Induction of IgG Antibodies Directed to a Mr 31,000 Melanoma Antigen in Patients Immunized with Vaccinia Virus Melanoma Oncylates

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ABSTRACT

Pre- and postimmunization sera from eight tumor-free melanoma patients undergoing vaccinia melanoma oncolysate (VMO) therapy were used to investigate the humoral response to antigens from infected and uninfected melanoma cells and from vaccinia virus. Immunodetection on Western blots showed that all patients, in addition to reacting to several other proteins, developed IgG antibodies to a Mr 31,000 protein antigen within 1 month of immunization. This Mr 31,000 antigen is expressed both on VMO and on melanoma metastases in situ, disappears in primary cultures of these metastases, and is absent in extracts from vaccinia virus, from human melanoma cell lines, and from normal melanocytes, suggesting that this Mr 31,000 protein is reexpressed following vaccinia virus infection of human melanoma cells. Periodate treatment of the blotted antigens abolished reactivity of patients' postimmunization sera with the Mr 31,000 antigen, thus showing that this antigen is a glycoprotein and that the relevant epitope is likely to reside on its carbohydrate moiety. These anti-Mr 31,000 IgG antibodies were absent in the sera of VMO-treated patients before immunization, absent in the serum of a normal donor hyperimmunized with vaccinia virus, and absent in normal human sera. In addition, these anti-Mr 31,000 antibodies appeared 1 week after the first VMO injection, remained stable during the treatment, and decreased when the treatment was stopped. Such antibodies can also be demonstrated in sera of melanoma patients bearing metastases but disappeared following resection of their metastases. Thus, in melanoma patients, immunization with VMO induces an antibody response directed against a Mr 31,000 glycoprotein likely to represent a new melanoma antigen. Further identification of this antigen could be of utmost interest for the further development of melanoma vaccines.

INTRODUCTION

Melanomas were among the first human tumors in which immune responses by the host were suspected to play a role in the clinical course of tumor growth. In vitro analyses demonstrated an immune response in patients, and both cell-mediated (1–4) and humoral (1, 5, 6) immune responses to melanoma antigens have been detected. These results provided a rational basis for the use of an active, specific immunotherapy in the treatment of the melanoma. Several groups have thus attempted either to prevent metastasis or to treat metastatic malignant melanomas by immunizing patients with various melanoma antigen preparations to induce an increase in their specific immune response (7–11; for a review, see Refs. 12 and 13).

Vaccination of melanoma patients with membrane preparations of allogeneic cultured melanoma cells infected with vaccinia virus (VMO) has been shown to be efficient with a total absence of toxicity and a great facility of treatment (14–16) and has even been claimed to increase by 20% the survival of treated patients (16, 17). Recent studies have shown that VMO can elicit an immune response to melanoma cell membrane antigens, a response which is only poorly induced by uninfected tumor cell lysates (15, 18). Although antibody production against melanoma cell surface antigens (19) and against melanoma gangliosides (20) has been demonstrated in patients immunized with VMO, the humoral response to melanoma-associated proteins in oncolysates has been little documented. Indeed, it has been reported that immunization of melanoma patients with lysates of virus-infected melanoma cells induces an IgG antibody response against proteins found in the infected lysates (21, 22). However, melanoma protein antigens in the VMO that are clinically relevant still remain to be identified.

We, therefore, tried to identify immunogenic melanoma protein antigens eliciting a specific immune response following VMO therapy, using the Western immunoblotting technique to analyze the response of stage II or recurrent stage II melanoma patients vaccinated with VMO. We demonstrated that repeated immunization of tumor-free melanoma patients with VMO induces the development of IgG antibodies directed to a glycoprotein of Mr 31,000, expressed both on VMO and on melanoma metastases in situ. This antigen is absent from lysates of vaccinia virus, normal melanocytes, and uninfected cultured human melanoma cells. Antibodies directed against the Mr 31,000 glycoprotein may also be detected in sera of melanoma patients with metastases.

MATERIALS AND METHODS

Human Melanoma Cell Lines and Culture. Four established and well-characterized human malignant melanoma cell lines, Mel2, Mel3, Mel4, and Mel B (23, 24), were cultured as confluent monolayers in 5A McCoy's medium (Gibco, Grand Island, NY) supplemented with 10% FCS (Flow, Irvine, UK), 2 mM glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. In some experiments, melanoma cell lines were cultured in the presence of 125 units/ml IFN-γ, according to Hendrix et al. (25), prior to solubilization. Normal adult human melanocytes were cultured without tumor promoters as described elsewhere (26). Cultures were maintained at 37°C in 5% CO₂ and routinely checked for Mycoplasma contamination.

VMO Production. The allogeneic VMO was prepared at the Institut Mérix as previously detailed (15, 16). Briefly, cells from human melanoma cell lines Mel2, Mel3, Mel4, and Mel B were harvested by treatment with 0.25% trypsin-0.02% EDTA (Puck's solution; Gibco), pooled, and infected with vaccinia virus for 20 h at 37°C at a multiplicity of infection of 10 infectious units of virus per melanoma cell in 5A McCoy's medium containing 1% FCS. Then, they were centrifuged at 500 × g for 2 h at 4°C. The cell pellet was sonicated in phosphate-buffered saline at 4°C and again centrifuged. The resulting pellet was discarded, while the supernatant was retained. In the meantime, the supernatant of the first centrifugation was spun at 30,000 × g for 2 h at 4°C, giving a pellet which was combined with the supernatant of the second centrifugation to make the oncolysate by further sonication and homogenization to eliminate intact cells. The oncolysates were adjusted to 2 mg/ml of total protein. They were submitted to controls to ascertain that they are free of adventious agents, such as Mycoplasma, hemadsorbing viruses, and hemagglutinin, by testing them directly and in other cellular systems in eggs and animals. The VMO lots met the standards of general safety and sterility tests as outlined in sections 610–11, 610–12, and 610–30 of the Food and Drug Regulations. The presence of intact melanoma antigens was confirmed with the use of monoclonal antibodies in a radioimmunoassay technique (27). Final VMO were aliquotted and stored at −80°C before injection to patients. As...
control, cell homogenates of uninfected melanoma cells were prepared as above described for VMO from the same pool of melanoma cell lines.

Immunization of Patients with VMO. Patients with stage II or recurrent stage II malignant melanoma, i.e., a Breslow level greater than 1.5 mm depth of invasion and a Clark’s level IV or V, were selected for inclusion in a clinical Phase II study of immunization with VMO. All patients had previously been rendered tumor free by appropriate surgery. They received a boost with small pox vaccine 1 week before initiation of the VMO immunotherapy. VMO (2 mg protein) were injected intradermally, without adjuvant, weekly for 3 months and then bi-monthly for a further 21 months or until relapse (28).

Sera. Sera were sampled from eight melanoma patients undergoing VMO immunotherapy, prior to and at various times following immunization. In addition, sera were obtained from three melanoma patients with lymph node metastasis prior to and following surgery. Sera from healthy donors and serum from one healthy individual hyperimmunized with vaccinia virus were used as controls. All these sera were stored at −80°C until used.

Melanoma Cells from Metastatic Specimens. From three patients with malignant melanoma, we obtained melanoma cells in metastatic lesions. Briefly, lymph nodes were obtained at the time of elective surgical removal, and their surface portion was removed to eliminate fibrotic tissue. After exclusion of the necrotic portions, each of these specimens was immediately mechanically dissociated in 5A McCoy’s medium. The resulting cell suspension was inoculated on culture flasks and maintained in 5A McCoy’s medium. The supernatant was removed by aspiration at least three times over a period of 24 h. The monolayer of melanoma cells was then scraped into a test tube with a rubber policeman and centrifuged at 1600g for 5 min. The supernatant was removed and the melanoma cells were resuspended in 5A McCoy’s medium supplemented with 10% FCS, 2 mM glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. Five days later, monolayers of human melanoma cells were isolated from other cell types, as assessed by the presence of premelanosomes and melanosomes, the absence of staining for leucine aminopeptidase (29), and the absence of staining with monoclonal antibody KL4 and directed against whole epidermal keratin polypeptides (30).

Lymph nodes, taken from healthy individuals shortly after accidental death and with no history of melanoma disease, were used as normal controls.

Preparation of Melanoma Antigenic Extracts. One ml of VMO, placed in a tissue culture plate of which the cover was removed, was irradiated with a UV light source (General Electric, 8W germicidal lamp; n° G1518) at a fixed distance of 14 cm to inactivate vaccinia virus. Then, the cellular proteins were solubilized in cold 10 mM Tris-HCl buffer (pH 7.4) containing 0.15 M NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 0.1 mM EDTA, 200 KIU/ml aprotinin, and 1 mM diisopropyl fluorophosphate and ultracentrifuged at 100,000 × g for 5 min (Airfuge; Beckman, France). Alternatively, melanoma cell lysates were prepared from pooled uninfected melanoma cell lines in the same way as for VMO production and were solubilized as described above in 10 mM Tris-HCl buffer.

UV-irradiated vaccinia virus preparations, individual melanoma cell lines Me12, Mel3, Mel4, and MelB, cultured with or without IFN-γ, normal melanocytes, melanoma cells from metastatic lesions, and normal lymph nodes were processed for protein extraction as described above.

Total protein concentrations of the different antigenic extracts were measured by the Bradford method (31) using a commercial kit and bovine gamma globulin as a standard (Bio-Rad Laboratories, Paris, France). AboIutions of solubilized antigenic extracts with equivalent protein contents were subjected to SDS-polyacrylamide slab gel electrophoresis (30 µg/lane).

Polyacrylamide Slab Gel Electrophoresis. SDS-PAGE was performed under reducing or nonreducing conditions in 20% or 5–15% gradient polyacrylamide gels using the method of Laemmli (32) as previously described (33). Myosin (M, 200,000), Escherichia coli β-galactosidase (M, 116,000), rabbit muscle phosphorylase b (M, 97,000), bovine serum albumin (M, 66,000), egg white ovalbumin (M, 42,000), bovine carbonic anhydrase (M, 31,000), soybean trypsin inhibitor (M, 21,000) were run as molecular weight markers on all slab gels (Amersham, Les Ulis, France).

Western Blot for Identification of Antigens Recognized by Serum Antibodies. Following SDS-PAGE, proteins were electrotransferred from gels to nitrocellulose sheets (Hybond-C; Amersham) according to the methods previously described (34–36) in a Tris-HCl buffer (pH 8.3) containing 0.19 M glycin and 20% methanol (35) using a transblot unit (Bio-Rad) connected to a power supply (Bio-Rad Model 200/2) at 70V for 1 h 30 min with a constant refrigeration (Cryofermostat Ministat; Bioblock Scientific). After transfer, to detect antibody–antigen complexes, the nitrocellulose sheets were washed three times with PBS containing 0.05% (v/v) of Tween 20. The coloration of the reactive bands was observed within 10 min and stopped by an extensive wash with deionized water. The strips were dried overnight between sheets of filter paper and subsequently photographed.

Staining of Proteins on Western Blots. For the staining and the detection of proteins after the Western blots, a commercial kit (Amersham) was used. Briefly, primary and secondary amino groups of the proteins bound to the nitrocellulose were biotinylated with a N-hydroxysuccimide derivative of the biotin containing an hydrophobic spacer arm. After washing to remove all excess of the biotinylation reagent, the nitrocellulose membranes were incubated with a solution of dried milk (5%) to block and prevent further nonspecific binding. The biotinylated proteins were then detected using a streptavidin–biotinylated alkaline phosphatase preformed complex with the detection signal generated by using a combination of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

RESULTS

Detection of Antibodies Specifically Induced by VMO in Treated Melanoma Patients. The VMO proteins on SDS gels followed by Western blots recognized by either preimmunization or postimmunization sera of eight malignant melanoma-bearing patients vaccinated with VMO are shown in Fig. 1. Before immunization with VMO, the serum antibodies of all but one patient bound antigens with molecular weights of 60,000 to 70,000, and in the majority of patients, antigens of molecular weights 170,000, 97,000, and 40,000 (Fig. 1A). Following oncolysate immunization, most patients had developed or increased antibodies to antigens of molecular weights 170,000, 9,000, 60,000 to 70,000 and 40,000. In addition, all immunized patients also developed antibodies to a group of proteins of M, 40,000 to 31,000 (Fig. 1B).

Some of these protein antigens evidenced in VMO by the sera of immunized patients could represent either cellular antigens recognized by naturally occurring antibodies or vaccinia virus antigens (in
the French population, most adult people have been immunized with vaccinia virus). Thus, we reacted sera with VMO antigens from 13 healthy donors and from a normal individual hyperimmunized with vaccinia virus (Fig. 2). Whereas the normal sera had antibodies directed against proteins of Mr, 60,000 to 70,000 and 40,000 (Fig. 2, Lanes 1–3), the serum of the vaccinia virus-hyperimmunized donor reacted with the Mr, 170,000, 97,000, 60,000–70,000, and 40,000 proteins (Fig. 2, Lane 4). None of the normal human sera, or the serum from the normal donor hyperimmune to vaccinia virus, reacted with the Mr, 31,000 protein which was only recognized by the serum of an immunized patient (Fig. 2, Lane 5).

To investigate the possibility that sera from immunized patients may react with FCS antigens, we separated FCS proteins by SDS-gel electrophoresis followed by Western blotting, and we incubated the corresponding blots with immunized patients’ sera. A representative pattern (Fig. 3, Lanes 2–3) shows several bands recognized in the Mr, 60,000 to 70,000 range but no band neighboring Mr, 31,000. Further studies of the reactivity of immunized patients were, therefore, focused towards the antibodies reacting with the Mr, 31,000 protein of the VMO.

To identify the isotypes of these specifically induced antibodies, Western blots of oncolysates were allowed to react with the postimmunization patients’ sera. The Mr, 31,000 protein was then revealed by sheep antisera to human total immunoglobulin or to human IgG or to human IgM. The eight melanoma patients tested in this study developed IgG antibodies against the Mr, 31,000 protein. No IgM antibodies to this protein could be detected, although some IgM antibodies to higher molecular weight antigens ranging from 60,000 and 70,000 were detected (data not shown).

To investigate whether antibodies to the Mr, 31,000 VMO antigen detected in postimmunisation sera react with a protein or carbohydrate epitope, VMO proteins bound to nitrocellulose sheets were subjected to periodate treatment. Drastic periodate oxidation (5 to 10 mM sodium metaperiodate) abolished the recognition of the Mr, 31,000 antigen by postimmunization patients’ sera, whereas the reactivity to the other antigens was unchanged (Fig. 4, Lanes 1–2). In addition, the reactivity of postimmunization patient serum with the Mr, 31,000 antigen was unchanged when VMO proteins were submitted to SDS-PAGE under nonreducing conditions, which indicates that this antigen is a single chain glycoprotein (Fig. 4, Lane 3).

These results indicate that the immunization of malignant melanoma patients with VMO induces the development of IgG antibodies which specifically react with a carbohydrate epitope of a Mr, 31,000 glycoprotein. These antibodies are not detected in sera from normal individuals, in the serum from a vaccinia virus hyperimmunized donor, or in preimmunization patients’ sera.

**Time Course of Antibody Response to the Mr, 31,000 VMO Antigen.** The development of antibodies to the Mr, 31,000 VMO antigen was monitored in sera of four patients undergoing immunization with VMO over a 2-year period. A representative individual response against VMO lysates is illustrated in Fig. 5. Before VMO treatment, no antibody to the Mr, 31,000 antigen could be detected (Fig. 5, Lane 1). During oncolysate treatment, the anti-Mr, 31,000 antibodies appeared as soon as 1 week after the first immunization, then increased after 1 month of vaccination (three injections), remained stable in their titer all along the immunization, and began to

![Fig. 2. Reactivity of normal human sera with VMO proteins. SDS-PAGE was performed in 5–15% gradient polyacrylamide gel under reducing conditions. Bound immunoglobulins were revealed using sheep antibodies to total human immunoglobulins. Lanes 1–3, typical profiles of 3 normal human sera representative of 13 normal sera; Lane 4, human serum hyperimmune to vaccinia virus; Lane 5, serum from immunized patient.](image)

![Fig. 3. Reactivity of postimmunization sera with FCS proteins. SDS-PAGE was performed in 5–15% gradient polyacrylamide gel under reducing conditions. Bound immunoglobulins were revealed using sheep antibodies to total human immunoglobulins. Postimmunization serum from patient Gal. was reacted with VMO antigens (Lane 1), with separated FCS proteins: Lane 2, 30 μg/lane; Lane 3, 100 μg/lane.](image)

![Fig. 4. Detection of antibodies specific for carbohydrate epitopes of VMO antigens in the serum of an immunized patient. SDS-PAGE was performed in 5–15% gradient polyacrylamide gel under reducing (Lanes 1–2) and nonreducing (Lane 3) conditions. Bound immunoglobulins from patient Gal.’s postimmunization serum were revealed using sheep antibodies to total human immunoglobulins. VMO antigens in Lane 2 were treated with 10 mM sodium metaperiodate prior to reaction with patient serum.](image)
It is worthy of note that, although preimmunization sera from tumor-free patients were always devoid of activity to the Mr 31,000 VMO antigen, in the sera of four unimmunized melanoma patients with metastases, antibodies to the Mr 31,000 VMO antigen were detectable (Fig. 6, Lanes 1—4); these antibodies disappeared 3 months after the metastasis resection (Fig. 6, Lanes 5—8).

The Mr 31,000 Glycoprotein Is a Melanoma Antigen. We investigated whether the Mr 31,000 antigen, present in vaccinia virus-infected melanoma cells and specifically recognized by the serum of patients following immunization with VMO, could be associated with either the human melanoma cells or the vaccinia virus. Since the VMO were prepared from a pool of four human melanoma cell lines infected by vaccinia virus, the presence of the Mr 31,000 glycoprotein was searched in antigen extracts of the four uninfected melanoma cell lines, processed either as a pool, similarly to the VMO preparation, or individually. Protein (30 μg) from lysates were analysed by SDS-PAGE and Western blotting. Numerous proteins were solubilized from human melanoma cells infected or not by vaccinia virus (Fig. 7, Lanes 1—2). No difference in the cellular protein profiles was seen between infected and uninfected melanoma cells taken as a pool (Fig. 7, Lanes 1—2) or individually (data not shown). It should be stressed that the VMO antigens detected on the immunoblots by patients postimmunization sera (Fig. 7, Lane 4) are not clearly individualized on the blot by the protein staining procedure (Fig. 7, Lanes 1—2). The Mr 31,000 protein found in oncolysates (Fig. 7, Lane 4) is not detectable in lysates of uninfected human melanoma cells, pooled cells (Fig. 7, Lane 5), or those cells taken individually (Fig. 7, Lanes 7–10), even following IFN-γ pretreatment of the melanoma cells (Fig. 7, Lanes 11–12), as well as in lysates of vaccinia virus (Fig. 7, Lane 6) and of normal melanocytes (Fig. 7, Lane 13).

Since the Mr 31,000 glycoprotein was detected only in VMO lysates and not in extracts of noninfected, cultured melanoma cells and of vaccinia virus, additional experiments were performed, searching for the presence of this antigen in lysates of melanoma cells isolated from three malignant melanoma metastases freshly obtained from surgery. As shown in Fig. 8, the lysates of melanoma cells from metastases in lymph nodes all contained an antigen with an electrophoretic mobility similar to that of the Mr 31,000 seen in VMO (Fig. 8, Lane 1) and reactive with all postimmunization sera of VMO-treated patients (Fig. 8, Lanes 2–4), in contrast to lysates from normal lymph nodes (Fig. 8, Lane 5). In addition, the autologous sera of the patients from whom the metastases were removed were also able to react with a Mr 31,000 antigen present both in lysates of melanoma cells from metastases (Fig. 8, Lanes 6–8) and in VMO lysates (Fig. 6, Lanes 1–4) but not in normal lymph nodes (Fig. 8, Lane 9).

**DISCUSSION**

Newcastle disease virus and vaccinia virus lysates of melanoma cells have been used by several groups in the adjuvant treatment of high risk melanoma patients (12) with demonstrable clinical benefit (15–17). However, in spite of the fact that several reports have shown a positive correlation between the serological response and the clinical response of the immunized patients (18–20), the antigen(s) in the oncolysates capable of inducing a protective response in the patients are far from being fully characterized (21, 22).
In the present study, we demonstrated that specific IgG antibodies, directed against a Mr 31,000 antigen expressed in VMO but absent from noninfected human melanoma cell lines and from vaccinia virus lysates and reacting with a similar protein in melanoma cells isolated from fresh lymph node metastases, can be found in the serum of tumor-free melanoma patients undergoing VMO immunization as a treatment of their tumor.

These antibodies are not detected in sera of tumor-free melanoma patients before VMO immunization, in the sera of 13 healthy donors, or of a normal individual hyperimmunized with vaccinia virus. This antibody response can be detected as soon as 1 week after the beginning of the vaccination, which is reminiscent of a secondary response in a previously sensitized individual and remains stable all along the treatment. These antibodies are naturally present in the sera of patients bearing melanoma metastases but disappear 3 months after surgical removal of the metastasis. In addition, periodate oxidation of VMO antigens bound to nitrocellulose sheets decreased reactivity of postimmunization patients’ sera with the Mr 31,000 band, suggesting that antibodies elicited in the patients during VMO immunization bind to a carbohydrate epitope rather than to an epitope in the amino acid sequence of the Mr 31,000 antigen. All these results enable us to hypothesize that the Mr 31,000 glycoprotein recognized by these antibodies is a melanoma protein induced under vaccinia virus infection of cultured melanoma cells.

We first investigated whether the Mr 31,000 glycoprotein expressed by vaccinia virus-infected melanoma cells was actually associated with melanoma cells or with the vaccinia virus. Since VMO were prepared from four allogeneic melanoma cell lines infected with vaccinia virus, we prepared lysates from the same pool of melanoma cell lines but uninfected and from vaccinia virus alone. These extracts were analyzed by polyacrylamide gel electrophoresis and blotted onto nitrocellulose sheets incubated with sera from VMO-immunized patients. The analysis of the serological response of these patients shows that the Mr 31,000 glycoprotein is undetectable in pooled, uninfected melanoma cells or in vaccinia virus lysates. Since Livingston et al. (38) detailed an interesting difference in the immunogenicity of various cultured melanoma cell lines, we also analyzed the serological response of immunized patients to each of the four individual, uninfected, cultured melanoma cell lines; no reactive band was seen in the Mr 31,000 region. In addition, no band was revealed in this region when the serum from a normal individual hyperimmune to vaccinia virus was reacted with blotted VMO proteins. Thus, the vaccinia virus-infected melanoma cells express a Mr 31,000 melanoma antigen not expressed by the noninfected melanoma cells and not related to the vaccinia virus. Hence, this antigen is likely to represent either a virus-induced or a virus-modified antigen.

FCS proteins are highly immunogenic in man and appear to be responsible for many of the immune responses induced by human tumor vaccines prepared from cultured tumor cells (38, 39). However, we showed here that no FCS proteins of Mr 31,000 was recognized by the patients’ postimmunization sera. Thus, the anti-Mr 31,000 antibodies are not likely to be directed against a FCS protein.

Among the numerous melanoma antigens recognized either by patients’ sera or by monoclonal antibodies, few are in the Mr 30,000 range. Therefore, we looked for the possibility that the Mr 31,000 antigen could be related to major histocompatibility complex class II antigens. No band in the Mr 31,000 region could be recognized by various postimmunization sera in various melanoma cell lines with known high or low expression of HLA-DR. In addition, melanoma cells were grown in culture in the presence of low concentrations (125 units/ml) of IFN-γ to induce and enhance the expression of class II antigens without altering cell proliferation (25, 40). This treatment did not affect the recognition of the Mr 31,000 glycoprotein by sera from melanoma patients and VMO-immunized patients.

Sera from VMO-treated patients, as well as autologous and allogeneic sera from melanoma patients before resection of metastases, also react against a protein with an apparent molecular weight of 31 kilodaltons extracted from melanoma cells isolated from fresh metastases. However, further biochemical properties of the 31-kilodalton antigen should be investigated before further comparison can be done. Nevertheless, the tumor origin of the Mr 31,000 antigen is suggested by the fact that a similar band is recognized both in VMO and in melanoma cells from metastases by sera from tumor bearing patients. Hence, the Mr 31,000 antigen is unlikely to be related to MHC class II antigens and is likely to be a melanoma antigen, expressed in melanoma cell lines only after infection by vaccinia virus and in melanoma cells in situ. At present time, it is unclear how infection by vaccinia virus will lead to the synthesis of the Mr 31,000 antigen in the cell lines used for preparation of VMO. Nevertheless, early after infection, the Mr 31,000 glycoprotein was detectable on melanoma-infected cells.

It is well documented that infection of cells with vaccinia virus results in profound cytopathic effects (41), alterations in membrane permeability (42), and a dramatic decrease in DNA, RNA, and protein synthesis (43–45) of host cells. Thus, we could postulate that the viral infection increases the synthesis of the Mr 31,000 melanoma protein, already expressed by cultured melanoma cells in a low amount that our serological methods cannot detect. The low expression of the Mr 31,000 antigen in cultured melanoma cells, in contrast to its high expression in fresh collected melanoma cells, could be the result of adaptation to culture or long term cultivation. Freshly isolated melanoma cells from metastases which contain the Mr 31,000 glycoprotein do not express any more this antigen after 5 days in a primary culture. This suggests that this antigen, lost in culture, can be reexpressed following viral infection of melanoma cells. Because the recognized epitope is a carbohydrate, the culture conditions might also induce a modification in the glycosylation of this antigen. Among the numerous events associated with the vaccinia virus infection, the production of enzymes by the virus is largely documented (45). Some of these enzymes could have a pronounced effect upon cellular protein phosphorylation and glycosylation in infected melanoma cells. Thus, the Mr 31,000 glycoprotein could also be the result of enzymatic cleavage, representing a virus-modified cellular antigen. At present, we cannot rule out any of these possibilities, suggesting that the one or another applies. Additional in vitro investigations of the mechanisms of action...
of vaccinia virus may improve our understanding of the newly expressed or modified M, 31,000 antigen.

In conclusion, we provide here evidence that the immunization of melanoma patients with viral oncolysates results in the development of a specific antibody response directed against a new and, until now, unreported melanoma antigen, a glycoprotein of M, 31,000; this melanoma antigen is apparently also recognized by the immune system of melanoma-bearing patients. Our results are in agreement with the data of Savage et al. (22) who reported antibody induction in six melanoma patients following 6 weeks immunization with allogeneic melanoma oncolysates prepared from three Newcastle disease virus-infected melanoma cell lines and that most of the elicited antibodies reacted with antigens found in extracts of virus-infected but not in extracts of uninfected tumor cells. Nevertheless, their findings, as well as those of Hersey et al. (21), show a complex pattern of reactivity of these antibodies against infected melanoma cell antigens, with a great diversity in the response from patient to patient, in contrast to ours. Indeed, we clearly showed that one of the induced antibodies is specifically directed against a M, 31,000 antigen present in the lysate of vaccinia virus-infected melanoma cell lines. However, careful examination of the blots presented by Savage et al. (22) shows that two of six patients do actually respond to a band in the oncolysate in the same molecular weight region. No such band could be seen in the blots presented by Hersey et al. (21) who immunized melanoma patients with an oncolysate prepared from a single melanoma cell line. Differences in the cell lines used, in the viruses used for oncolysate preparation, and in the methods used for the solubilization of the antigens from the cells could account for these discrepancies.

The significance of these specific antibodies as mediators of the patients’ immune responses to the tumor has yet to be assessed; and further experiments are needed to investigate whether the M, 31,000 antigen is able to induce a clinically effective immune response in humans and whether a correlation can be drawn between individual antibody titers reached in the patients and their clinical outcome.

There is a general consensus that T-cells are involved in the control of tumor growth, and there is now considerable interest in defining melanoma antigens recognized by T-cells. Using Western blot analysis, Hersey et al. (46) have characterized antigens ranging from M, 43,000 to 50,000 recognized by cytotoxic T-cells. Recently, Notter and Schirrmacher (47) reported that a Mr 24,000 melanoma protein fraction could be able to stimulate T-cell responses for proliferation of CD8+ T-cells. In this context, it would be of interest to determine whether the M, 31,000 glycoprotein could represent a possible target antigen for T-cells.

Further studies are needed to identify the M, 31,000 antigen as a specific melanoma-associated antigen or a normal tissue antigen on melanoma cells. The characterization of this M, 31,000 antigen, against which the host could be able to trigger an immune response, could have an important impact in the formulation of a melanoma vaccine, enhancing immune response of tumor-bearing patients to their own malignant cells.

ACKNOWLEDGMENTS

We are greatly indebted to Dr. A. Weissbrod (Centre Léon Bérard) for providing the patients’ sera used in these studies. We express our special gratitude to patient Gal., who generously provided us with an abundant sample of his own postimmunization serum. The human serum hyperimmune to melanoma-associated antigens defined by murine monoclonal antibodies in allogeneic and xenogeneic hosts. Cancer Res., 47: 5284—5289, 1987.


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