Localization of Neuroblastoma in Vivo with Tumor-specific Antibodies

David S. Terman, Ian Stewart, Arnold Tavel, and Dennis Kirch

The Department of Medicine, University of Colorado [D. S. T.], and Veterans Administration Hospital [D. S. T., I. S., A. T., D. K.]
Denver, Colorado 80220

SUMMARY

Studies of the mouse C-1300 neuroblastoma were undertaken in order to isolate tumor-specific antibodies and harness them for detection of tumors in vivo. Preliminary investigations demonstrated the strain-growth specificity of the neuroblastoma in A/Jax male mice and established the requirement for tumor viability for successful adoptive passage. Intradermally passed tumor permitted extended survival of mice so that serum could be sampled at intervals for the presence of tumor-specific antibodies. By means of an indirect radioimmunoassay with glutaraldehyde-fixed tumor cells as antigen, tumor-specific antibodies were identified in the serum of tumor-bearing hosts 6 days after inoculation, with a steady increase in antibody levels observed through Day 22. An eluate in which immunoglobulin G antibodies were identified by immuno-electrophoresis was obtained from purified tumor cells by acid buffer incubation. These antibodies were labeled with 125I, absorbed with normal tissues, and injected into tumor-bearing mice. A selectively collimated, single-probe isotope localization was positioned over the intradermal tumor, while the rest of the animal was shielded with lead. With this device, 125I-neuroblastoma eluate was significantly taken up in the tumor. We herein describe some of the growth characteristics and immunological activity of the mouse neuroblastoma. We characterize the evolution of the host's humoral immune response with radioimmunoassay and demonstrate that tumor-specific antibodies may be eluted from neuroblastoma. Most important, we demonstrate that these eluted antibodies, when radiolabeled, absorbed with normal tissues, and injected into tumor-bearing mice, may be detected with a sensitive external scintillation detector and show incremental uptake specifically in the neuroblastoma. The study thus suggests that tumor-specific antibodies may be harnessed to detect the presence of primary and perhaps occult neoplastic tissue.

MATERIALS AND METHODS

Animals. A/Jax male mice and CBA female mice 6 weeks of age (The Jackson Memorial Laboratories, Bar Harbor, Maine), A/He male mice 6 weeks of age (Cumberland Farms, Clinton, Tenn.), and CF-1 female mice (Carworth Farms, Portage, Mich.) were utilized for the foregoing experiments. They were maintained on commercial food pellets and tap water.

Tumors. The C-1300 neuroblastoma growing in A/Jax male mice was obtained from Dr. Kedar Prasad, and the reticulum cell sarcoma growing in BALB/c female mice was from Dr. Barbara Jacobs. Both tumors were maintained in their respective hosts by i.d. and s. c. passage.

Preparation of Cell Suspensions and Sonic Disruption. At appropriate intervals, tumor-bearing mice were exsanguinated and their sera were stored at −20°C until ready for use. Tumors were extirpated, trimmed free of fat and skin, minced with scissors, and gently pressed through a No. 80 stainless steel wire mesh into chilled nutrient mixture F-12 with glutamine (Grand Island Biological Co., Grand Island, N. Y.). The tumor cell suspension was washed 5 times in media, counted, and injected in 0.03 to 0.5 ml media by an appropriate route. Washed tumor cells suspended in 3 ml of media were held in an ice bath while studies demonstrating the presence of blocking antibodies and immune complexes in the sera of patients and relatives of tumor-bearing hosts (4, 5), there have been few reports of the immunological behavior of the C-1300 tumor.

Accordingly, we herein describe some of the growth characteristics and immunological activity of the mouse neuroblastoma. We characterize the evolution of the host's humoral immune response with radioimmunoassay and demonstrate that tumor-specific antibodies may be eluted from neuroblastoma. Most important, we demonstrate that these eluted antibodies, when radiolabeled, absorbed with normal tissues, and injected into tumor-bearing mice, may be detected with a sensitive external scintillation detector and show incremental uptake specifically in the neuroblastoma. The study thus suggests that tumor-specific antibodies may be harnessed to detect the presence of primary and perhaps occult neoplastic tissue.

INTRODUCTION

The mouse C-1300 tumor arose spontaneously in the body cavity of an albino male A/Jax mouse and was tentatively diagnosed as neuroblastoma (1). The biochemical and morphological characteristics of the mouse neuroblastoma have demonstrated a striking resemblance of the human neuroblastoma, a major neoplasm in children (10). While there have been reports of the presence of surface membrane antigens on the mouse neuroblastoma (7, 12) and...
undergoing sonic disruption with a Model W185 ultrasonic cell disrupter (Heat Systems-Ultronics, Inc., Plainview, N. Y.) calibrated at 50 watts for 3 min.

Trace Labeling of Proteins. Rabbit γ-globulin for use in radioimmunoassay, tumor eluate IgG, and MOPC 141 IgG were labeled with 125I by the chloramine-T method (8) and dialyzed against PBS for 3 days. The mean specific activities of these proteins were 5 × 10⁴ cpm/mg protein, 3 × 10⁴ cpm/100 µg protein, and 6 × 10⁴ cpm/mg protein, respectively.

Immunodiffusion and Immunoelectrophoresis. These procedures were performed on microscope slides with 2% agar (Lonagar; Wilson Diagnostics Inc., Glenwood, Ill.) in sodium barbital buffer, pH 8.6, utilizing 25 V and 5 ma/slide. The slides were developed with rabbit antisera to mouse immunoglobulins that were provided by Dr. Howard Grey.

Preparation of Antisera for Radioimmunoassay. Rabbit antisera to mouse γ-globulin were prepared by repetitive immunization of a 2.5-kg New Zealand female rabbit with mouse immunoglobulins incorporated in incomplete Freund's adjuvant. Rabbit immunoglobulins were isolated by precipitation with 50, 40, and 33% saturated ammonium sulfate at 4° (15). The final precipitate was dialyzed against PBS for 3 days prior to trace labeling with 125I as previously described. The specificity of the rabbit antibodies for mouse IgG and IgM was demonstrated by immunoelectrophoresis.

Radioimmunoassay for Detection of Tumor-specific Antibodies. Tumor cell suspensions were prepared from 8 large neuroblastomas in F-12 media. The cells were washed once and then resuspended in F-12 medium with 5% γ-globulin-free newborn calf serum (Grand Island Biological Co.) at pH 7.4 and held at 25° for 2 hr, after which the cells were washed again in media. Erythrocytes in the mixture were lysed with 10 ml of distilled water held at 25° for 5 min, and the remaining tumor cells were washed again in media. Twenty-five ml of 0.075% glutaraldehyde in PBS were added to the tumor cells and held at 20° for 5 min. After 2 washes, the tumor cells were suspended in PBS, and 1.2 ml of suspension containing 0.8 ml of tumor cells were added to experimental and control test tubes. Undiluted serum (0.3 ml) from tumor-bearing mice was incubated with tumor cells for 12 hr at 4°, and 0.3 ml of control normal mouse serum was added to tubes containing the same number of tumor cells and similarly treated. The cells were then washed twice in PBS, and 1 ml of 125I-rabbit anti-mouse immunoglobulin serum containing 265,000 to 270,000 cpm was added to test tube and incubated for 12 hr at 4°. Each test tube was counted, after which the cells were washed twice with PBS, and the tubes were finally counted for 1 min in a Picker counter. The percentage of total counts bound relative to the total number of counts added to each tube was calculated and the results are expressed as the percentage of total counts bound in experimental tubes above the normal serum control tubes.

Elution Studies. Nine C-1300 tumors approximately 3 cm in diameter were extirpated, and single-cell suspensions were prepared. Cells were washed once in media, after which erythrocytes were lysed with distilled water for 5 min at 25°, and the cells were washed 5 times in media. Twenty ml of glycine-HCl buffer, pH 2.7, were incubated with the tumor cell suspension and held at 4° for 30 min (16). The mixture was frozen at -20°, thawed, then centrifuged at 2000 rpm for 7 min and the sediment was discarded. The precipitate was then discarded. The supernatant was dialyzed against 0.01 M NaCl for 6 hr at 4°, concentrated in a Model 12 ultrafiltration cell with an XM100A diaflow ultrafilter (Amicon Corp., Lexington, Mass.) and dialyzed against PBS for 24 hr. The eluate quantitated by the method of Lowry et al. (6) contained 100 µg of protein. As a control, IgG was isolated from sera of BALB/c mice bearing the MOPC 141 tumor by starch block electrophoresis in sodium barbital buffer at pH 8.6. It was then dialyzed against 0.01 M NaCl for 6 hr, concentrated, dialyzed in a manner similar to that for the eluate, and shown to contain 2 mg of protein (6).

Assay for Localization in Vivo of Radiolabeled Antibodies. Labeled neuroblastoma eluate or labeled MOPC 141 immunoglobulins were absorbed in succession with 2 ml of normal A/Jax male liver cells and 0.5 ml of spleen cells for 50 min at 25°. The cells were then removed by centrifugation and the supernatants were dialyzed against PBS for 2 days. Either 125I-eluate or MOPC 141 125I-IgG in 0.3- or 0.5-cu cm volumes representing 6 to 7 × 10⁷ cpm as measured in a Picker γ counter were injected i.v. into mice anesthetized with pentobarbitol-bearing neuroblastoma or reticulum cell sarcoma. The animals were placed on 0.01% potassium iodide water for 5 days prior to the experiment. Continuous monitoring of the amount of radioiodine contained in the tumor or head was carried out with a Model 235, selectively collimated, single-probe isotope-localization monitor (D. A. Pittman, Ltd., England). Schematic representation of this experimental system is shown in Chart 1. Selective counting of the tumor or head was facilitated by the use of a lead body shield. Count rate measurements were made at 1-min intervals with the probe positioned 0.5 cm above the animal. The total amount of injected 125I-IgG exceeded the measuring capacity of the isotope monitor; therefore, the results of selective uptake by the tumor of 125I-IgG were expressed as cpm recorded over the tumor at various intervals after tumor injection. The

Chart 1. Experimental design for localizing injected radionuclide in the tumor is shown. Mouse was anesthetized, secured to mobile plate, and shielded with lead, except for the tumor and head. The isotope-localization monitor was placed over the tumor or head, and the mouse was precisely positioned with guides to permit alternate counting of each area.
RESULTS

Allogeneic Transfer Studies. Initial studies were aimed at establishing the strain specificity of the C-1300 tumor to determine whether the tumor retained its histocompatibility antigens in serial passage. Accordingly, 10⁶ C-1300 cells were transferred s.c. to strains of mice in groups of 7, which mice differed from the A/Jax at both major and minor histocompatibility loci. Tumor growth was followed over the ensuing 30 days. Whereas the tumor was palpable between Days 12 and 15 in the A/Jax strain, it failed to appear after s.c. injection in CBA female, CF-1 female, and A/He female mice.

Growth of C-1300 Tumor Injected by Various Routes. The pattern of tumor growth was studied in 6-week-old A/Jax mice by injecting 3 × 10⁶ C-1300 tumor cells into groups of 7 mice i.v., i.d., s.c., and i.p. Animals receiving the tumor by the i.p. and s.c. routes succumbed to the tumor between Days 16 and 20, while those receiving i.d. and i.v. tumor cells survived an average of 10 days longer.

Tumor Transfer with Viable and Non-viable Tissue. Studies were carried out to determine whether viable tumor cells were required to adoptively passage the tumor. Accordingly, 10⁶ neuroblastoma cells in 0.03 ml of media were transferred i.d. into 10 A/Jax male mice immediately after tumor extirpation and washing in media. A corresponding number of cells were treated sonically and stored at 4⁰ for 4 days before injection, and an additional aliquot of cells was disrupted sonically before undergoing 6 cycles of freezing at −20⁰ and thawing at 25⁰. Sonically treated cells (0.03 ml) stored at 4⁰ and 0.03 ml of sonically treated cells that were frozen and thawed were injected i.d. into groups of 10 A/Jax male mice, and the course of tumor growth was followed. Light microscopic examination of sonically treated frozen, and thawed cells revealed nearly total cell disruption and considerable cell debris. Tumors appeared 8 to 12 days after injection in the group receiving tumor cells that were immediately transferred after extirpation. Those groups receiving sonically disrupted tumor cells and sonically treated, frozen and thawed cells showed swelling at the site of injection, sometimes progressing to the size of a small nodule by Day 10. However, these nodules ultimately regressed and by Day 20 there was no sign of tumor growth in any animal in these groups.

Radioimmunoassay for the Presence of Tumor-specific Antibody in the Serum of Tumor-bearing Hosts. The evolution of the humoral antibody response to neuroblastoma was studied by radioimmunoassay as previously described. Sera from 4 A/Jax male mice were collected and pooled on Days 6, 10, 14, 18, and 22 after i.d. injection of 10⁶ neuroblastoma cells, and the quantity of neuroblastoma specific antibody in 0.3-ml aliquots was assayed. A tumor nodule was present by Day 10 in all animals; however, sera that were obtained from mice on Day 6 when no tumor was apparent at the inoculation site showed tumor-specific antibodies. There appeared to be an increase in the quantity of circulating antibody in sera collected over the ensuing 22 days (Chart 2) with no experimental sample binding less than the normal mouse serum control.

Identification of Immunoglobulin Eluted from Neuroblastoma Cells. The presence of immunoglobulin on the neuroblastoma might provide further evidence of a host-immune response to the tumor. Tumors from mice that received i.d. injections of 10⁶ neuroblastoma cells 25 days earlier were extirpated, single-cell suspensions were prepared, and elution was carried out as described in “Materials and Methods.” Immunoelectrophoretic studies of the concentrated eluate with rabbit antisera to mouse immunoglobulins demonstrated the presence of IgG antibodies in the neuroblastoma eluate (Chart 3). Similar immunoelectrophoretic studies of the immunoglobulins obtained from mice bearing the MOPC 141 tumor also showed only IgG. In addition, immunoelectrophoretic analysis of the eluate and MOPC 141 immunoglobulins with rabbit antisera to whole mouse serum revealed only precipitin arcs corresponding to mouse IgG.
In Vivo Eluate Studies. The specificity of the 125I-eluted antibodies for the neuroblastoma was demonstrated in vivo. The 125I-eluate (0.3 ml) was injected i.v. into a neuroblastoma-bearing mouse. Count rate over tumor and mouse head was monitored, as described in “Materials and Methods.” The results (Chart 4) showed a steady increase in 125I count rate over the neuroblastoma in contrast to an insignificant change in isotope uptake over the head of the mouse. MOPC 141 125I-IgG (0.3 ml) injected into a smaller neuroblastoma-bearing mouse failed to show an incremental uptake over the tumor (Chart 4). This experiment was repeated twice with similar experimental findings.

In a 2nd experiment, 0.5 ml of neuroblastoma eluate was injected into 2 mice, 1 bearing a reticulum cell sarcoma, the other a neuroblastoma of comparable size. The 125I count rate did not increase over that of the reticulum cell sarcoma, in contrast to an incremental rise in count rate over that of the neuroblastoma (Chart 5). In neither animal was there an incremental rise in counts over the mouse head (Chart 5). MOPC 141 125I-IgG failed to show increasing uptake when injected into neuroblastoma- or reticulum cell sarcoma-bearing mice.

DISCUSSION

This study addresses itself to a most important problem in clinical oncology, i.e., the ability to detect primary and occult neoplasia in vivo. This investigation suggests that tumor-specific antibodies may be utilized to localize tumor in vivo. The specificity of this uptake was clearly shown by (a) the demonstration of increasing uptake of neuroblastoma eluate over the neuroblastoma but not over the mouse head, (b) the failure of MOPC 141 IgG to show incremental uptake in the neuroblastoma, and (c) the inability of the neuroblastoma eluate to show incremental uptake in a reticulum cell sarcoma.

The present study further suggests that the tumor itself may provide a rich source of tumor-specific antibodies which, after purification and radiolabeling, may be utilized for diagnostic purposes. The external scintillation detector used in this study has been used clinically to detect radioactive fibrinogen in deep vein thrombosis. We have demonstrated that an analogous system may show the in vivo uptake of tumor-specific antibodies by tumor. Moreover, we have now visualized radiolabeled tumor-specific
antibody in the neuroblastoma with the sensitive Anger scanning camera. Thus it should be feasible to detect occult or metastatic tumors with radiolabeled antitumor antibodies and sensitive external scintillation detectors.

Knowledge of the location of occult tumors might permit the specific destruction of neoplastic foci with precision external radiation delivery systems and the specific removal of tumor with surgery. Thus the usefulness of tumor-specific antibodies may not be limited to diagnosis but may theoretically play an important role in therapy. Indeed, there have been several reports of tumor-specific antibodies as carriers of anticancer agents (2, 3, 9, 11) that have demonstrated tumorcidal activity in vivo.

The foregoing studies clearly demonstrate that the neuroblastoma-bearing host is capable of mounting a humoral immune response to his own tumor. This evidence derives from several sources, namely, (a) demonstration by radioimmunoassay of the presence of immunoglobulin with tumor specificity in the sera of tumor-bearing animals, (b) the identification of immunoglobulin eluted from tumor cells by immunoelectrophoresis, and (c) the demonstration of the tumor specificity of the eluted antibody by in vivo localization in the neuroblastoma. The continued detection of tumor-specific antibody during the course of tumor growth may have been due to the i.d. growth location in the mouse, where the tumor appeared to be effectively sequestered. Minimal release of tumor specific antigens into the circulation, which might neutralize tumor-specific antibodies, might have occurred under these circumstances, permitting the detection of free tumor-specific antibodies in the serum by radioimmunoassay. Indeed, metastatic lesions were not grossly apparent in mice that succumbed to the tumor by Week 4, and tumor-specific antigens were not detectable by immunodiffusion in pooled serum of terminal tumor bearing mice using a potent heterologous antibody to the mouse neuroblastoma.

It appears from radioimmunoassay that tumor-specific antibodies may develop within 6 days following tumor inoculation and prior to the appearance of the tumor nodule. Whether these antibodies correspond to the blocking or enhancing antibodies described (4) is yet to be determined. The presence of tumor-bound immunoglobulins in some tumor systems has been described (17) and their function has been partially characterized in others (14). While it is conceivable that the tumor-specific antibody eluted from the neuroblastoma has enhancing activity and may potentiate tumor growth, trace quantities of heavily labeled tumor-specific antibodies may minimize this risk when these are used for diagnostic purposes. An evaluation of the antibodies detected in the serum and eluted from the tumor cells for membrane and/or cytoplasmic specificity is presently in progress.

At least 5 antigens have been described on the mouse neuroblastoma (7, 13). The nature of the host-immune responsiveness to these antigens is an important question for future investigation.

response to each or any of these surface specificities on the tumor is as yet unknown. However, the capacity of eluted antibody to localize in the neuroblastoma in animals in which tumor-specific antibodies were demonstrated in the serum would suggest that some variation in the avidity of tumor-specific antibodies exists. Tumor-bound immunoglobulins elutable from the tumor may represent the more avid tumor-specific antibodies. This possibility, as well as the kinetics of their development, is presently under investigation.

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