° and at 2000 rpm for 10 min. The cells were then washed twice with ice-chilled PBS. One drop of fluorescein isothiocyanate-conjugated goat antiserum to IgG (Hyland Labs, Costa Mesa, Calif) at a dilution of 1:8 was then added to each cell pellet, mixed gently, and incubated in an ice bath for 20 min. The cells were then washed twice with ice-chilled PBS. One drop of 90% glycerol was then applied to each pellet, and the cells were resuspended. The cells were then transferred onto a slide and covered with a coverslip. The slides were observed for fluorescence with a Lietz Wetzler fluorescent microscope (200 W, UG-1 filter and K430 barrier filter; Mercury Lamp Osram HBO) with a built-in phase-contrast light source. All slides were kept at 4°, except the one being observed to minimize movement of membrane antigens.

Indirect Immunofluorescence after Fixation. CaCL 74-36 cells were fixed with methanol prior to indirect immunofluorescent staining for comparison with membrane immunofluorescent patterns. Detailed procedures have been described elsewhere (12).

Effect of Vinblastine on Capping. TU-M-BW cells were used in this study. Prior to membrane immunofluorescence as described above, each test tube of cells (3 × 10⁵) was treated with 2 drops of vinblastine sulfate (Lilly, Indianapolis, Ind.) at 10⁻² and 10⁻³ M for 30 min. Control test tubes were treated with PBS. The cells were then washed in PBS and stained by the membrane immunofluorescence technique.

Photography. Kodak Tri-X 135 was used for photography. Each field was taken by UV and phase-contrast light. Exposure intervals are shown in the legend.

Viability Count. Percentage of viable cells were obtained by counting 200 cells using trypan blue exclusion method. One drop of the dye (0.4%; Grand Island Biological Co., Grand Island, N. Y.) was mixed with 5 drops of cell suspension. The cells were counted in a hemocytometer under a light microscope.

RESULTS

Absorption Studies. Results of absorption experiments are shown in Table 1. These data indicate that 3 human melanoma cell lines (TU-M-BW, CaCL 74-36, and RPMI-8322) shared common membrane antigens. Furthermore, human skin fibroblasts, HeLa cell line, BCG, Earle’s minimal essential medium, and fetal calf serum were not able to absorb out the antibody against the melanoma antigens.

Demonstration of Capping. All 3 melanoma lines showed full-surface fluorescence, sequential capping, polarization, and extrusion of antigen-antibody complexes over the incubation period of 2 to 30 min with RL postautoimmune antimelanoma plasma (October 13, 1975). No fluorescence was seen when control sera and PBS were used.

Figs. 1 to 7 show the fluorescent patterns of CaCL 74-36 cells and simultaneous photographs taken by phase-contrast examination over a period of 30 min. Full-surface fluorescent cells with a heavy beading appearance (Fig. 1a) are maximal at 2 min of incubation. The cytoplasm and nucleus are too dark to be seen by immunofluorescence, but the phase-contrast image (Fig. 1b) reveals an intact cell with distinct cytoplasm and nucleus. By phase-contrast microscopy, it can be seen that the periphery of the cell is granular. Capping (Figs. 2 to 3) is maximal after 12 to 20 min of incubation (Chart 2). It should be noted that the circumferential granular substances seen in the phase microscopy during this time period (Figs. 2b and 3b) have now accumulated at the site of the fluorescent antigen-antibody complexes. The rest of the cell membrane is relatively smooth. After 20 min of incubation, polarization (Fig. 4a) and extrusion (Fig. 5a) of the fluorescent tag are maximal (Chart 2). When viewed by phase microscopy, the polarized and extruding granular substances are again located in the same area as the fluorescent materials (Figs. 4b and 5b). Fig. 5 (a and b) shows extruded materials on one pole and dotty fluorescent complexes on the other. Fig. 6a shows nonfluorescent cells. Note the smooth cell surface on phase contrast (Fig. 6b). Fig.
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Table 1
Specificity of RL postimmune plasma for common melanoma membrane antigens

Absorption. Number of cells: plasma volume = 1.5 to 2.0 x 10^6:1/30 ml, 1 hr at room temperature and 23 hr at 4°C. Volume of medium or BCG: plasma volume = 1:1, same incubation time.

<table>
<thead>
<tr>
<th>RL plasma Absorption</th>
<th>TU-M-BW</th>
<th>CaCl 74-36</th>
<th>RPMI-8322</th>
<th>Two allogeneic skin fibroblasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preimmune (1:4)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Postimmune (1:8) absorbed with</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TU-M-PK melanoma</td>
<td>-</td>
<td>NT</td>
<td>NT</td>
<td>-</td>
</tr>
<tr>
<td>TU-M-BW melanoma</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>NT</td>
</tr>
<tr>
<td>CaCl 74-36 melanoma</td>
<td>-</td>
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<td>-</td>
<td>NT</td>
</tr>
<tr>
<td>RPMI-8322 melanoma</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>NT</td>
</tr>
<tr>
<td>Two allogeneic skin fibroblasts separately</td>
<td>+</td>
<td>+</td>
<td>NT</td>
<td>NT</td>
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<tr>
<td>HeLa</td>
<td>+</td>
<td>NT</td>
<td>NT</td>
<td>-</td>
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<tr>
<td>BCG</td>
<td>+</td>
<td>+</td>
<td>NT</td>
<td>-</td>
</tr>
<tr>
<td>Earle’s minimal essential medium—</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td>100% fetal calf serum</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>NT</td>
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</table>

* - , no fluorescence; +, bright membrane fluorescence; NT, not tested.

Increased. There was a subsequent increase and then a decline in the number of cells which exhibited fluorescent capping, followed by sequential increase in cells which were either extruding the antigen-antibody complex or had lost the fluorescent label entirely.

Using viable and methanol-fixed cells for comparison, it was found that after 2 min of incubation, only 25 to 30% of viable cells showed detectable fluorescent label while 60 to 70% of methanol-fixed cells were found to be labeled with “ring” membrane fluorescence (Fig. 8).

Inhibition of Capping. Inhibition of capping was noted when the cells were kept at 0° or treated with vinblastine at 10^-2 M for 30 min. No appreciable inhibition of capping was seen with vinblastine at 10^-3 M.

DISCUSSION

The studies reported here demonstrate the presence of common antigens on the plasma membrane of at least 3 cultured melanoma lines. Using postimmune antimalanoma serum, we demonstrated that the membrane antigens went through a time-dependent sequence of capping and extrusion from the cell surface. The antibody did not detect any cytoplasmic antigen under these circumstances. The common tumor antigens on the cell surface were highly mobile. This phenomenon is similar to studies (6—9, 17, 23, 27—29) of other surface antigens. A similar report has recently demonstrated shedding of membrane antigens by human breast cancer cells after incubation with human antisera (22).

The antiserum utilized in these experiments appeared to be specific for melanoma antigens. There was no loss of antibody activity after absorption with nonmelanoma cells. In addition, this antiserum had been used in a large number of experiments with fixed cells obtained from several other melanoma patients (12). Using membrane immunofluorescence technique, we could not detect the presence of adequate quantities of antibody in the plasma or sera of 3 melanoma patients who had not been specifically immunized or in the sera of 2 normal patients. The plasma and sera from the 5 negative humans had been treated like the immunized human plasma, and no alteration of proteins had occurred which might produce nonspecific binding to the test melanoma cells.

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studied had been maintained in culture for a large number of cell generations, away from the influences of immunogenic modulation of the tumor antigens, and therefore were free to express tumor antigen without any host- or immune-induced selection. Tumor cells obtained directly from patients as primary surgical specimens are under the influence of continuous host resistance reactions and thus may alter their surface antigen expression. This may be one factor in the confusing reports about the presence and/or distribution of tumor antigens. Accurate control of time and temperature in this study allowed us to observe events which might have been obscure to other investigators who examined melanoma cells at later time points or who worked at temperatures which permit movement and polarization of surface antigens (14). We believe that this is another technical condition which may also be partly responsible for the confusing results regarding the presence of common membrane antigens in melanoma cells.

In our experiments, only 25 to 30% of the viable cells contained detectable fluorescent label at maximum during the early min after addition of antimelanoma antibody. When the cells were fixed with methanol, approximately 60 to 70% of the cells demonstrated labeling with the fluorescein-tagged anti-human IgG. These data are consistent with a previous study (11). We have no experimental explanation for this phenomenon. However, there are at least 2 theoretical explanations. (a) The quantity of surface antigen is relatively small and widely dispersed in the majority of tumor cells. Its density is insufficient to be detectable by the relatively insensitive experimental methodology of the fluorescent microscopy. When the cells are fixed and collapsed down on the surface of a slide, the density of antigen increases and may then be detectable by microscopy. (b) Alternatively, the cell population which is not visualized in the viable state with fluorescent anti-IgG may represent a population of cells in which the surface antigen is embedded within the membrane (11) or stericly obstructed such that the human antibody directed against the melanoma antigen is physically prevented from binding. This may or may not be related to the cell replication cycle. Under these circumstances, cells which may be in a selected part of the cell replication cycle may be the only cells capable of binding the human antibody. However, we have not yet determined experimentally if any of these explanations for the difference in antigen detection between fixed and viable cells are valid.

Examining the early time periods after the addition of antibody, we found that about 2% of the cell population appeared to be undergoing spontaneous extrusion of antigen. This suggests that cultured melanoma cells may be shedding antigen spontaneously without any dependence upon the presence of antibody. In support of this conclusion, we (10) and others (1, 5, 25) have found that, in tissue culture, tumor antigen is found in cell-free supernatants. Our observations in this study merely confirm by fluorescent visualization that antigen may be shed without apparent cell lysis.

In summary, our data support the idea that common melanoma antigens exist on the surface of malignant melanoma cells. The antigen-antibody complexes on the cell surface are mobile and may be extruded from the plasma membrane. This mobility may be inhibited when the cells are kept at 0° or pretreated with vinblastine. Since capping is inhibited at 0° and with pretreatment with vinblastine, it can be concluded that the redistribution of antigen-antibody complexes is an active microtubular process. The shedding of antigen-antibody complexes by melanoma cells in vitro may represent a mechanism of antigenic modulation in vivo; this may explain the survival of tumor cells in patients despite tumor-specific immune reactions which might prevent tumor growth.

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Fluorescein-labeled goat anti-human IgG (1:8) was used for fluorescent staining throughout. All photographs were taken on Kodak Tri-X 135 black-and-white film at 30-sec exposures.

- Fig. 1. A, unfixed viable human melanoma cells showing full-surface speckled fluorescence after incubation with postimmune RL antimalanoma plasma and fluorescent staining. b, phase-contrast image of a. × 400.
- Fig. 2. a, unfixed viable human melanoma cells showing two-thirds capping of antigen-antibody complexes after incubation with postimmune RL antimalanoma plasma and fluorescent staining. b, phase-contrast image of a. × 400.
- Fig. 3. a, unfixed viable human melanoma cells showing one-fourth capping of antigen-antibody complexes after incubation with postimmune RL antimalanoma plasma and fluorescent staining. b, phase-contrast image of a. × 400.
- Fig. 4. a, unfixed viable human melanoma cells showing polarization of antigen-antibody complexes after incubation with postimmune RL antimalanoma plasma and fluorescent staining. b, phase-contrast image of a. × 400.
- Fig. 5. a, unfixed viable human melanoma cells showing extrusion of antigen-antibody complexes after incubation with postimmune RL antimalanoma plasma and fluorescent staining. b, phase-contrast image of a. × 400.
- Fig. 6. a, unfixed viable human melanoma cells showing no fluorescence after incubation with postimmune RL antimalanoma plasma and fluorescent staining. b, phase-contrast image of a. × 400.
- Fig. 7. a, unfixed viable human melanoma cells showing no fluorescence after incubation with preimmune RL plasma and fluorescent staining. b, phase-contrast image of a. × 400.
- Fig. 8. Methanol-fixed human melanoma cells showing full-surface fluorescence after incubation with postimmune RL antimalanoma plasma and fluorescent staining. × 400.

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