Antibody Development to Viral and Allogeneic Tumor Cell-associated Antigens in Patients with Malignant Melanoma and Ovarian Carcinoma Treated with Lysates of Virus-infected Tumor Cells

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ABSTRACT

Pre- and postimmunization sera from six malignant melanoma and six ovarian carcinoma patients were used to investigate the humoral immune response to antigens expressed in extracts of allogeneic tumor cells and lysates of these same cells infected with virus. Nitrocellulose paper replicas of cell extracts, fractionated by polyacrylamide gel electrophoresis, were used as antigenic targets. Antibodies that bound to tumor cell antigens of defined molecular weight were identified with enzyme-linked probes specific for human immunoglobulins G, A, and M. Prior to therapy, all sera reacted with one or more antigens expressed by the unmodified tumor cells. Postimmunization sera from two malignant melanoma patients and one ovarian carcinoma patient reacted with antigens in extracts of uninfected tumor cells. These same antigens were not detected by preimmunization sera. Most postimmunization antibody responses were directed against antigens associated with the infecting virus itself and antigens found in extracts of virus-infected but not in extracts of uninfected tumor cells. These results suggest that treatment with viruses of virus-infected allogeneic human tumor cells elicits humoral immune responses against: (a) tumor cell-associated antigens; (b) antigens that are specifically virus associated; and (c) antigens that may be virus induced or virus modified cytoplasmic or nuclear antigens.

INTRODUCTION

Observations by Koprowski (1) as well as Lindenmann and Klein (2, 3) demonstrated that lysates of virus-infected tumor cells augmented the immunological response of mice to growing tumors. Lindenmann (4) postulated that these “oncolysates” made weakly immunogenic surface membrane antigen determinants more immunogenic by a process he called “viral adjuvanticity.” Syngeneic animals have been successfully immunized against tumors using membrane extracts obtained from tumor cells in which viruses completed replication and demonstrated surface budding (5). Several clinical investigations of this phenomenon have used various viral oncolysates (6–10).

The rationale for utilizing viral oncolysates in the management of malignant melanoma (6, 8, 11) and gynecological cancers (12, 13) has been reported previously. That these treatments enhance cell-mediated responses to tumors has been suggested (3, 14–18). Enhanced cell-mediated responses have been specifically demonstrated in malignant melanoma (16, 19–21) and gynecological cancers (22, 23).

While cellular responses which have developed during oncolysate therapy have been studied, the humoral response to oncolysates has not been well documented. This paper provides direct evidence in favor of the hypothesis that immunization with viral oncolysates results in the development of novel antibody responses in malignant melanoma and ovarian carcinoma patients to viral and cell-related antigens that were not previously recognized by the immune systems of these patients. The methodologies used effectively discriminate among antibodies developed to antigens which are intrinsically associated with tumor cells and those which are virus associated or virus induced.

MATERIALS AND METHODS

Immunization of Patients and Collection of Sera. The malignant melanoma patients (Patients 1–6) received 5.0 mg of G and 2.5 mg of PHL oncolysates during wk 1–6, as well as 5.0 mg of M-40 oncolysate, wk 2, 4, and 6. Oncolysates were injected s.c. in the anterior thigh. In contrast, ovarian carcinoma patients (Patients 7–12) received a single 5.0-mg injection i.p. of oncolysate in wk 1. Malignant melanoma patients had their preimmunization sera and the following postimmunization sera tested: Patient 1, 5 wk; Patient 2, 2 wk; Patients 3 and 4, 3 wk; and Patients 5 and 6, 6 wk. The ovarian carcinoma patients all had preimmunization and 4-wk postimmunization serum samples analyzed.

Tumor Cell Cultures. Human malignant melanoma cell lines, PHL, G, and M-40, were obtained from Dr. W. A. Cassel, Emory University, Atlanta, GA, at passages 35, 34, and 65, respectively (23). The cells were grown at 35°C in humidified air in L-15 medium supplemented with 10% fetal calf serum without antibiotics. The ovarian carcinoma cells, MDAH 2774, were maintained in the same medium containing 50–70 μg of gentamicin per ml. Tumor cells were free of Mycoplasma as demonstrated with Hoescht No. 33285 stain.

Preparation of Lysates of Virus-infected Cells (Oncolysates). Newcastle disease virus was added to cultures of the malignant melanoma cell lines, while PR8/A/34 influenza virus was used to infect the ovarian carcinoma cells. Cells were grown to confluent monolayers in T-150 flasks. The medium was then replaced with L-15 without serum, and incubation was continued for another 47 h at 37°C. The medium was then decanted, and 10 ml of virus suspension containing approximately 1 × 10⁸ chick embryo infectious doses were added and incubated 15 min at 22°C and for another 45 min at 35°C with agitation every 5 min. This medium was then replaced with 30 ml of fresh serum-free medium, and incubation was continued for 20 h at 35°C. Cells were then harvested with a rubber policeman. Virus-infected cells were washed with phosphate-buffered saline (pH 7.3), centrifuged at 1000 × g for 15 min, washed with 0.15 M NaCl, and resuspended in one packed cell volume in 0.15 M NaCl containing 1 mM MgCl₂. After 2 min at 22°C, the cells were vortexed for 1 min and incubated another 2 min at 22°C. DNase, 1 μg/ml in 0.15 M NaCl, was added volume for volume. After 5 min at 37°C, 2 packed cell volumes of 0.15 M NaCl were added, and the cells were sonicated twice for 1 min each in a 10-kHz Ratheon cup sonicator at full power. Aliquots of the sonicate (1.5 ml) were dispensed in 60-mm Petri plates and incubated with UV light for 15 min at 40 ergs/s/m² in a laminar flow hood. The irradiated suspension was diluted, aliquots were removed for sterility testing, and the remainder was dispensed in sterile glass vials and stored at 5 mg total protein per ml at −70°C.

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Cell Extractions. Confluent monolayers were harvested by scraper, washed 3 times with Tris saline, and then solubilized with 5 packed cell volumes of 1.0% NP-40 in the same buffer (24). One mm phenylmethylsulfonyl fluoride and 100 kallikrein inactivator units of aprotonin (Trasylol; FBA Pharmaceuticals, New York, NY) were added to the solubilization buffer to inhibit enzymatic proteinolysis.

Polyacrylamide Gel Electrophoresis. NP-40 cell extracts and the oncolysates were fractionated by SDS.PAGE under reducing conditions using the system of Laemmli (25) as adapted by this laboratory for the analysis of tumor cell extracts (26). Ten % gels with an acrylamide:bisacrylamide ratio of 30:0.8 were used. Gels were run at 25 mA/gel at 22°C. Phosphorylase b (M, 94,000), bovine serum albumin (M, 67,000), ovalbumin (M, 43,000), carbonic anhydrase (M, 30,000), soybean trypsin inhibitor (M, 20,100), and RNase (M, 14,000) were run as molecular weight markers on all gels. Unless otherwise indicated, 250 µg of tumor cell extract were fractionated on each cm of gel.

Identification of Specific Immunologically Reactive Proteins in the SDS.PAGE Gels by Western Blotting. Proteins were transferred from the SDS.PAGE gels to nitrocellulose paper (HAWP; 0.45-µm pore size; Millipore Corp., Bedford, ME) at 150 mA for 3 h at 22°C in 25 mM Tris:192 mM glycine:20% (v/v) methanol, pH 8.3, as described by Towbin (27). The transfers were air dried overnight and then cut into 12 (7 x 125 mm) replicate test strips, another strip containing the molecular weight standards and a portion of the test extract, and a final strip containing an enzyme-linked immunosorbant assay standard and a portion of the test extract (26). Each of the 12 strips was used to test a single serum sample for antibodies of a specific isotype that react with one or more antigens in the tumor cell extracts. The test strips and enzyme-linked immunosorbant assay standards were incubated in undiluted calf serum for 1 h at 22°C to block nonspecific binding of patient immunoglobulin as previously described (26). The strips were then incubated in human serum diluted 1:50 in sealed tubes on a rocking platform for 2 h. They were then washed 3 times in Tris-buffered saline with 0.1% (v/v) Tween 20 (TWETS buffer). Each strip was washed 2 times with 10 ml of TWETS buffer, and then all the strips were combined through 3 washes of 300 ml each of TWETS in trays. Next, the strips were incubated for 30 min with a 1:200 dilution of rabbit antiserum specific for human IgG, IgA, and IgM (Calbiochem.Behring Corp., LaJolla, CA). These antisera were also diluted in calf serum. The strips were then washed 3 times with TWETS in trays as above. The strips were then incubated for 30 min in goat anti-rabbit IgG, conjugated to horseradish peroxidase (Miles Labs., Elkhart, IN). After another 3 washes in TWETS, the strips were developed in TWETS buffer containing 3,3-diaminobenzidine (20 µg/ml) (Sigma Chemical Co.), nickel chloride (20 µg/ml), and 0.01% (v/v) H2O2. Colored bands developed in the strips within 30 min at sites which previously bound human immunoglobulin and peroxidase-conjugated goat anti-rabbit IgG. The strips were then washed one time in TWETS and one time in deionized water and dried overnight between filter paper strips. Strips containing molecular weight marker proteins and a portion of the protein bands from cell extracts or viral oncolysates were stained in 0.1% (w/v) amido black in 2.0% acetic acid for 15 min. The strips were then rinsed in deionized water and destained in 2.0% acetic acid at 60°C for 10 min. The strips were washed once in deionized water, air dried, and reassembled for photography.

Protein Determinations. Total protein concentrations were performed by the Lowry (28) or Bradford methods (Bio-Rad Laboratories) (29) using bovine γ-globulin as a standard.

RESULTS

Identification of Antibodies in Preimmunization Sera to Antigens Present in Lysates of Uninfected Tumor Cells. Each patient had IgG, IgA, and IgM antibodies in preimmunization sera that reacted with antigens expressed by NP-40 extracts of cultured melanoma or ovarian carcinoma cells. Fig. 1 displays the preimmunization IgG antibodies of 5 malignant melanoma patients to an NP-40 extract of the G cell line as well as the preimmunization IgG antibodies of 5 ovarian carcinoma patients to the NP-40 extract of the 2774 ovarian carcinoma cell line. Each patient's antibodies had a specific pattern of reactivity. Some recognized the same antigens. For example, IgG of Patients 5 and 6 reacted with Band a, a M, 98,000 antigen; IgG of Patients 7, 8, 11, and 12 reacted with M, 113,000 molecule, Arrow c. Others (Patient 1, Lane B, and Patient 12, Lane L) identify antigens with molecular weights of 62,000, Arrow b, and 83,000, Arrow d, that are not detected by antibodies of other patients.

Some patients appear to have antibodies to the same proteins in these tumor cell extracts. For example, antibodies from Patients 5 and 6 (Lanes E and F) react with a M, 98,000 antigen, Arrow a. Patients 7, 8, 11, and 12 identify a M, 113,000 molecule, Arrow c. Others (Patient 1, Lane B, and Patient 12, Lane L) identify antigens with molecular weights of 62,000, Arrow b, and 83,000, Arrow d, that are not detected by antibodies of other patients.
Fig. 2. Comparison of antibodies in pre- (Subscript 1) and postimmunization sera (Subscript 2) to antigens expressed in uninfected malignant melanoma cells of the G line (Lanes A and B) and in MDA 2774 ovarian carcinoma cells (Lanes C to E). The positions of molecular weight marker proteins (M, × 10^3) are indicated at the left-hand margin. Bound IgG (Lanes A and C), IgA (Lane D), or IgM (Lanes B and E) was demonstrated with appropriate second antibodies. Melanoma Patient 4, Lane A, Arrow a, responded to a M, 96,000 antigen; Melanoma Patient 1’s serum reacted with a M, 49,000 antigen, Lane B, Arrow b, and a M, 32,000 antigen. Lane B, Arrow c. Patient 10, Lanes C to E, Arrow d, had a polyclonal response to a M, 13,000 molecule and an IgM response to a M, 54,000 antigen. Lane E, Arrow e.

Fig. 3. Characterization of pre- and postimmunization responses of 6 malignant melanoma patients to virus-modified PHL oncolysate. Subscript 1, pre-, and Subscript 2, postimmunization sera. Lane A, molecular weight markers; numbers in left margin, molecular weight × 10^3. Lane B, Amido black-stained transblot of the oncolysate. Lanes C–H, sera from Melanoma Patients 1–6, respectively. Arrow a, postimmunization response of all patients to a M, 56,000–58,000 antigen complex.

Fig. 4. Isotype (IgG, IgA, and IgM) of antibodies that develop after immunization in the circulation of patients with malignant melanoma against antigens in the PHL oncolysate. Shaded boxes indicate antigens identified by postimmunization sera, but not by antibodies in preimmunization sera.

Fig. 5. Isotype of antibodies to antigens in the G oncolysate detected by postimmunization sera of malignant melanoma patients.
of 6 made IgM responses to antigens in the G oncolysate. Finally, 5 of the 6 patients made IgG responses to the lysates of M-40. The new antigens recognized by postimmunization sera of the melanoma patients are summarized in Table 1. Patients 1 and 3 recognized more antigens than the others. Antibodies of all 3 major isotypes developed to a Mr 58,000 antigen that appeared to be commonly present in oncolysates of the 3 malignant melanoma cells. In contrast, certain antigens, such as the Mr 127,000 and 137,000 antigens, which were uniquely present in the PHL oncolysate (Fig. 4) were identified by antibodies of only 1 or at most 2 donors.

Responses of the ovarian carcinoma patients to the 2774 oncolysate were in sharp contrast to the responses of the malignant melanoma patients to their respective oncolysates. Novel antibody responses to the 2774 oncolysate actually used to immunize the patients were not detected. However, when 2774 cells were infected with virus and extracted 20 h later with NP-40 in the presence of phenylmethysulfonyl fluoride and Trasylol, antibody responses to a Mr 28,000 antigen were detected with IgG in sera from Patients 7 and 8, and a Mr 79,000 antigen was detected with IgG in sera from Patients 9 and 10 (Fig. 12).

Antibody Responses to Virus-infected Allantoic Fluid. Virus-infected allantoic fluids were studied to identify which antigens were specifically associated with the infecting virus. Fig. 7 shows that IgG antibodies in the sera of the 6 malignant melanoma patients recognized 4 antigens with molecular weights of 42,000, 52,000, 56,000, and 58,000 in allantoic fluid infected with NDV. These same antigens were recognized by IgA antibodies in these patients' sera, but IgM responses were found only to the Mr 58,000 antigen. To validate that these were virus-associated antigens, a chicken anti-NDV reference antiseraum was tested against the Western blots of the fractionated allantoic fluid. The chicken antibodies also reacted with the Mr 42,000, 53,000, 56,000, and 58,000 antigens (Fig. 8).

A direct comparison of antigens recognized by the malignant melanoma patients' antibodies and those recognized by an NDV reference antiseraum in Western blots of the PHL oncolysate is shown in Fig. 9. This figure demonstrates antigens with molecular weights of 68,000, 49,000, 34,000, 33,000, and 32,000 that...
The serum of the normal individual who had been hyperimmunized with influenza PR8/A/34 had IgG antibodies to these same Mr 27,000 and 28,000 antigens as well as to other antigens in the molecular weight range between 24,000 and 26,000 (Fig. 11).

DISCUSSION

These results provide evidence in favor of the hypothesis that immunization with viral oncolysates stimulates antibody responses to antigens in allogeneic human tumors and their corresponding viral oncolysates. Certain of the tumor cell-associated antigens were recognized by antibodies in preimmunization sera. It is unlikely that these represent nonspecific reactions (15, 27). The antibodies in preimmunization sera could represent normal autoantibody responses to normal cytoplasmic or cytoskeletal proteins (30-33) or to cell surface antigens (34, 35). Some may be directed against fetal (36) or tumor cell antigens (37-39). This "background" response, present in preimmunization sera, served as a control for the identification and characterization of humoral responses that developed subsequently upon immunization with the viral oncolysates.
Three types of antigens were identified by the newly developed antibodies in postimmunization sera: (a) Antigens categorically associated with uninfected tumor cells which were not recognized by antibodies in preimmunization sera; (b) antigens specifically associated with virus-infected fluids; and (c) antigens found in oncolysates but not in uninfected tumor cells nor in virus-infected allantoic fluids. The last category may represent virally induced antigens, virus-modified cellular antigens, or nuclear antigens specifically associated with the virus-infected tumor cells. Note that nuclear antigens were likely to be found in the viral oncolysates but not in the NP-40 extracts used in this study.

Following immunization (Fig. 2), 3 patients, 2 with malignant melanoma and 1 with ovarian carcinoma, developed antibodies to antigens in extracts of uninfected tumor cells. The identification of these antigens indicated the ability of viral oncolysates to stimulate immune responses to epitopes that are intrinsically associated with uninfected allogeneic tumor cells. Response to this type of antigen may be particularly noteworthy to those interested in enhancing immune response of tumor-bearing patients to their own malignant cells (1-4, 40).

In both malignant melanoma and ovarian carcinoma patients, the majority of new antibodies were made to virus-associated molecules (Figs. 7-10). The most diverse postimmunization responses developed against virus-modified cell extracts (Figs. 3-6 and 12; Table 1). The response of the melanoma patients was qualitatively greater than that of the ovarian carcinoma patients with respect to both the numbers of antigens that induced antibody responses and the diversity of antibody isoforms generated. The more diverse antibody response of the melanoma patients may reflect the greater frequency of immunization or the larger number of oncolysates used in the immunization schedule. Indeed, postimmunization sera of the ovarian carcinoma patients showed novel responses to antigens expressed in NP-40 extracts of the virus-infected ovarian carcinoma cells but did not identify such antigens in the oncolysates actually used for immunization. Conceivably, the inclusion of proteolytic enzyme inhibitors during and after NP-40 extraction may have protected antigens sensitive to degradation by lysosomal enzymes in these tumor cell extracts.

All 6 melanoma patients produced antibodies to a M, 56,000-58,000 NDV antigen complex, as shown in Figs. 3, 7, and 9. While the viral proteins that induced these antibody responses have not been fully characterized, the molecular weights of several are similar to previously identified components produced by NDV. For example, the molecular weight of fusion protein, F, is 56,000 (41, 42). The molecular weight of P proteins are 53,000 and 55,000 (42), and M, a matrix protein, has a molecular weight of 41,000 (43). Two ovarian carcinoma patients produced antibodies, and 3 others had preexisting antibodies to a M, 28,000 viral antigen shown in Figs. 8, 10, and 12. The three antigens of PR8/A/34 with molecular weights of 24,000-26,000, 27,000, and 28,000, recognized by sera of ovarian carcinoma patients, have molecular weights identical to known influenza viral proteins 6 and 7 (44) as well as viral glycoprotein 4 (45).

In the malignant melanoma patients, not all the responses to the viral oncolysates were directed against virus or tumor cell antigens. Some of these, including the M, 34,000, 127,000, and 137,000 antigens shown in Fig. 4, the M, 34,000 and 122,000 antigens shown in Fig. 5, and the M, 33,000, 34,000 and 68,000 antigens shown in Fig. 9, could be cell membrane, cytoplasmic, or cytoskeletal antigens induced by or modified by the virus. Alternatively, they could represent nuclear antigens not present in NP-40 extracts. Postimmunization sera did not identify these antigens in extracts of the 3 tumor lines, not infected with virus, nor did they detect these in virus-infected allantoic fluid.

The production of antibodies of viral components in the oncolysates and to the virus-induced or -modified antigens conceivably could alter the cell-mediated response of the host to autologous tumor (46, 47). For example, antigens released by the viral oncolysates may form complexes with circulating antibodies in vivo which could modify the activities of natural killer cells. However, this and other effects of immunization with viral oncolysates may vary, depending upon the levels of preexisting antibody to the virus used to modify the tumor cells. For example, Boone et al. noted that preexisting antibodies to the virus antigens reduced the immunizing effect of oncolysates (14), while Lindenmann (3) found the opposite result.

The results of the present study contrast with those of Livingston et al. (48) who found that vesicular stomatitis viral lysates of both autologous and allogeneic melanoma cells were not effective in evoking humoral immune responses to Class 1 and Class 2 malignant melanoma antigens. However, certain differences in our work and that published by Livingston et al. (48-50) make comparison of the studies difficult. Different viruses were used to infect the cultured tumor cells, different fractions of oncolysate were used for vaccination, and different antigenic targets and different methods for detecting the antibodies recognized by the serum antibodies were used to evaluate the humoral responses that developed in the recipients. Clearly additional investigations which comprehensively identify antigens stimulated by immunization with viral oncolysates are needed.

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