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Perspectives and Commentaries

Melanoma-associated Antigens: Prospects for Clinical Use

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MALIGNANT melanomas, although less common than most malignancies, account for 1% of all cancers, and have been the subject of numerous new biological and clinical approaches reported in thousands of publications over the last 15 years. Their strong biological aggressiveness when they attain as little as 1.5 mm in thickness, and their persistent resistance to therapy when they spread — 80% failure to chemotherapy — make malignant melanomas one of the biggest challenges to therapy.

A new approach could be the therapeutic use of the immunological reaction of the host.

Why do melanomas interest immunologists? Clinical observations suggest that an immunological control might exist and could be induced by antigenic stimulation by the tumour. Such observations are: (1) occasional spontaneous regression in superficial spreading melanoma (SSM); (2) vitiligo appearing in metastatic melanomas (antibodies to melanocytes); and (3) alternating long-lasting remissions and sudden spread.

Attempts to demonstrate the existence of melanoma-associated antigens were made in the early seventies and were followed by specific immunotherapy trials, using vaccines prepared from melanoma cells. We shall see that no definite evidence of melanoma immunogenicity was ever obtained. However, monoclonal antibodies definitely demonstrate the presence of melanoma-associated antigenic structures at the cell surface.

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We shall consider the question from three different angles: (1) antibodies to melanomas; (2) melanoma antigens; and (3) clinical application.

ANTIBODIES (Ab)

As melanomas may be immunogenic tumours, the production of antibodies to tumour antigens has been examined in three different situations: (1) the existing Ab in patient sera (autologous reaction); (2) the induction of Ab in patients' sera (autologous and homologous reaction); and (3) the induction of Ab in animals (heterologous reactions).

The first report with regards to the existence of anti-melanoma antibodies in melanoma patients' sera appeared at the end of the sixties. Several groups attempted to show that these antibodies were specifically cytolytic for circulating melanoma cells but no such evidence was found.

Another investigation attempted to induce the production of specific antibodies by injecting the patient with his own irradiated melanoma cells (autologous approach). Although antibodies with variable specificity were detected, no clinical response was observed [1].

The same procedure was applied to homologous melanoma cell lines [2], that is, from other patients. An antibody-dependent cellular cytotoxicity (ADCC) test (where cytotoxicity is produced by killer cells and antibodies) showed that 35% of patients' sera lysed the immunizing cells. Unfortunately, no correlation was found between the presence of antibodies and the clinical evolution. In addition, these antibodies also lysed other tumour cells than melanoma and were thus proved to be non-specific. Finally, by testing 104 normal sera, 17% were shown to

contain antibodies non-specifically lytic to melanoma cells.

In order to assess the specificity of antibodies detected in patients' sera — that is, the reaction to autologous tumour — a wide investigation programme was organized by the Immunology Subgroup of the EORTC Melanoma Group. A selection of 53 sera, based on defined clinical data (remissions, vitiligo), were tested for their specificity on melanoma cell lines by means of different techniques, including immunofluorescence, immunoadherence, cell-dependent cytotoxicity, antibody-dependent cellular cytotoxicity and hemadsorption. Only 18 out of 53 sera were found to contain antibodies reacting to melanoma cells, but the reaction was not entirely specific [3].

Other authors [4] suggested the existence of antigen common to melanoma and the foetal brain.

Many research groups also investigated the cellular immunity of melanoma patients. A cytolytic cell-mediated test (CCM) showed that 13 out of 25 patients had lymphocytes cytotoxic to their autologous tumour cells and 31 out of 56 patients had lymphocytes cytotoxic to allogenic melanoma cells. Although no toxicity was found against normal skin fibroblasts, these experiments did not use enough controls [5] and their conclusions were therefore not entirely acceptable.

After immunization of melanoma patients with autologous irradiated tumour cells, cytotoxic lymphocytes were found in five out of 12 patients, but no correlation was observed with the secretion of autologous antibodies. Such cytotoxic lymphocytes were also detected in healthy people [6].

In order to be able to isolate and purify tumour antigens, it is essential to obtain far more antibodies than were found in patients' sera. Therefore many laboratories began to produce heteroantisera, immunizing many different animals with cultured or fresh tumour cells. Rabbits and guinea pigs were used first, but many groups immunized monkeys and especially chimpanzees for obvious reasons.

The problem which then arose was whether or not to purify the possibly specific antibodies out of animals' sera, by performing successive cellular or tissular absorptions. In order to attest the specificity of these purified preparations, they were tested either on melanoma cell lines or for the detection of circulating antigens in patients' sera. However, attempts to compare reactivities from such a large panel of antibodies was found difficult.

For instance, Carrel's group [2] checked a monkey antiserum on melanoma cell lines using three different detection methods: complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC) and mixed hemadsorption (MHA). They concluded that under identical conditions MHA seemed to be the most sensitive method, but that each method detected different antigens.

The major difficulty in interpreting such immunological screening comes from the polyclonality of the heteroantisera, which are directed against several different antigenic determinants.

The advent of monoclonal antibodies (Mabs) provided a new and valuable tool in the field of tumour immunology.

The first experimental step was to define the monoclonal antibodies' specificity using an assortment of melanomas, other tumours and non-tumour cell lines.

To the best of our knowledge, no monoclonal antibody recognizing an antigen exclusively specific of the surface of tumour cells has yet been obtained. However, some of these Mabs are directed against structures preferentially expressed on the membrane of melanoma cells. Moreover, some of them show cross-reactivity with glioma and neuroblastoma lines. Reciprocally, a monoclonal antibody directed against glioma cells cross-reacted with melanoma and neuroblastoma lines [7]. This clearly demonstrates the existence of differentiation antigens of neuroectodermal origin that are common to these tumours.

The comparative analysis of melanoma cell lines with a panel of anti-melanoma monoclonal antibodies recognizing different membrane antigens has shown great heterogeneity between the different cell lines and within individual cell populations [8]. The RIA demonstrated large quantitative differences between positive lines; on autoradiography the maximal labelling with one monoclonal antibody was 50% [8]. Similar heterogeneity has been found in the *in vivo* situation, when frozen sections of melanoma were incubated in the presence of a panel of anti-melanoma monoclonal antibodies [2].

ANTIGENS

Since monoclonal antibodies directed against melanomas can recognize membrane antigens, they represent a powerful tool for isolating and purifying tumour-associated antigens.

The anti-melanoma monoclonal antibodies currently produced have been elicited from different immunizing cell lines, selected by different techniques. They seem to identify a great variety of antigens. Although the immunizing material consists of melanoma cells from long-

term tissue cultures, monoclonal antibodies recognize antigens in tissue sections and sometimes in melanoma cells at early passage levels. This promises few tissue culture artefacts and little antigenic drift.

Despite their great diversity, several antigens have now been defined, their molecular profile characterized and, sometimes, their tissular distribution analysed by immunoperoxidase or immunofluorescence techniques on frozen or paraffin-embedded tissue sections. Only relatively few monoclonal antibodies reacted in this last case, suggesting that most determinants are irreversibly destroyed by fixation. Most of these antigens are membrane-bound glycoproteins, a few being of glycolipid nature.

Until now, as already mentioned, no monoclonal antibody restricted to melanomas has been obtained, and thus no melanoma restricted antigen identified. This is not due to the lack of specificity of monoclonals but the lack of recognizable antigen.

One can classify the melanoma-associated antigens in 4 groups, with respect to their biochemical nature and/or their specificity (Table 1).

High-molecular-weight antigens (HMW antigens) are glycoproteins having a mol. wt of 260 kD or more and they have been isolated and analysed by several groups.

Wilson *et al.* [9] found an HMW melanoma antigen by sulphododecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). A large number of glycosylated polypeptides were revealed (80-280 kD), in addition to a very heterogeneous group of polypeptides distributed over 400 kD in apparent mol. wt. The authors suggested that this complexity in mol. wt was a consequence of a multichain composition of the antigen and could thus reflect combinations of several base units. Indirect immunofluorescence analysis on cryostat thin sections showed the distribution of the different antigens in different tissues: melanomas, naevi and some skin carcinomas. It was not detectable on normal melanocytes and other normal and tumoural tissues. The results obtained by other groups with monoclonal antibodies directed against HMW antigens are similar, and these antigens are neuroectoderm-associated.

Glycoproteins of mol. wt under 150 kD were described by Rümke. Those glycoproteins of mol. wt ranging from 80 to 140 kD [10] were recognized by monoclonal antibodies directed against melanoma. The latter were tested on various frozen thin tissue sections by an immunoperoxidase technique. One group of monoclonal antibodies reacted with the following metastatic melanomes: mammary, lung, brain and bladder tumours. Only foetal tissue showed a slight positivity. Another group of monoclonal antibodies reacted positively with most controls [10].

Nevertheless, it must be pointed out that one monoclonal antibody of this group shows a much more restricted specificity when used on paraffin tissue sections [11].

Khosravi and Liao [12] found a glycoprotein of 87 kD in the spent medium and cell homogenate of cultured human melanoma cells. They also showed data suggesting several cleavages in the antigenic chain. The relationship of this antigen to the others deserves future investigation.

A sialoglycoprotein of 97 kD, structurally related to transferrin, was described by Brown *et al.* [13]. By using monoclonal antibodies to different antigenic determinants on the GP97 molecule, a very sensitive 'double determinant immunoassay' was developed, and extensively used to analyse the presence of GP97 in various culture lines and tissue biopsies.

This antigen is present in most tumours and, in smaller amounts, in most normal tissues; however, some melanomas have about 100 times more antigen GP97 than normal cells.

Pukel *et al.* [14] developed a monoclonal antibody that recognized a glycolipidic melanoma-associated antigen. By comparing the composition and structure of this antigen to the original GD3 by thin-layer chromatography (TLC), the authors showed that it was the same

Table 1. *Melanoma antigens recognized by monoclonal antibodies*

Class	Mol. wt (kD)	Nature	Tissue specificity
1. HMW	>260	glycoproteins	neuroectoderm
2. MMW	<150	glycoproteins	neuroectoderm and endothelial cells
3. GP97	97	sialoglycoproteins	ubiquitous
4. GD3	—	glycolipids	ubiquitous

molecule and that their monoclonal antibodies were directed specifically against the GD3 ganglioside. A wide range of cells and tissue was thus examined for the presence of GD3. Melanomas (cultured cells or tumour tissues) were shown to have GD3 and GM3 as major gangliosides.

Other cells and tissues examined also contained GD3, but usually only in small amounts. Melanomas and a T-cell line were characterized by a simplified ganglioside profile with GD3 and GM3 as major components.

The use of monoclonal antibodies has also revealed that melanomas express major histocompatibility class I (HLA-ABC) and class II antigens (HLA-DR). The presence of HLA-DR is a prominent characteristic of melanomas [15]. Normal cells should not normally express it *in vivo*, except lymphocytes and macrophages.

CLINICAL USE

As the use of neuroectodermal antigens in the clinical application already involves several techniques which are closely interrelated, it represents an almost comprehensive approach. These techniques include the immunohistochemical study of the binding of several Mabs to different melanomas, scintigraphy using radiolabelled Mabs and melanoma drug-targeting i.e. complexes of Mabs with cytotoxic drugs. This development is very recent and only very few published works can be referred to.

Neuroectodermal antigens can help to solve two major clinical problems: (1) the diagnosis of undifferentiated metastases from unknown and primary tumour; and (2) the recognition of individual melanomas by certain monoclonal antibodies and not by others.

It is well known that 4% of disseminated melanomas are diagnosed without any known primary tumour. An anaplastic disseminated tumour of unknown origin can be a disseminated melanoma. Since many metastases are amelanotic, the use of anti-melanoma monoclonal antibodies in the differential diagnosis will become a very useful test as shown by van Duinen *et al.* [11] when large quantities of Mabs will be available for routine clinical use.

The relationship between pre-existing naevus and melanoma has been questioned recently. It seems that only 10% of primary melanomas contain residues of pre-existing naevus and that this relationship has been over-estimated in the past. However, melanomas do not arise *de novo* but rather from precursor lesions. They have been identified as dysplastic naevi [16]. Such skin lesions show evidence of melanocytic proliferation in the epiderm together with atypia. The latter can be either slight, mild or severe and lead to malignant melanoma in a progressively irreversible way. Today, detection of such early precursor lesions lack specific criteria. Anti-melanoma monoclonal antibodies should be useful in this approach.

Technically immunoperoxidase methods have been much improved and adapted to malignant melanomas. The use of the Biotin-Avidin system gives a red reaction product which can be readily differentiated from the melanin pigment [2]. Some anti-melanoma monoclonal antibodies recognize intracytoplasmic antigens whose identification is more pronounced in melanoma [10]. Therefore they can be used on formalin-fixed and paraffin-embedded specimens in retrospective studies. For membrane antigens the use of frozen sections of the tumour is a prerequisite condition.

Most melanoma metastases cannot be detected by common radiology, scanning and scintigraphy. The use of labelled monoclonal antibodies is expected to lead to a new way of detecting small melanoma foci. To date, only large metastases have been found, which would have been detected anyway by classical tests [17].

Methodology for monoclonal antibodies scintigraphy is not fully optimized. Besides the fact that a background radioactivity is found in the vascular compartment, the mouse immunoglobulins are also pinocytosed and taken up in the liver. New approaches using F(ab) are likely to reduce monoclonal antibodies' unspecific sequestration in the reticulo-endothelial system [17].

Since at least four groups of melanoma-associated antigens have been isolated by immune absorption techniques, it seems worthwhile developing RIA for detecting circulating melanoma antigen in patients' sera. The obvious usefulness would be to monitor patients' responses to therapy and recurrences after remission.

A recent paper by Khosravi and Liao [12] clearly shows that this approach is feasible.

The best available chemotherapy regimen does not give more than a 20–25% remission rate in melanoma. In such a resistant tumour, it would seem worthwhile trying to develop 'missiles' made of monoclonal antibodies linked to cytotoxic drugs. This approach has already been attempted by Vennegoor *et al.* [18] using a xenogeneic polyclonal immune serum but was not successful *in vitro*.

Nevertheless, the available tests of immunohistochemistry and immunoscintigraphy should, in the near future, bring predictive results for selective drug targeting in melanoma.

Several difficult problems can be foreseen. Normal neuroectodermal tissues might be damaged. The currently available monoclonal antibodies are of murine origin and therefore would not be appropriate for repeated administration to humans. The efficient concentration of most cytotoxic drugs will need enormous amounts of monoclonal antibodies. In addition, the maximum binding of 50% of cells *in vitro* may predict a limitation for targeting [8].

It can be anticipated that the new generation of monoclonal antibodies will be found by human hybridomas. Promising results were obtained by Warenius *et al.* [19], who hybridized lymph node lymphocytes from a melanoma patient with a plasma cell leukaemia-derived cell line.

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