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# REPORT OF THE IMMUNOLOGY WORKING PARTY OF THE EORTC MELANOMA GROUP

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Although patients with metastatic malignant melanoma rarely respond to conventional therapy, melanoma is one of the tumors which has a high incidence of spontaneous regression of the primary tumor or of metastatic lesions. These two observations explain the interest of experimentalists and clinicians in this tumor, especially in its immunology and the possibility of immunotherapeutic control of it. Interests in these matters among members of the EORTC Melanoma Group lead to the formation of an Immunology Subgroup. Since its foundation at a meeting in Amsterdam in May 1977, investigators from 10 laboratories (Appendix 1) have participated in the Subgroup's activities. Clinicians of eight other centers listed in the footnote of Appendix 1 have assisted the Subgroup in donating sera.

The goal of the working party is to facilitate research on the determination and characterization of melanoma associated membrane antigens by interlaboratory cooperation.

Initially seven laboratories tested six monkey antisera against human melanoma and two human sera from melanoma patients against seven human melanoma cell lines distributed by one laboratory, as well as on various other cell lines, available locally to each laboratory. Despite extensive absorption with non-melanoma cells, and in some cases cross-absorption with melanoma cells, none of the sera were found to contain melanoma specific antibodies. In particular the sera reacted with a number of non-malignant cell lines when examined by the immunofluorescence test and the mixed-hemadsorption test. With complement-dependent techniques (immune-adherence and cytotoxicity) the reactions seemed to be more specific although showed no absolute specificity. Though there were recognizable parallels in results from different laboratories, for instance the strongest reactions obtained were generally between antisera and the homologous cells that had been used for immunization, there were still considerable quantitative and even occasional qualitative differences between the results from different laboratories. Membrane immunofluorescence and mixed-hemadsorption appeared to be more sensitive techniques, giving the highest titers and the highest incidence of positive results. With immune-adherence and cytotoxicity as measured with $^{51}Cr$ release the results were less often positive, but in general correlated with the strength of immunofluorescence positivity. Complement-dependent cytotoxicity was the least sensitive test but provided the most tumor-specific results. Interpretation of the results obtained with antibody dependent cellular cytotoxicity (ADCC) was difficult, mainly due to strong prozone effects. The ADCC results were not congruent with any of the other techniques. The four laboratories applying immunofluorescence tests had quantitatively different results, although they tended to detect the same patterns of serum-target cell interaction.

Since some of the differences in results could possibly be attributed to differences in the conditions of the target cells at the time of testing, in spite of their recent origin from a single culture, a workshop was held involving three laboratories employing identical target cells simultaneously. At this workshop the immunofluorescence, immune-adherence, complement-dependent detachment and the mixed-hemadsorption techniques were compared. Eight standard antisera were used, some of which had been extensively absorbed, including absorption with cross-reacting melanoma cells. Despite these measures, melanoma specificity was not clearly demonstrated and the sera still reacted with many types of cells other than melanoma. Some sera reacted preferentially with the cell line used for immunization but also cross-reacted to a lesser degree with other melanoma cell lines. This was particularly seen with the immune-adherence technique.

A second workshop in which four laboratories participated led to the adoption of a standard technique in which the immunofluorescence test was directly applied to the viable adherent cells of monolayer cultures grown on microscope slides (see Appendix 2). Agreement was reached on the description of various patterns of membrane fluorescence.

A further cooperative study tested 30 human sera, derived from 28 patients with "interesting" melanocytic conditions and one healthy control. Two samples from a patient with spontaneous regression of multiple skin metastases were included. The other serum donors were 12 patients with vitiligo, three patients with Sutton's naevi, five patients with depigmentation after successful therapy of melanoma, three patients with spontaneous regression of melanomas and five other patients with malignant melanoma. Eight laboratories tested these sera by the following techniques: membrane binding of IgG detected by radiolabeled staphylococcus protein A (Prot. A), immune-adherence, immunofluorescence on melanoma monolayers and on cells in suspension, ADCC and inhibition of horse erythrocyte agglutination by a ganglioside. On this occasion it was not possible to provide uniform target cells. Eight sera reacted detectably in a number of tests with various melanoma cell lines, but all reacted additionally with lines from tumors other than melanoma and/or with normal cell lines. The most reactive sera were from three vitiligo patients, two Sutton naevi patients, two patients with possible spontaneous regressions, and one melanoma patient shortly after perfusion chemotherapy. The last patient had poly-specific HLA antibodies. None of the sera reacted exclusively with melanoma cells, and the reactions were weak in absolute terms. In view of the failure to demonstrate melanoma specificity in highly absorbed xeno-antisera and patient-derived sera, it was felt that for the time being further studies with human sera should be limited to highly specific situations such as patients with spontaneous regression, or regressing Sutton naevi.

Monoclonal antibodies seemed to be a more promising approach to the characterization of melanoma associated antigens. A workshop was therefore held in which six laboratories participated, and in which 15 monoclonal antibodies were tested on three melanoma and three non-melanoma cell lines

using radiolabeled antibody (or Prot. A) binding techniques (5 laboratories) and the immunofluorescence technique (2 laboratories). The results showed that the various modifications of the radiolabeled antibody techniques and the immunofluorescence technique measured essentially the same antibodies, at least when strongly positive antibodies are under test. Only three monoclonal antibodies, which were already known to be predominantly reactive with melanoma cells, were found selectively positive with the three melanoma cell lines.

In the near future the working party hopes to develop a panel of 10-20 monoclonal antibodies with predominant reactivity with melanoma cells. Such a panel will then be used to determine and characterize melanoma-associated antigens on primary tumors and metastases, as well as antigens free or antibody-complexed in the serum or urine of melanoma patients.

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APPENDIX 1

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## APPENDIX 2

The membrane immunofluorescence technique on cultured melanoma cells grown in monolayers.

The membrane immunofluorescence technique on monolayers of melanoma and other cells, as described by The et al. (Ann. N.Y. Acad. Sci. 254:528, 1975) and slightly modified thereafter, is carried out as follows: Tumor cells are cultured in monolayers. When confluent, the cultures are trypsinized, washed and brought into suspension in MEM containing 10% FCS at a concentration of 4 x $10^5$/ml. Amounts of 0.025 ml are seeded in each of 12 wells of Teflon coated microscopic slides which are then kept overnight in a humid box at 37°C in a $CO_2$ incubator. The attached cells are once washed with fresh medium and then fixed with paraformaldehyde (one drop of a 1% solution in RPMI freshly prepared from a 4% stock solution, kept in the dark at 4°C) for 5 minutes. The cells are then washed three times as described above and subsequently incubated with the serum sample under investigation in twofold dilutions starting with 1/10 or 1/20. After 20 minutes incubation at room temperature the slides are washed for 3 x 2 minutes in PBS, then dried with filterpaper in-between the wells and incubated with the conjugate for another 20 minutes. After 3 x 2 minutes washing, fixation occurs in absolute methanol for 10 minutes after which the slides are air-dried and mounted in phosphate-buffered glycerol.

Fixation with paraformaldehyde was shown not to impair the stainability of melanoma cell lines, HeLa cells and fetal fibroblasts by various antisera, such as rabbit anti-β2 microglobulin, rabbit anti-α1 fetoprotein, human anti-HLA and anti-melanoma and monoclonal anti-HLA, anti-Ia like antigen and anti-melanoma-associated antigens.