Immunofluorescent Detection of Common Melanoma Membrane Antigens by Sera of Melanoma Patients Immunized against Autologous or Allogeneic Cultured Melanoma Cells

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SUMMARY

Five melanoma patients were immunized against their autologous irradiated melanoma cells plus Bacillus Calmette-Guérin. Four of them developed membrane fluorescent antibody to five of seven melanoma cell lines but not to human skin fibroblasts, human kidney, monkey kidney, WI-38, HeLa, and leukemic T-lymphocyte cell lines. The highest antibody titer was 1/128. Antibody activity was completely absorbed by positive melanoma lines but not by negative melanoma lines, nonmelanoma lines, Bacillus Calmette-Guérin, or culture medium containing fetal calf serum. Five other melanoma patients were immunized against allogeneic melanoma cell lines plus Bacillus Calmette-Guérin. All developed membrane-reacting antibody to the five positive melanoma lines described above at titers up to 1/32. Two of the nine positive postimmune sera were tested in an autologous system in which they were reactive to their own melanoma cells but not to their skin fibroblasts. One of three primary melanoma imprints was positive for fluorescence, but three of three metastatic imprints were negative. The predominant antibody was immunoglobulin G, and it fixed C3. With viable melanoma cells, sequential capping, polarization, and extrusion of antigen-antibody complexes could be seen after incubation with postimmune antimalanoma sera.

Negative sera included those from preimmune melanoma patients, 24 nonimmunized melanoma patients, 27 nonmelanoma cancer patients; and 110 of 112 noncancer patients. In the last group of patients two sera were positive, one at a dilution of 1/2 and the other at 1/4. The immunofluorescent data presented in this study strongly suggest the presence of common melanoma membrane antigens on cultured human melanoma cells.

INTRODUCTION

The presence of common human malignant melanoma antigens has been controversial. Common melanoma antigens have been observed on the plasma membrane (3-4, 17), in the cytoplasm (11, 17-20), and in the nucleolus (14). It is generally assumed that tumor membrane antigens are the probable target for specific cellular or humoral immunotherapy. In this study, an attempt has been made to demonstrate the presence of common melanoma membrane antigens by immunofluorescence on cultured human melanoma cells and tumor imprints from melanoma biopsies. The antisera were prepared from melanoma patients immunized against autologous or allogeneic irradiated cultured melanoma cells mixed with BCG.

MATERIALS AND METHODS

Culture Cell Lines

Melanoma Cell Lines. Seven established human malignant melanoma cell lines maintained in tissue culture were used in this study: TU-M (Tulane University-melanoma)-BG (passage 22, 23, and 24); TU-M-BW (passage 9) (7); TU-M CY (passage 8); TU-M-FP (passages 6, 8, 11, and 13); TU-M-MR (passage 15); TU-M-PK (passages 29 to 30, 32, 33 to 34, and 35); and TU-M-PU (passage 1). These cell lines were established by mincing fresh surgical specimens and were grown as monolayers in plastic bottles (Falcon Plastics; 75-sq cm 250-mi flasks) with RPMI Medium 1640 containing 10 to 20% FCS and antibiotics (penicillin, 100 units/ml, and streptomycin, 100 μg/ml) at 37°. The plastic caps were screwed on tightly during incubation.

Skin Fibroblasts of Melanoma Patients. TU-SF (Tulane University-skin fibroblasts)-BG (passage 4 to 5) and TU-SF FP (passage 2 to 4) were used in this study. They were established and grown as the melanoma cell lines.

Other Cell Lines. Human kidney, monkey kidney WI-38, and HeLa cell lines were obtained commercially from Flow Laboratories (Rockville, Md.). Molt cells (T-cells from acute lymphocytic leukemia, passages 3 and 4) were grown in suspension with the same media as for melanoma cell lines. The commercially obtained cell lines were not kept in the same incubator as were the cultured cell lines.

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Immunization of Patients

Melanoma Patients

**Autologous Immunization.** Five patients with either Stage II or III malignant melanoma that could be resected free of all gross disease were immunized with their autologous irradiated cultured melanoma cells mixed with BCG. Approximately 1 to 4 x 10^8 irradiated (2 to 7 x 10^8 rads) cells suspended in 0.5 ml of RPMI 1640 plus Glaxo BCG (1 to 3 x 10^6 viable organisms) were injected intradermally at approximately 2-week intervals (Chart 1).

**Allogeneic Immunization.** Five patients with Stage I, II, or III malignant melanoma that could be resected free of all gross disease were immunized with irradiated allogeneic cultured melanoma cells. Cell lines used were TU-M-BG, TU-M-FP, TU-M-MR, and TU-M-PK. TU-M-PK was used only on 3 occasions during allogeneic immunization of Patient C. N. Doses and schedules were similar to those in autologous immunization (Chart 2).

Melanoma cells for autologous or allogeneic immunization were trypsinized (0.25%) prior to irradiation.

**BCG Immunotherapy**

Six patients undergoing BCG immunotherapy were included. Their diagnosis, routes, intervals, and doses of injections are summarized in Table 1.

**Immunofluorescent Studies**

Cultured melanoma cells were harvested mechanically with a rubber policeman. The cells were then washed 3 times in PBS. The cells were resuspended in PBS, and 1 droplet was pipetted onto a glass slide to give a final cell count of 25 to 35 per high field (x400). The droplet was allowed to dry and was then fixed with 95% methanol. One drop of appropriate serum or plasma was applied onto the cell smear and incubated in a moist chamber at room temperature. Sera or plasmas were diluted out by PBS. The slides were placed in a glass container and thoroughly washed in PBS, 3 times with each washing of 5 to 10 min. The cell smear was dried and incubated with 1 drop of fluorescent reagent at room temperature for 30 min. Fluorescein isothiocyanate-conjugated FGAH IgG, IgM, and Complement 3 at a dilution of 1/8 (Hyland Laboratories, Costa Mesa, Calif.) were used. After thorough wash of the slides in PBS as previously described, they were mounted with 90% glycerol and observed for fluorescence with a Leitz Wetzler fluorescent microscope (Osram HBO mercury lamp).
Table 1

<table>
<thead>
<tr>
<th>Name</th>
<th>Diagnosis</th>
<th>Type of BCG and route of injection</th>
<th>Interval</th>
<th>Total BCG dose</th>
<th>Fluorescence on positive melanoma cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>V. D.</td>
<td>Malignant melanoma</td>
<td>Glaxo, intralesional</td>
<td>Day 1, 36, 57</td>
<td>2.2-8.8 x 10⁴</td>
<td>–</td>
</tr>
<tr>
<td>J. D.</td>
<td>Malignant melanoma</td>
<td>Glaxo, intralesional</td>
<td>Day 1, 8, 28, 37, 56, 64</td>
<td>4.5-18 x 10⁴</td>
<td>–</td>
</tr>
<tr>
<td>B. M.</td>
<td>Malignant melanoma</td>
<td>Tice, tine</td>
<td>Day 1, 120, 131</td>
<td>0.9-3.6 x 10⁴</td>
<td>–</td>
</tr>
<tr>
<td>N. R.</td>
<td>Malignant melanoma</td>
<td>Glaxo, intralesional</td>
<td>7/70 (10 lesions 1.6 to 6.4 x 10⁶ injected)</td>
<td>1.6-6.4 x 10⁴</td>
<td>–</td>
</tr>
<tr>
<td>I. R.</td>
<td>Malignant melanoma</td>
<td>Glaxo, intralesional</td>
<td>Day 1, 8, 14, 56, 70</td>
<td>1.25-5 x 10⁴</td>
<td>–</td>
</tr>
<tr>
<td>A. C.</td>
<td>Squamous cell of hypopharynx</td>
<td>Glaxo, tine</td>
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<td></td>
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Lamp, 200 watts, UG-1 filter, and K430 barrier filter). Two clear-cut patterns were seen: (a) bright membrane fluorescence present in greater than 30 to 50% of the cell population designated by + to 2+; and (b) no fluorescence in greater than 95% of the cell population designated by a minus (−). When NaCl, 8.8 g in 1000 ml distilled water, was used instead of PBS, the same results were obtained.

Demonstration of Complement-fixing Antibodies by Immunofluorescence

The double-sandwich method was used. Test sera or plasmas were heated at 56° for 30 min and used in complement-fixing experiments. After initial antigen-antibody reaction was completed as described under indirect immunofluorescent studies, nonheated normal human sera were applied as source of complement for 30 min at room temperature. The slides were washed, and FGAH C reagent was then applied. Reading criteria were the same as those listed under indirect immunofluorescent studies.

Membrane Immunofluorescent Studies

The technique of Möller (16) was used with the modification that living cells were incubated with appropriate sera or plasmas, washed, and fixed with methanol on the slide before the fluorescent reagent was applied.

Sera or Plasmas Tested

All preimmune and postimmune sera or plasmas were tested. In addition, sera from patients undergoing BCG immunotherapy were tested. Control sera were obtained from melanoma patients not undergoing immunotherapy, patients with other cancers not undergoing immunotherapy, random hospital patients, and blood bank donors.

Trypsinization of Melanoma Cells

The monolayers of melanoma cells were dispersed by means of trypsinization (3 to 5 ml of 0.25% trypsin in RPMI Medium 1640 for 1 to 5 min). The cells were then washed with PBS and prepared for immunofluorescent studies.

Absorption Studies

Positive sera or plasmas were absorbed with different cell lines, BCG, culture medium, and FCS by incubating equal volumes of sera or plasma with the appropriate absorbent for 1 hr at room temperature and 23 hr at 4°. Nontrypsinized cells were used for absorption studies.

Tumor Imprint Studies

Fresh melanoma nodules removed from surgery were cut into 4 × 4-mm pieces. Several monolayer tumor imprints were applied onto a slide. The imprints were fixed with methanol, and an indirect immunofluorescent technique was performed using 1 positive postimmune autologous antimalanoma serum. Three primary melanoma nodules and 3 metastatic melanoma nodules were tested.

Immunodiffusion

With the use of immunodiffusion plates (Hyland), Ouchterlony diffusion was set up between positive sera at dilutions of 1/2 and 1/4 versus RPMI Medium 1640 containing 20% FCS and against antibiotics (penicillin and streptomycin).

RESULTS

Indirect Immunofluorescent Studies

Antibody Titers of Patients during Their Courses of Autologous or Allogeneic Immunization. Both IgG and IgM antibody titers were followed during the course of autologous or allogeneic immunization in 10 melanoma patients. The results are shown in Charts 1 and 2. The predominant
S. P. L. Leong et al.

antibody was lgG. The highest postautoimmune lgG titer of 1/64 was that of RL in reference to TU-M-PK melanoma line. The highest postalloimmune lgG titer of 1/32 was that of FP in reference to the same melanoma line. All preimmune sera or plasmas were negative for fluorescence (Fig. 1). In general, repeated immunizations were required to raise antibody titers and their drop was usually associated with cessation of immunization or progression of disease. A clinical correlation with the antibody titers will be discussed in depth in another paper.6

Positive Melanoma Cell Lines. Four of 5 autologous postimmune sera were reactive to 5 of 7 melanoma cell lines at the membrane (Fig. 2). The 5 positive melanoma cell lines were as follows: TU-M-BG (passage 22 to 24); TU-M-BW (passage 9); TU-M-FP (passages 6, 8, 11, and 13); TU-M-MR (passage 15); and TU-M-PK (passages 29 to 30, and 32 to 35). Five of 5 allogeneic postimmune sera were positive to the same 5 melanoma cell lines as shown above. All 9 positive postimmune antisera were positive to irradiated melanoma cell lines: Tu-M-BG (passage 24) and TU-M-MR (passage 15).

Negative Cell Lines. Two of 7 melanoma cell lines, namely, Tu-M-CY (passage 8) and TU-M-PU (passage 1), were negative when they were tested with the previously mentioned 9 positive postimmune melanoma antisera. Other negative cell lines tested were: 2 skin fibroblasts, TU-SF-BG (passages 4 to 5) and TU-SF-FP (passage 2 to 4) (Fig. 3); WI-38; HeLa cell lines; human kidney cell line; monkey kidney cell line and Molt cell lines (leukemic T cells, passages 3 and 4).

Two Melanoma Patients: An Autologous System. Both BG and FP postimmune sera were positive to their autologous melanoma cell lines (Fig. 2) but not to their own skin fibroblasts (Fig. 3). In addition, their skin fibroblasts could not absorb the antibody activity. Both TU-M-BG and TU-M-FP cell lines would absorb the antibody activity of BG and FP sera (Table 2).

Membrane Immunofluorescent Studies

When viable melanoma cells were incubated with postimmune antimelanoma sera, sequential capping, polarization, and extrusion of antigen-antibody complexes could be seen (Fig. 4). These results have been published elsewhere (10).

Effect of Trypsin on Melanoma Membrane Antigens

Trypsinized melanoma cells showed patchy or dotty membrane fluorescence after incubation with postimmune autologous antimelanoma sera (Fig. 5).

Absorption Studies

Results are summarized in Table 2. A dilution study with PBS as well as culture medium (RPMI 1640) containing streptomycin, penicillin, and 100% FCS showed no significant difference in antibody titers.

Type of Antibodies

Results of monospecific staining by FGAH IgG, IgM, and Complement 3 are shown in Table 3. Results of the double-sandwich method to show Complement 3-fixing antibodies are also included.

Seroepidemiological Studies

Sera from nonimmunized patients or blood bank donors were tested against positive melanoma cells. The immunofluorescent results are shown in Table 4.

Immunodiffusion Experiments

Repeated immunodiffusion studies showed no precipitant cell lines between positive postimmune antimelanoma sera versus the fresh culture medium of RPMI Medium 1640 plus 20% FCS plus penicillin and streptomycin.

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Table 3

<table>
<thead>
<tr>
<th>Type of antibody reactive to melanoma membrane antigens as determined by monospecific fluorescent staining</th>
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<tbody>
<tr>
<td>Postimmune antimalanoma sera (1/6)</td>
</tr>
<tr>
<td>------------------------------------</td>
</tr>
<tr>
<td>IgG⁺</td>
</tr>
</tbody>
</table>

* Stained by fluorescent goat anti-human IgG, IgM, and C₃, respectively.
† Double-sandwich method: heat-inactivated antisera + melanoma cells + normal fresh serum + fluorescent goat anti-human C₃.

** Tumor Imprint Studies

One of 3 primary melanoma biopsy imprints showed bright membrane fluorescence (Fig. 6). Two of 3 such biopsy imprints showed no fluorescence. Three of 3 metastatic melanoma biopsies showed no fluorescence (Fig. 7).

DISCUSSION

The immunofluorescence data in this study showed that 5 of 7 melanoma cultured cells were positive for membrane fluorescence (Fig. 2). The antibody activity was absorbed by positive melanoma cells but not other negative cell lines. Furthermore, 2 melanoma cell lines, obtained from Dr. S. K. Liao (Hamilton, Ontario, Canada), was positive for membrane fluorescence when tested against the same antisera (unpublished data). These findings strongly suggest the presence of common melanoma membrane antigens on most if not all of the cultured melanoma cells, and they are consistent with previous monkey (13, 15) and chimpanzee immunization studies (9, 21) as well as with other immunofluorescent studies (4, 17). Further evidence that these melanoma antigens were located on the membrane was unequivocally demonstrated when viable melanoma cells were incubated with specific human antimelanoma sera (10). Sequential steps of capping, polarization, and extrusion of antigen-antibody complexes could be vividly seen (Fig. 4). Also, when viable cells were treated with trypsin and then fixed, patchy fluorescence could be seen (Fig. 5), indicating that trypsin had digested portions of membrane antigens.

Melanoma cells used for immunization were trypsinized. However, nontrypsinized cells were used for immunofluorescent and absorption studies. Although trypsinization resulted in partial digestion of melanoma membrane antigens (Fig. 5), the antisera developed after immunization with trypsinized cells were able to bind with the antigens on nontrypsinized cells. Thus, the active melanoma antigenic determinants were apparently retained after trypsinization.

Two of 7 melanoma cell lines in this study were negative for fluorescence. The reasons may be 2-fold. First, the number of antigenic determinants on these 2 cell lines could be fewer than the others so that their presence was not detected by immunofluorescence. Recently, we have tested 5 established human melanoma lines from different laboratories in the United States and Canada. Three of them were negative for membrane fluorescence when the postautimmune antimelanoma sera were used. However, when microcomplement fixation assay was used, all 5 melanoma lines were found to bind with the antisera at titers up to 1/2500 with subsequent fixation of guinea pig complement. Appropriate controls showed no complement fixation, indicating that these melanoma cells did not fix complement spontaneously. Other negative lines included WI-38, VA13 (transformed WI-38 with SV40), leukemic B-cell, HeLa, lung adenocarcinoma, and prostatic carcinoma cell lines. The negative melanoma lines in this study, namely, TU-M-CY and TU-M-PU, have not been tested by microcomplement fixation assay because they have been lost at this time. Second, these 2 negative melanoma lines could have been in a nonantigenic phase of their cell cycle when they were tested for membrane fluorescence in this study. We have preliminary evidence from synchronizing experiments to show that melanoma membrane antigens are phase dependent (unpublished data).

Bright membrane fluorescence could be seen in 1 of 3 primary melanoma tumor imprints (Fig. 6). However, 3 of 3 metastatic melanoma imprints were negative for fluorescence (Fig. 7) by both direct and indirect methods.

Since the tumor cells were grown in cultured medium and the patients were immunized with irradiated tumor cells plus BCG, one might argue whether the antibody activity was directed against the medium and BCG. BCG, culture medium containing 20% FCS, and 100% FCS could not absorb the antibody. In addition, no antibody activity was demonstrated in patients on BCG immunotherapy (Table 1). Kerbel and Blakeslee (6) pointed out that FCS adsorbed on cell surface could be a source of potentially serious misinterpretations in immunological studies of cell-associated antigens with antisera produced by the injection

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of cells grown in FCS-containing cultures. FCS could be ruled out to be an antigenic source in this study because: (a) culture medium containing 20% FCS or 100% FCS alone could not absorb the fluorescent antibody activity; (b) Negative melanoma and nonmelanoma cell lines were grown in the same medium containing FCS; and (c) antibody activity was retained after absorption with negative cell lines including autologous skin fibroblasts.

Standardization of antigens for antibody titer determination was achieved by using 1 positive melanoma cell line throughout. Thus, different antibody titers from different patients can be compared more meaningfully. TU-M-PK cell line (passages 33 to 35) was chosen because it was growing well and could be obtained in large quantities during this study. Also, it was not used in this immunization protocol except on 3 occasions for Patient C. N. As shown in Charts 1 and 2, the highest antibody titer (RL) versus TU-M-PK passage 34 (Chart 1) is 1/64. When TU-M-FP passage 8 was tested, the RL titer went up to 1/128. Thus, it can be seen that the amount of antigens on melanoma cell membrane vary from one cell line to another. Furthermore, when viable melanoma cells were used the RL titer dropped to 1/32 (10). This drop could be explained by the presence of embedded antigens in the phospholipid bilayer of the melanoma membrane (10). After fixation by methanol the embedded antigens were exposed, thus increasing the antibody titer. From the 2 autologous systems (BG and FP) in which autologous melanoma and fibroblast cells are used (Table 2), it is highly unlikely that the melanoma membrane antigens as demonstrated in this study could be HLA antigens.

Cells in this study were fixed with methanol for 2 to 5 min after drying. The difference between positive and negative fluorescence was clear-cut (Figs. 1 and 2). No cytoplasmic or nuclear fluorescence was seen. In contrast, Morton et al. (17), Muna et al. (18), Oettgen et al. (19), and Romsdahl et al. (20) used acetone, whereas Lewis et al. (11) used isopentane to fix their cells; these authors were able to show cytoplasmic fluorescence. Using frozen sections of melanoma tissue and fixing with acetone, McBride et al. (14) demonstrated antinuclear antibody in melanoma patients. To complicate the issue further, Whitehouse (22) reported antibodies to cytoplasmic microtubules in these patients. No attempt was made in this study to investigate the difference between these fixatives with regard to fluorescent patterns. Further studies will be pursued to answer the question of whether different fixatives would yield different patterns of fluorescence.

Recently, Laprevotte et al. (8) reported essentially negative immunofluorescence results of sarcoma sera versus fixed sarcoma cells using different antigenic preparation techniques as well as fixatives. Their results were consistent with ours in that all our preimmune melanoma sera or plasmas were negative for fluorescent antibodies.

Reports on the presence of fluorescent autoantibodies in the sera of nonimmunized melanoma patients by several authors (4, 11, 14, 17–20) seem to contradict some of our data with sera from unimmunized patients. Although different fixatives were used in these studies, our findings raise the question about the apparent failure of melanoma patients to develop melanoma membrane antibodies until immunized with autologous or allogeneic irradiated cells. One possible explanation is the presence of circulating antigen-antibody complexes that could cause specific immunological paralysis. When the tumor was removed and the melanoma patients were immunized with nongrowing tumor cells, this type of immunological paralysis was abolished, and the production of antimelanoma antibody resulted. Whatever the reason, other investigators (5) have also reported the appearance of antimelanoma antibody in melanoma patients immunized against their autologous cells when no antibodies were detectable prior to immunization.

The humoral response in the patients was elicited only after multiple immunization as shown in Charts 1 and 2, suggesting that the melanoma membrane antigens are weakly antigenic. Of the 10 melanoma patients studied, 1 patient showed no humoral responses after repeated immunizations (Chart 1). This lack of response could be explained by fewer immunization doses for this patient than for other patients.

Autoimmunization study by Ikonopisov et al. (5) showed that melanoma autoantibody activity by membrane immunofluorescence could be demonstrated within 1 week after initial immunization and would disappear after 14 days. From 1 to 4 x 10⁶ cells were used in each injection in our study, whereas Ikonopisov et al. used 60 to 500 x 10⁶ in each injection and the patients were immunized only on 1 or several occasions. No definitive documentation of cross-reactivity of membrane fluorescence was made in their study. In another study (11), 2 kinds of antibodies were described. One acting against the cell membrane was specific and cytotoxic to each tumor; the other was reactive to cytoplasm antigens, which appeared to be present in most or all melanoma patients.

From the above-mentioned autoimmunization study by Ikonopisov et al. (5), no apparent effect on the course of the disease in their patients following autoimmunization was noted. Exact clinical correlation with the antibody titers as presented in Charts 1 and 2 will be discussed in another paper. Although follow-ups of the melanoma patients are still short, in general, high titers were associated with no evidence of progression of disease. Titers usually dropped after immunization was discontinued or when the disease progressed. The exact role of these antibodies has not been determined. Since the antibodies were predominantly IgG and were found to fix complement, they may be cytotoxic, either by themselves or in collaboration with cellular effectors (antibody-dependent cellular cytotoxicity). They may prevent the formation of blood-born metastases (2, 12). Alexander and Hall (1) showed that formation of blood-born metastases was achieved after the removal of tumor-specific antibody in rats with primary chemically induced fibrosarcoma. Since the postimmune antimelanoma sera are able to detect melanoma membrane antigens, they may be used to isolate these antigens by current immunobiological methods. Their isolation should facilitate the development of a radio- or enzymoimmunoadsorbs, which would permit sensitive and quantitative measurement of antigen levels in different stages of the disease.
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REFERENCES


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. Methanol-fixed human malignant melanoma cells showing no fluorescence after incubation with preimmune autologous melanoma serum and fluorescent staining. x 400.

Fig. 2. Methanol-fixed human malignant melanoma cells showing full-surface fluorescence after incubation with postimmune autologous antimalanoma serum and fluorescent staining. x 400.

Fig. 3. Methanol-fixed human skin fibroblast showing no fluorescence after incubation with postimmune autologous antimalanoma serum and fluorescent staining. x 600.

Fig. 4. Human malignant melanoma cells showing extrusion of antigen-antibody complexes after incubation with postimmune autologous antimalanoma serum, methanol fixation, and fluorescent staining. x 600.

Fig. 5. Methanol-fixed human malignant melanoma cells with prior trypsin treatment showing dotty or patchy fluorescence after incubation with postimmune autologous antimalanoma serum and fluorescent staining. x 400.

Fig. 6. Methanol-fixed tumor imprint from a primary malignant melanoma biopsy showing bright membrane fluorescence after incubation with postimmune antimalanoma serum and fluorescent staining. x 400.

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