Reactivity of Human Brain Antiserum with Neuroblastoma Cells and Nonreactivity with Thymocytes and Lymphoblasts

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SUMMARY

Brain-associated antigens have been detected on human and mouse thymocytes. Also, murine neuroblastoma and brain cells have common antigens. In this study we compared the reactivity of rabbit anti-human brain (HB) serum with neuroblastoma and brain cells. The binding of HB antiserum to viable cells was assessed by immunofluorescence and an indirect radiolabeled antibody assay. HB antiserum reacted with >80% of neuroblastoma cells and with 0.1% of human thymocytes, bone marrow lymphoid cells, and lymphocytic leukemia cells. HB antiserum also reacted with 5 to 10% of peripheral blood lymphocytes. Absorption with neuroblastoma cells did not alter this reactivity. Rabbit antiserum raised against normal human thymocytes and leukemic T-cells specifically bound to thymocytes but did not bind to neuroblastoma cells. The reactivity of anti-HB serum against SK-N-SH neuroblastoma cells was removed by absorption with HB, but not with human kidney or liver, or mouse and guinea pig brain.

We conclude that human neuroblastoma cells possess cell-surface antigens that are present on HB. These antigens appear to be species specific and are not present on normal or malignant thymic cells. Conversely, thymus-associated antigens are not expressed on neuroblastoma.

INTRODUCTION

Neuroblastoma, the most common malignant solid tumor of childhood, still carries a poor prognosis despite recent advances in chemotherapy and radiotherapy (22). However, cases of spontaneous regression have been reported (4, 19). This phenomenon occurs most frequently in infants less than 12 months of age. The biological differences between neuroblastomas in young and older children are unknown. Some children with neuroblastoma have antibodies directed against membrane antigen(s) present on autologous or allogeneic neuroblasts (18, 20). It is unclear, however, whether these antigenic determinants are related to malignant transformation or represent cell differentiation or embryonic antigen(s). It is possible that variable expression of these antigens affects prognosis.

In mice, antigens present in normal mouse brain are also expressed by the neoplastic cells of the C1300 neuroblastoma cell line (24, 32, 33). The presence of normal HB antigen(s) on human neuroblastoma cells has not been investigated. However, HB antigen(s) is expressed on human T-lymphoid cells derived from normal thymus (11, 17), normal peripheral blood (12, 36), T-cell leukemias (11, 12) and a human T-cell line (35). Therefore, the aim of this study was to establish whether HB antigen(s) is expressed by tumor cells derived from patients with neuroblastoma and to determine the relationship, if any, between this antigen(s) and those present on normal or neoplastic T-cells.

To answer this question, we prepared antiserum against human fetal brain. The binding of this antiserum to human neuroblastoma and normal and neoplastic lymphoid cells was determined by immunofluorescence and an indirect RAA. The data indicate that there is brain-associated antigen(s) on human neuroblasts that is not present on human thymocytes or neoplastic T-cells.

MATERIALS AND METHODS

Source and Preparation of Neuroblastoma Cells

Cell line SK-N-SH, used in these studies, was established at the Sloan-Kettering Institute for Cancer Research, New York, N. Y. Its characteristics were described by Biedler et al. (3). The other neuroblastoma cell line, IMR-32, was obtained from the American Type Culture Collection, Rockville, Md. Neuroblasts were grown as monolayers in 250-sq cm Falcon tissue culture flasks. The culture media consisted of RPMI 1640, (Grand Island Biological Co., Grand Island, N. Y.), 10% FCS, and 1% penicillin-streptomycin (Grand Island Biological Co.). The flasks were tightly screw-capped and incubated in dry air at 37°. Neuroblasts were used when they had reached a confluent growth. Adherent cells were removed by the addition of calcium- and magnesium-free Hanks' balanced salt solution containing 0.125% trypsin (Sigma Chemical Co., St. Louis, Mo.) and 0.02% EDTA (Fisher Scientific Co., Fairlawn, N. J.). The flasks were...
were then agitated until the cells were in suspension (about 1 to 2 min). FCS was added to stop the activity of the trypsin. The cells were washed 3 times in PBS, pH 7.4, and the viability was determined by the cellular exclusion of trypan blue. Greater than 90% of the neuroblasts used in these assays were viable.

Neoplastic cells from neuroblastoma patients were obtained from the primary tumor (1), lymph node metastases (2), bone marrow (2), and pleural fluid (1). Tissue from the primary tumor or lymph node metastases was minced with scissors and passed through progressively smaller-gauge needles to obtain single-cell suspensions (25).

When possible, 3 ml of heparinized bone marrow [0.1 ml heparin (Fellows Medical Manufacturing, Oak Park, Mich.)/1 ml bone marrow] were obtained from areas that were almost completely replaced with neuroblasts (>80%) and separated from erythrocytes by gravity sedimentation at 37°. Neoplastic cells from 1 child with pleural effusion were also separated by gravity sedimentation. Cell suspensions were washed 3 times with Hanks' balanced salt solution and adjusted to the proper concentration. Viable cells from 1 patient were stored in the frozen state according to the method of Netzel et al. (26).

The human cell lines, cultured as above and used as controls, were from: carcinoma of the pharynx (Detroit 562), fibrosarcoma (HT 1080), embryonic lung (L 132), embryonal rhabdomyosarcoma (RD), Wilms' tumor (TuWi), and Chang liver (obtained from the American Type Culture Collection). Raji cells were obtained from St. Jude's, Memphis, Tenn. Glioblastoma A 172 was obtained from Dr. J. Fogh, Sloan-Kettering.

Source of Normal and Leukemic Cells

Blood was obtained from healthy volunteers, and the lymphocytes were separated by centrifugation through Ficoll (Pharmacia, Piscataway, N. J.)-Hypaque (Winthrop Laboratories, New York, N. Y.) (9). Bone marrow was obtained either from children with ALL who had been in continuous remission for 1 to 4 years or from children in whom a bone marrow had been performed in the work-up of a suspected hematological disorder. The nucleated cells were separated from erythrocytes by gravity sedimentation at 37°. The proportion of lymphocytes in these samples varied considerably, from 10 to 60%. Thymus tissue was obtained from children undergoing cardiac surgery, and single-cell suspensions were prepared (25).

Leukemic cells were obtained from the bone marrow of 21 children with ALL at the time of diagnosis. The diagnosis of ALL was made by cytomorphology of bone marrow samples and was based on the concurrent opinion of 3 hematologists. All samples tested had >90% lymphoblasts. Bone marrow leukemic cells were separated by gravity sedimentation at 37° (6).

Preparation of Antisera

HB Antiserum. Brain tissue from a 14-week-old fetus was homogenized and emulsified with an equal amount of complete Freund's adjuvant (Grand Island Biological Co.). New Zealand White rabbits received injections of 1.5 ml of emulsion into the footpad and hip. The animals were given booster injections with frozen homogenized brain in complete Freund's adjuvant on Days 7 and 14 and bled on Day 21. The antiserum was heat inactivated at 56° for 30 min. The antiserum for the assays was derived from a single rabbit and absorbed 3 times with human AB RBC, once with Raji cells, once with autochthonous liver, and once with chronic myelogenous leukemia cells. The liver used for absorption was obtained from the same fetus, homogenized, washed with RPMI 1640, and stored at ~70°. The cells for absorption were derived from a human lymphoblastoid B-cell line (Raji) and from the peripheral blood of a patient with chronic myelogenous leukemia in blast crisis. All absorptions were performed at a serum:cell or homogenate ratio of 1:1 (v/v). For each absorption, serum and cells or homogenate were incubated at room temperature for 1 hr in Falcon plastic tubes that were gently rotated on a Lab-Tek rotator. The mixture was centrifuged at 2500 rpm for 30 min at 4° in an International PR-6 centrifuge. The supernatant was used immediately or stored at ~20°.

Rabbit Anti-Human Thymocyte and Anti-E ALL Sera. The preparation of the anti-thymus serum used in this study has been described previously (25). Rabbits were given 2 injections of thymus serum and bled 7 days after the last injection. Aliquots of the sera were absorbed with human AB erythrocytes, Raji cells, normal bone marrow cells, and lymphoid cells from patients with chronic lymphocytic leukemia.

E ALL blasts to be used for immunization were obtained from the bone marrow and peripheral blood of an 11-year-old boy with ALL. Clinical and hematological features of this child have been reported (Ref. 7, Patient 2). Rabbits were given injections in the footpad of 1.5 × 10⁸ leukemic cells in complete Freund's adjuvant. By cytomorphology, 93% of cells used for immunization were lymphoblasts, and half of them formed erythrocyte rosettes after 1 hr incubation at 4° or 37°. Cells were injected i.p. into the same rabbits 4 weeks and 12 weeks after the 1st injection, and the animals were bled 7 days after the last injection. The dose of the booster immunizations was 2 × 10⁸ viable cells/rabbit.

After complement inactivation at 56° for 30 min, the anti-E ALL serum was absorbed 4 times with packed human AB erythrocytes at a cell:serum ratio of 1:1 (v/v). The serum was subsequently absorbed with Raji cells (a B-cell line) (1:1 ratio) 2 times and with bone marrow cells from different patients with ALL in remission (1:1 ratio) 3 times.

Control Sera. For control sera we used normal rabbit serum or serum from a rabbit immunized with cells from a cell line derived from a neoplastic lymph node of a patient with Hodgkin's disease. The preparation of this antiserum has been described previously (25). The hyperimmune serum was used for a control to eliminate the possibility that positive results were due to nonspecific binding of serum containing high levels of IgG.

Immunofluorescence with HB Antiserum

The reactivity of the HB antiserum at dilutions ranging from 1:50 to 1:800 was determined by indirect immunofluorescence. Cells (1.5 × 10⁶) were incubated for 30 min at 4°
with 50 µl of the antisera or control sera. After the 1st incubation, the test cells were washed with PBS and incubated for 30 min at 4° with 50 µl of fluorescein isothiocyanate-conjugated goat anti-rabbit Ig (Meloy Laboratories, Springfield, Va.) diluted 1:5 with PBS. The cells were then washed and mounted with PBS-glycerol under a coverslip and viewed using a fluorescence microscope. The results were expressed as percentage positive immunofluorescent cells. The HB antiserum reacted strongly with the neuroblasts (>90% positive) at dilutions of 1:50 and 1:100. At higher dilutions, there was a reduction in the percentage and intensity of positive cells.

In order to distinguish whether peripheral blood cells reactive with anti-HB serum were lymphocytes or monocytes, the peroxidase method of Preud'Homme and Flandrin (28) was used to identify monocytes. Precipitated peroxidase-positive material was seen only in the monocytes. This method did not interfere with immunofluorescence of positive lymphocytes. A T-cell-enriched fraction of blood lymphocytes was obtained by filtration through glass-wool columns as previously described by Greaves and Brown (16).

### RAA of HB Antiserum Binding to Neuroblasts

Expression of HB antigen(s) on neuroblasts was also sought utilizing a RAA. The 1st step was similar to that used for immunofluorescence: 1.5 × 10⁶ cells were incubated with the test antisera or control sera at dilutions of 1:50 to 1:800. All samples were run in duplicate. The cells were then washed twice in 0.1% bovine serum albumin (Sigma Chemical Co.) in PBS and incubated for 30 min at 4° with 50 µl of ¹²⁵I-labeled goat IgG containing 25 µg of antibodies to rabbit IgG. The preparation of this reagent and determination of the optimal concentration for this assay have been described previously (25). The cells were washed 3 times in 0.1% bovine serum albumin in PBS, the pellet was resuspended in 1 ml of normal 0.9% NaCl solution, and the radioactivity was counted in a Packard autogamma scintillation spectrometer. The specific binding was expressed as cpm of test antiserum minus cpm of control serum. The specific binding of anti-HB serum to SK-N-SH cells was 1500 ± 200 and 1450 ± 300 at 1:50 and 1:100 dilutions, respectively. Specific binding decreased proportionally at higher dilutions. Therefore, HB antiserum was used at 1:50 or 1:100 dilutions in all experiments.

### Regeneration of Antigen(s) on Neuroblastoma Cell Surface Cultured with and without FCS

Neuroblastoma cells were cultured as described above. When a confluent growth had been reached (usually Day 5), the culture media and nonadherent cells were decanted from the flasks. Two ml of 0.1% Pronase (Calbiochem, San Diego, Calif.) in PBS, pH 7.4, were added to the flask, the flask was quickly agitated, and the Pronase-PBS was decanted. One ml of 0.1% Pronase-PBS was added, and the flask was incubated at 37° for 30 min. Ten ml of media (RPMI 1640 with 10% FCS) were added to stop the proteolytic activity of the Pronase. The cells were then washed 3 times in RPMI 1640 alone. An aliquot of these cells was tested for binding of HB antiserum by immunofluorescence. The rest of the cells were cultured with and without 10% FCS with RPMI 1640 and 1% Pen-Strap at a concentration of 25 × 10⁹/ml in loosely capped plastic tubes (Falcon Plastics, Oxnard, Calif.) in a 5% CO₂ incubator at 37°. After 2, 4, 8, 12, 16, or 24 hr of incubation, the cells were washed 3 times with PBS and assayed for brain-associated antigen(s) by immunofluorescence.

### Absorption Experiments

HB, liver, and kidney were obtained at autopsy from a 5-year-old boy. Brain was also obtained from C3H mice and guinea pigs (Camm Research Institute, Wayne, N. J.). The various tissues were minced with scissors and then homogenized. Two-ml aliquots of the homogenates were placed in glass bottles, lyophilized, and stored at −20°.

All absorptions were carried out at 37° for 30 min and 4° for 30 min. Fifty µl of antiserum were absorbed with the cell number or tissue weight as specified in each experiment. After absorption the antiserum was centrifuged at 2500 rpm for 30 min at 4° in an International PR-6 centrifuge.

### RESULTS

#### Reactivity of HB Antiserum with Neuroblasts

By immunofluorescence and a RAA, antiserum to HB reacted with neuroblasts from 2 cell lines (Table 1). By immunofluorescence, we consistently demonstrated the presence of HB cell-surface antigen(s) on more than 90% of neuroblasts derived from the cell line SK-N-SH. The reactivity of anti-human brain serum with SK-N-SH neuroblasts was also demonstrated by the RAA. The antiserum also bound to cells from another human neuroblastoma cell line, IMR-32.

<table>
<thead>
<tr>
<th>Cell source</th>
<th>No. of experiments</th>
<th>Immunofluorescence (% positive cells)</th>
<th>Specific binding (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK-N-SH</td>
<td>15</td>
<td>&gt;90</td>
<td>1316c (1010–1625)d</td>
</tr>
<tr>
<td>IMR-32</td>
<td>1</td>
<td>&gt;90</td>
<td>ND*</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>3</td>
<td>&gt;80</td>
<td>ND</td>
</tr>
<tr>
<td>Pleural fluid</td>
<td>3</td>
<td>&gt;80</td>
<td>1188f (1114–1263)</td>
</tr>
<tr>
<td>Primary tumor</td>
<td>3</td>
<td>&gt;80</td>
<td>ND</td>
</tr>
<tr>
<td>Lymph node</td>
<td>1</td>
<td>&gt;80</td>
<td>ND</td>
</tr>
<tr>
<td>Metastases</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frozen primary</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor</td>
<td>1</td>
<td>&gt;80</td>
<td>ND</td>
</tr>
</tbody>
</table>

* From 0 to 3% of target cells incubated with control antiserum (normal rabbit or anti-Hodgkin's disease cell line) were positive by immunofluorescence.

f Specific binding is expressed as cpm of test antiserum minus cpm of control sera. The binding of control serum ranges from 371 to 416 cpm.

d Mean of 5 separate experiments.

e Number in parentheses, range.

f ND, not determined.

f Values from 1 patient.
Positive reactivity of antisera to cells maintained in culture media containing FCS may be due to FCS protein(s) absorbed onto the cell membrane. If binding of anti-HB serum were due to the presence of antibodies against human proteins which may cross-react with antigens present in FCS, one would expect that different cell lines grown in FCS-containing media would exhibit binding with anti-HB serum. By immunofluorescence, 2 normal cell lines (Chang liver and embryonic lung) and 5 of 6 lines derived from various other neoplasms (glioblastoma, fibrosarcoma, pharyngeal carcinoma, Wilms' tumor, and Raji) were nonreactive with the anti-HB serum. The only cell line reacting with the HB antisera was an embryonal rhabdomyosarcoma line in which 78% of the rhabdomyoblasts were immunofluorescent positive. Preliminary data from quantitative absorptions indicate that neuroblasts and embryonal rhabdomyosarcoma cells express the same antigen(s) but at a different cell-surface density. Absorption of HB antisera with $5 \times 10^6$ neuroblasts removed the serum's reactivity with neuroblastoma cells, whereas the antisera still reacted against neuroblasts after absorption with the same number of rhabdomyosarcoma cells. However, when the number of cells for absorption was increased to $20 \times 10^6$, both neuroblasts and rhabdomyosarcoma cells abolished the antibody binding to human neuroblasts. Experiments in progress should establish whether this antigen is also expressed on rhabdomyosarcoma cells from children with this cancer. We also tested for reactivity between unabsorbed HB antisera and FCS by double immunodiffusion. No precipitin lines were detected.

Further evidence that the antigen(s) detected by HB antisera was synthesized by the SK-N-SH cells was obtained by treatment of the cells with the proteolytic enzyme Pronase, as described under "Materials and Methods." By immunofluorescence, we found that anti-HB serum did not react with neuroblasts immediately after treatment with Pronase. However, within 12 to 18 hr of incubation at 37°C in a culture medium devoid of FCS, 75 to 80% of the neuroblasts exhibited surface immunofluorescence, indicating the reappearance of HB antigen(s). The kinetics of regeneration of HB antigen(s) was similar in neuroblasts recultured in media with or without FCS.

The positive immunofluorescent reactivity against SK-N-SH was abolished by absorption of anti-HB serum with 0.5 mg of brain tissue obtained from a 5-year-old child at autopsy. Less than 1% of the neuroblasts were reactive with HB antisera after absorption with the brain tissue. However, there was no detectable alteration in reactivity between HB antisera unabsorbed or absorbed with 5 times as much (2.5 mg) kidney or liver from the same person. More than 90% of the neuroblasts were positive. The specificity of the antisera was investigated by comparing reactivity of anti-HB serum with neuroblasts after the antisera had been absorbed with lyophilized human, mouse, or guinea pig brain. As already described, binding of the HB antisera to neuroblasts could be abolished by prior absorption with 0.5 mg of lyophilized HB (Table 2).

This was demonstrated using both the immunofluorescence and RAA techniques. Absorption of the antisera with guinea pig or mouse brain antigen did not reduce the number of immunofluorescent-positive neuroblasts. However, the RAA demonstrated a reduction in specific binding. Approximately half of the activity remained after absorption of the antisera with the same amount (2.5 mg) of mouse or guinea pig brain. This suggests that the remaining activity of HB antisera against SK-N-SH cells is species specific.

It has been suggested that a basic protein of nervous tissue may bind IgG via the Fc position (1). It is therefore possible that antibody absorption on brain tissue or neuroblastoma cells may be due to nonspecific binding of IgG on this tissue or cells. Therefore brain, liver, and neuroblasts were used to absorb anti-thymus serum for which reactivity with thymocytes and circulating T-lymphocytes has been described (8, 25, 34). The anti-thymus serum was absorbed with 0.5 or 2.5 mg of brain or liver from the same person, or $20 \times 10^6$ SK-N-SH cells or tumor cells from a child with neuroblastoma. The reactivity of anti-thymus serum against human thymocytes did not differ before or after absorption with any of these cells or tissues as described above. By immunofluorescence, the number of positive thymocytes

### Table 2

<table>
<thead>
<tr>
<th>Tissue for absorption</th>
<th>None</th>
<th>HB</th>
<th>Mouse brain</th>
<th>Guinea pig brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain tissue (mg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immunofluorescence (% positive cells)*</td>
<td>&gt;90</td>
<td>ND*</td>
<td>ND &gt;90</td>
<td>ND &gt;90</td>
</tr>
<tr>
<td>Specific binding (cpm)*</td>
<td>(1537–1778)</td>
<td>0</td>
<td>889</td>
<td>760</td>
</tr>
<tr>
<td>% Reactivity*</td>
<td>100</td>
<td>54</td>
<td>46</td>
<td>47</td>
</tr>
</tbody>
</table>

* From 0 to 3% of target cells (SK-N-SH) incubated with control antiserum (normal rabbit or anti-Hodgkin’s disease cell line) were positive by immunofluorescence.

ND, not done.

* Specific binding is expressed as cpm of test antiserum minus cpm of control sera. The binding of control serum ranged from 458 to 522 cpm.

* Numbers in parentheses, range.

* Percentage reactivity = (specific binding absorbed/specific binding unabsorbed) × 100.
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with absorbed or nonabsorbed antisera ranged from 70 to 90%. In a separate experiment we also tested the binding of these antisera against an enriched fraction of circulating T-lymphocytes. Again, no difference was detected; the proportion of positive cells ranged from 60 to 80%. Thus, under these experimental conditions and with these antisera, brain absorbs the activity of HB antigen(s) expressed on neuroblasts, but not the binding of anti-thymus to T-cells, indicating that the absorption is specific and not due to the removal of IgG antibodies by nervous tissue basic protein.

Anti-HB serum was also tested against neoplastic cells obtained from children with neuroblastoma. As shown in Table 1, the results with tumor cells obtained either from the bone marrow, pleural fluid, or tissue biopsy specimens in 6 children with neuroblastoma indicate that HB antigen(s) is not expressed solely by long-term cultured neuroblasts. Regardless of the source, we found that more than 80% of neuroblasts obtained from these patients reacted positively with the anti-HB serum. This was confirmed by the RAA with cells obtained from the biopsy specimen of 1 patient. Viable neuroblasts obtained from 1 child were frozen and upon thawing were still capable of binding anti-HB serum.

As shown in Table 3, absorption of the anti-HB serum with 20 x 10^6 neuroblasts from the SK-N-SH cell line or a patient’s tumor removed essentially all reactivity against SK-N-SH cells as determined by immunofluorescence (<1% positive cells) and RAA (0 to 100 cpm). These results suggest that the antigen(s), detected by the anti-HB serum, is not an HLA antigen(s).

Reactivity of HB Antiserum with Normal and Leukemic Lymphoid Cells. Having demonstrated that anti-HB serum detects an antigen(s) on neuroblasts, we asked whether this antisera would also react with normal and leukemic lymphocytes. Less than 1% of the thymocytes were positive by the immunofluorescence assay (Table 4). Normal bone marrow lymphoid cells were also nonreactive. However, anti-HB serum was bound to a small but consistent population (5 to 10%) of normal PBL. It is unlikely that these positive immunofluorescent cells were monocytes, because by phase microscopy, they were smaller than monocytes and also peroxidase negative. Absorption experiments were performed to determine whether the anti-HB serum was detecting the same antigen(s) on neuroblasts and this subset of PBL. Anti-HB serum was tested against lymphocytes derived from normal donors and from a child with neuroblastoma at the time of diagnosis. The assay was performed before and after absorption of the antisera with either 20 x 10^6 SK-N-SH cells or neuroblasts derived from the same patient. By immunofluorescence, the number of positive lymphocytes (5 to 10%) did not change after absorption. By the RAA, the specific binding to allogeneic lymphocytes was low (100 to 167 cpm) but was similar whether absorbed or unabsorbed sera were tested. This indicates that antibodies to HB react with a different antigen(s) expressed on neuroblasts and a subset of human PBL.

We had also studied bone marrow blasts from 21 patients with untreated ALL. Five of these patients had T-cell ALL. This was established by the fact that lymphoblasts from these patients formed rosettes with sheep erythrocytes and reacted with heterologous antisera to human thymus (anti-T) and to erythrocyte rosette-forming ALL blasts (anti-E^+ ALL). The other 16 children had E^- (Non-T, Non-B) ALL. Table 4 shows that anti-HB serum did not react with bone marrow blasts from patients with either E^+ or E^- ALL. Lymphoblasts from an adult with B-cell leukemia (chronic lymphocytic leukemia with 80% of the cells bearing μ-heavy- and κ-light-chain markers) also failed to bind anti-HB serum.

These experiments did not exclude the possibility that antigenic determinants present on thymus and not on HB may be expressed by neuroblasts. Therefore, we tested anti-E^+ ALL and anti-thymus sera against neuroblasts and human thymocytes. It has been previously shown that both antisera react with thymus-associated antigen(s) (8, 25, 34). Data from a representative experiment are shown in Table 5. By immunofluorescence, greater than 80% of thymocytes were positive with anti-T and anti-E^+ ALL sera. Neuroblasts were negative. Similar results were obtained in additional experiments using thymocytes from 3 different donors and...
The HB antigen(s) expressed by neuroblasts was not detected in the mouse brain antigens studied, all were present in adult tumors by an antiserum prepared against human fetal brain. The antigen(s) detected by the HB antiserum is not present when these same antisera were incubated with neuroblasts. However, this HB antiserum did detect, on a small population of PBL and thymocytes from a 14-week-old fetus. Expression of different antigen(s) could be explained by the age of the fetal brain used for production of the antiserum. Brouet and Toben used the brain of a 21-week-old fetus, whereas our brain tissue was from newborn mice. Birnbaum (5) demonstrated that brain tissue from newborn mice did not share a common antigen(s) with thymocytes. By immunofluorescence, Brouet and Toben (10) found that HB antiserum prepared with fetal brain did not react with human thymocytes. None of these authors investigated whether brain antigen(s) was present on mouse or human malignant neuroblasts.

An interesting observation by Brouet and Toben was that, although thymocytes were nonreactive with HB antiserum, there was a small population of PBL that had this brain antigen(s). We obtained similar data. However, the number of reactive lymphocytes reported by these investigators was approximately 3 times the number detected by us. This could be explained by the age of the fetal brain used for production of the antiserum. Brouet and Toben used the brain of a 21-week-old fetus, whereas our brain tissue was from a 14-week-old fetus. Expression of different antigen(s) by fetal brain may correlate with maturation and cell differentiation. Thus, the detection of brain antigen(s) on different subpopulations of PBL and thymocytes may depend on the age of the brain. There is evidence for this in mice (5, 23).

The RAA confirmed these results. The specific binding of E⁺ ALL and thymus antisera with thymocytes was 917 and 1010 cpm, respectively. Specific binding was not detected when these same antisera were incubated with neuroblasts. In contrast, the binding of HB antiserum to neuroblasts was 1408 cpm but only 33 cpm with thymocytes, which is within the variation of control values. Therefore we conclude that the antigen(s) detected by the HB antiserum is not present on normal or malignant T-cells and, conversely, that thymus-associated antigens are not present on the cell surface of neuroblasts.

**DISCUSSION**

This study demonstrates that a brain-associated antigen(s) is detected on malignant neuroblasts derived from established human cell lines and children with neuroblastoma by an antiserum prepared against human fetal brain. The HB antigen(s) expressed by neuroblasts was not removed by absorption with kidney or liver, suggesting that it is not present in these tissues. This antigen(s) was not present on normal thymocytes, normal bone marrow lymphoid cells, or E⁺ or E⁻ lymphoblasts from children with ALL. However, this HB antiserum did detect, on a small population of lymphocytes, a brain-associated antigen(s) that was different from antigen(s) expressed on malignant neuroblasts.

Several studies in mice have attempted to define brain-associated antigens expressed by malignant neuroblastomas (2, 21, 31). Schachner et al. (32) have recently summarized the antigenic complexity of mouse brain. They reported that of 6 mouse brain antigens studied, all were present in adult brain and only 2 on fetal brain. These 2 fetal mouse brain antigens were designated “nervous system antigen” and NS-4 and were not detectable on the murine C1300 neuroblastoma cell line. Thus, these mouse fetal brain antigens do not appear to correspond to the HB antigen(s) described in our study. On the other hand, Seeds (33) has reported that antiserum raised against mouse cerebellum killed C1300 neuroblasts and that this reactivity was not absorbed with human neuroblasts. This suggests that there is a differentiation antigen(s) on C1300 cells that is species specific. HB antigen(s) present on human neuroblasts appears to be species specific because absorption of HB antiserum with mouse or guinea pig brain did not abolish its reactivity against SK-N-SH neuroblasts.

The data presented do not exclude the possibility that the antigens detected by the HB antiserum are expressed on other fetal cells, i.e., fetal or embryonic antigens. This is unlikely, because (a) reactivity against neuroblasts was completely absorbed with nonfetal brain, (b) the HB antiserum had been previously absorbed with autochthonous fetal liver as described above, and (c) the HB antiserum did not bind to cells from an embryonic cell line. It has previously been shown in both the murine and human systems that brain and thymus share common antigen(s) (5, 14, 23). Consequently, we expected that the HB antigen(s) expressed by neuroblasts would also be detected on the membrane of thymocytes. This did not occur. There are precedents for the lack of reactivity of brain antiserum with thymocytes. Birnbaum (5) demonstrated that brain tissue from newborn mice did not share a common antigen(s) with thymocytes. By immunofluorescence, Brouet and Toben (10) found that HB antiserum prepared with fetal brain did not react with human thymocytes. None of these authors investigated whether brain antigen(s) was present on mouse or human malignant neuroblasts.

In mice, Thy-1 antigen has been detected in low concentration on the surface of a transplantable tumor derived from the C1300 cell line (30). Our data with the HB antiserum indicate that the HB antigen(s) detected on neuroblasts is not present on thymocytes. However, this does not exclude the possibility that other thymocyte antigen(s) may be expressed by human neuroblastoma cells. To examine this possibility we investigated whether 2 antisera raised against normal thymocytes and thymus-derived ALL blasts would react with human neuroblastomas. By immunofluorescence and a RAA, we demonstrated that the T-antigens detected by these antisera (8, 25, 34) were not present on human neuroblasts (Table 5).

There have been reports delineating an immune response to human neuroblastoma cells in children with this tumor,
in their relatives, and in unrelated controls (15). Hellström et al. (18) and Jose and Sesmadri (20) have demonstrated the presence of antibodies to neuroblastoma cells and antigen-antibody complexes in the sera of these patients. The antigenic portion of immune complexes present in children with progressive disease appears to be a soluble, tumor-type-specific component (20). However, the specificity and chemical nature of these neuroblastoma-associated antigens have not been defined. A prerequisite to the understanding of the biological and clinical significance of cell-surface antigens on human tumors is the categorization of these antigens according to their specific characteristics. Are they fetal, differentiation, or tumor specific? In this study we have demonstrated that some of the antigens expressed by human neuroblastoma cells may not be related to neoplastic transformation, but instead reflect cell origin and differentiation. Therefore, more knowledge in this area would be required before rational immunotherapy can be planned for children with neuroblastoma and specific tests developed to monitor their response to treatment.

The identification of HB antigen(s) on malignant neuroblasts could have diagnostic value. Neuroblastoma may be confused with acute leukemia, especially in children with extensive bone marrow involvement (13, 27, 29). The immunological differentiation between these 2 diseases should now be possible by the recognition of antigens on neuroblasts that are not expressed on blasts from children with acute leukemia.

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Reactivity of Human Brain Antiserum with Neuroblastoma Cells and Nonreactivity with Thymocytes and Lymphoblasts

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