Suggestive Evidence for in Vivo Binding of Specific Antitumor Antibodies of Human Melanomas

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SUMMARY

Antibodies eluted from homogenates of human melanoma cells reacted against melanoma antigens in a complement fixation test. Before elution, sonically treated homogenate did not react significantly against autologous serum but, following elution, antigenic activity increased markedly (up to 32-fold). Eluate of one melanoma reacted with the sonically treated residue of other melanomas but not with similarly prepared residues of sarcoma, carcinomas, or normal tissues. Melanoma eluates contained more IgG than IgA. Traces of IgM were found in two melanoma eluates. Eluates of normal tissues (lung, kidney, and muscle) were devoid of serum proteins and did not react with the sonically treated melanoma residues. These results support the hypothesis that antitumor antibodies are bound to melanoma cells in vivo and that these antigens are cross-reactive.

INTRODUCTION

Antitumor antibodies that react with human tumor antigens prepared from biopsy tumor tissues and cultured human tumor cells have been demonstrated in the sera of cancer patients by a variety of in vitro immunological techniques (2, 3, 6, 11, 13, 17, 18, 29). If such antigen/antibody reactions occur in vivo, one would expect to find these antibodies on tumor cells obtained from biopsy and autopsy materials. Although immunoglobulins have been eluted from human and animal tumors by many investigators, the immunological reactivity of these immunoglobulin preparations has not been clearly established in human systems (15, 16, 21, 22, 26, 27, 28).

Eilber and Morton (8) showed that antisarcoma antibodies could be occasionally eluted from the biopsy tissue of human sarcomas. Subsequently, Phillips and Lewis (20) suggested that immunoglobulins having specific antimalanoma activity could be eluted from human melanoma cells. These results implied an in vivo reaction between serum antibodies and the antigens on the tumor cells.

This investigation was undertaken to determine whether antibodies could be eluted consistently from human malignant melanoma tissues and to determine the antitumor specificities of such antibodies.

MATERIALS AND METHODS

Tumorous and Normal Tissues

Melanoma, sarcoma, and carcinoma tissues obtained at biopsy or autopsy were either processed immediately for elution of antibodies or quick frozen in liquid nitrogen and stored until used. Normal tissues (muscle, kidney and lung) were obtained at autopsy from patients who expired from causes other than cancer and were processed in a like manner.

Elution of Antibodies

In order to determine the most efficient method for antibody elution, a human melanoma (Patient C. F.) tissue specimen (~20 g net weight) was cleaned thoroughly to remove all connective and adipose tissues. The tumor was minced finely and passed through a 60 mesh screen. The tissue that did not go through the screen was discarded. The cells obtained from the screening were washed 15 times with 9 to 10 volumes of PBS2 or until the final wash had an absorbance below 0.025 at 280 nm. The cells were weighed (~10 g net weight) and resuspended in 40 ml PBS to achieve a 20% (w/v) cell suspension. After an aliquot was removed, the remaining suspension was equally divided into 5 graduated conical centrifuge tubes marked A through E, and centrifuged at 2500 rpm in a GLC-1 centrifuge (Sorvall, Inc., Newton, Conn.) at room temperature for 10 min. The supernatants were discarded and the packed cells were treated as follows.

Tube A. Cells were resuspended in 9 volumes of PBS (pH 7.4) and exposed to 56° for 1.0 hr with intermittent shaking and then were centrifuged (Microfuge, Fisher Scientific Co., Pittsburgh, Pa.) at high speed for 30 min. Supernatant and sediment were saved.

Tube B. Cells were resuspended in 9 volumes of 15% NaCl solution (pH 7.4), incubated at 4° with intermittent shaking, and centrifuged at 10,000 × g for 10 min.

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Endnotes:
1 The abbreviation used is: PBS, 0.025 M phosphate-buffered saline (0.85% NaCl), pH 7.4.
Antigen titer

Sonically treated residue

Source of antibody Antigen dilution Homogenate\( ^{c} \) before elution Sonically treated residue\( ^{e} \) after elution by procedure

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\( ^{a} \) All eluates were dialyzed and concentrated to equal volumes.

\( ^{b} \) Antigen titers were derived by complement fixation test using eluates at 2 dilutions (1:3 and 1:9).

\( ^{c} \) Sonically treated suspension of 20\% (v/v) tumor cells before any treatment.

\( ^{d} \) Sonically treated suspension of 20\% (v/v) tumor cells after treatment with each procedure.

\( ^{e} \) Procedure A, exposure of melanoma cells to 56\(^{c}\) for 1.0 hr; Procedure B, exposure of melanoma cells to 15\% NaCl solution (pH 7.4) at 4\(^{c}\); Procedure C, exposure of melanoma cells to 15\% NaCl solution (pH 7.4) at 37\(^{c}\); Procedure D, exposure of melanoma cells to 15\% NaCl solution (pH 7.4) at 37\(^{c}\); Procedure E, exposure of melanoma cells to low pH (2.6) at 4\(^{c}\).

**Antitumor Antibodies from Human Malignant Melanomas**

Supernatant and sediment were saved.

**Tube C.** Cells were resuspended in 9 volumes of 15\% NaCl solution (pH 7.4), incubated at 37\(^{c}\) with intermittent shaking, and centrifuged as described in "Tube B." Supernatant and sediment were saved.

**Tube D.** Cells were resuspended in 9 volumes of 15\% NaCl solution (pH 7.4) and incubated at 56\(^{c}\) as in "Tube B." Supernatant and sediment were saved.

**Tube E.** Cells were resuspended in 9 volumes of 0.56 m glycin-HCl buffer (pH 2.6), stored at 4\(^{c}\) for 10 min with intermittent shaking, and centrifuged as in "Tube B." Supernatant was immediately adjusted to pH 7.4 with 0.1 N NaOH, and sediment was saved.

Supernatants A through D were dialyzed against a large volume of PBS and centrifuged at 36,000 \( \times g \) for 1.0 hr. Each was then concentrated by vacuum dialysis, adjusted to 1.0 ml/volume with PBS and used as the eluates (antibody).

Sediments A through D were washed 5 times with 9 volumes of PBS, resuspended in PBS (20\% v/v), and sonically disrupted at low frequency to form a fine suspension. These suspensions were used as antigens and are hereafter called "sonically treated residue."

The aliquot of cell suspension saved before the various treatments was adjusted to 20\% (v/v) in PBS and treated sonically as described above. This suspension was designated as the homogenate (before elution) and was also used as antigen.

The protein concentration in the 1st 5 eluates ranged from 15 to 20 mg/ml as determined by 280 to 260 nm absorption ratio. The protein concentration in the low-pH eluate was 8 mg/ml. In the 1st 5 eluates, the IgG was determined as 0.4 to 0.6 mg/ml and IgM at trace quantities (<0.005 mg/ml) by the radial immunodiffusion method. The IgG concentration in the low-pH eluate was 0.3 mg/ml.

Results (Table I) indicated that, although all the eluates contained IgG in measurable quantities and IgM in trace quantities, the low-pH eluate did not contain antibody activity by complement fixation against any sonically treated residues, whereas eluates of Procedures A through D showed complement-fixing antibody activity against all sonically treated residues, including the residue of low-pH treatment.

As seen in Table 1, active antibody elution was accomplished most efficiently by exposing the tumor cells to 15\% NaCl solution at 37\(^{c}\). This particular procedure, with modifications as outlined in Chart 1, was followed routinely thereafter to process 4 additional melanomas, 3 carcinomas, 1 sarcoma, and 3 specimens of normal tissues.

**Immunological Techniques**

Antibody activity of the eluates and antigenic activity of the sonically treated residues were determined by an ultramicrocomplement fixation test described by Colombani et al. (4). Essentially, the procedures were the same as those of the conventional microcomplement fixation test developed by Sever (23) and adapted by Eilber and Morton (9, 10) for tumor-antitumor systems except that the total volume/well was 8 \( \mu l \) instead of 25 \( \mu l \). Because the volume/well was so small, the tests were performed in
CANCER RESEARCH VOL. 35

R. K. Gupta and D. L. Morton

RESULTS

Table 2 summarizes the results of complement-fixing antibody activity from melanoma cells from Patient C. F. Before elution, the homogenate from this melanoma specimen did not react significantly with autologous serum and not at all with homologous sera. Following elution with 15% NaCl solution, the sonically treated residue showed antigenic activity against both the eluted antibodies and with autologous serum antibodies, but had lower reactivity with homologous serum antibodies.

Additional experiments were done on melanoma tissue specimens from 4 patients (S. N., M. M., R. C., and F. H.) to determine whether 1st, the eluted antibodies would cross-react with the sonically treated residues; 2nd, the antibodies would react with sonically treated residues of 3 carcinomas (M. A., P. R., and A. G.), 1 sarcoma (F. C.); or 3rd, with 3 normal tissue specimens of muscle, lung, and kidney obtained from a patient who expired from a nonmalignant cause. These results are presented in Table 3. Eluted antibody dilutions from 4 melanomas (S. N., M. M., C. F., and R. C.) were standardized at 1:10 because each was antigenic in the undiluted state. The eluted antibodies from the 5 melanomas reacted with autologous and/or homologous sonically treated tumor residues. The eluates did not react with sonically treated residue of the sarcoma, the 3 carcinomas, or the normal tissues, nor did the eluates of the carcinomas, sarcoma, and normal tissues react with the sonically treated residues of the 5 melanomas. One melanoma residue (R. C.) did not react with any of the eluted antibodies; however, antibodies from R. C. did react with the melanoma residues of M. M. and C. F.

Analyses of the eluates by immunodiffusion and immunoelectrophoresis with specific antiserum to human immunoglobulins are shown in Table 4. All melanoma eluates contained IgG and IgA. Immunoglobulin of Class G was predominant in each. Only 2 eluates (M. M. and R. C.) contained IgM. IgG was also demonstrated in the sarcoma and 2 of the carcinoma eluates. The eluates of normal tissues had none of these immunoglobulins. None of the melanoma eluates reacted with normal tissue residues, nor...
did eluates of normal tissues react against the melanoma residues. The antibodies eluted from each of the melanomas consistently cross-reacted with other melanoma residues but not with residues of other histological types of cancers studied.

**DISCUSSION**

Antibodies specifically reactive with melanoma antigens were eluted consistently from biopsy specimens of 5 human melanomas. These melanoma tissues contained antigens that reacted in vivo with antimelanoma antibodies. After the elution procedure, the sonically treated melanoma residue showed antigenic activity against both the eluted antibodies and autologous serum antibodies. These results suggested that the antigen-antibody complexes formed in vivo on the tumor cell surface were dissociated during the elution procedure and that active antigen and antibody were recovered separately. The recovered antibodies were probably similar to serum antibodies because the antigen titers of the sonically treated residues increased against both autologous serum and the eluted antibodies. In addition to the free antibodies, the eluates may have contained antigen-antibody complexes because some of the eluates were anticomplementary in their undiluted state. This possibility is under investigation.

It seems likely that the immunoglobulins demonstrated on biopsy specimens by Thunold et al. (27) were in fact antitumor antibodies. Since the antibodies eluted from human melanoma tissues are complement fixing, they may be directly cytotoxic in vivo; however, their significance in terms of tumor-host relationship, whether they play a role in blocking or augmenting cell-mediated immunity (12, 14, 24, 25), remains to be determined.

Apparently, there was a common antigen responsible for the reactivity among the melanomas, because 2 of the melanoma residues (M. M. and C. F.) reacted with antibodies eluted from all 5 melanomas (Table 3). However, the pattern of reactivity between the eluted antibodies and sonically treated residues suggested that antigens of more limited specificity were also present, because 2 residues (S. N. and F. H.) reacted only to their autologous eluted antibodies and to C. F. antibody.

The results (Table 3) also show that the amount of immunologically active antibody that could be eluted from melanoma tissues varied from patient to patient. The eluted antibodies of C. F. and S. N. were the strongest reactors by complement fixation, followed by those of F. H.; the antibodies of M. M. and R. C. reacted the least. These differences in complement-fixing antibody reactivity might be explained by variations in the antigenicity of the respective tumors. Furthermore, the cells derived from different patients may not carry the same number of antibody-binding sites.

A possible explanation for the nonreactivity of R. C.'s...
melanoma residue with the antibodies eluted from various melanomas may involve inactivation of the melanoma antigen. In subsequent experiments we found that repeated exposure to 15% NaCl solution destroyed all antigenic activity of the tumor cells and, also, although antibodies eluted from R. C.’s melanoma tissue did not react with the autologous sonically treated residue, these antibodies did react with the sonically treated residues of M. M. and C. F. These immunological reactions between eluted antibodies and melanoma residues cannot be explained by isoantibody activity against histocompatibility (HL-A and blood group) antigens in the melanoma tissue because the antibody eluted from each patient’s melanoma reacted with his corresponding autologous melanoma residue. Many of these eluted antibodies reacted with other melanomas but did not react against sonically treated residues of other histological types of neoplasms or normal tissues, nor did the eluate of the other neoplasms or normal tissue react against the sonically treated residues of melanoma. If the observed reactivities between eluted melanoma antibodies and sonically treated residues of melanoma were due to histocompatibility antigens, some reactivity between the antibodies eluted from melanoma and these other types of tissue residues would have been expected.

Therefore, it would appear that the human malignant melanomas certainly contain cell surface antigens which may react with antimeranoma antibodies in vivo.

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REFERENCES

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