Correlation of Immune Complexes in Disseminated Neuroblastoma with Serum Antibody to Bovine Serum Albumin

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ABSTRACT

To evaluate the relationship between tumor burden and circulating immune complexes (IC) in children with neuroblastoma (NBL), we studied sera collected at intervals from patients with disseminated (Stage III or IV) NBL. Sera from 10 of 12 patients contained IC by the Raji cell assay at some time during the first 9 to 11 months of the study. Higher IC levels were observed in sera of female patients. Fluid-phase C1q binding tests detected IC in only 16% of sera. IC measurements by either assay did not correlate with tumor burden. However, serum IC levels, as measured by the Raji cell assay, correlated significantly with serum antibody to bovine serum albumin (BSA) (r = 0.54; p < 0.001, r = r as determined by Spearman rank correlation test). Measurement of anti-BSA antibodies in sera from the 12 patients, tested serially for circulating IC, and from five additional patients revealed that these had significantly higher anti-BSA activity than was found in sera from 13 age-matched controls. Sera from females also had relatively high levels of anti-BSA. Levels of antibody to bovine γ-globulin and casein were not abnormal. Three sera with high IC levels (>800 μg equivalents of heat-aggregated IgG) and relatively low anti-BSA activity appeared to contain "hidden" antibodies to BSA. These were demonstrated by measuring the increase in the ability of sera to bind 125I-BSA after they had been briefly acidified and then neutralized in the presence of the labeled BSA. The possible relevance of these results to the pathophysiology of NBL is discussed in light of earlier work that reported idiotypic antibodies or other anti-immunoglobulins (21, 24, 25).

Once the tumor is diagnosed, one would suspect that subsequent changes in serum IC levels should also follow changes in tumor burden. We now report a longitudinal study of patients with disseminated NBL, followed for an average of 9 months. Previous studies have reported a correlation between serum IC and the staging of this tumor (6). We observed no significant relation between serum IC levels and tumor burden. Instead, we noted that serum IC levels correlated significantly with serum antibody to BSA. "Hidden" or "blocked" antibodies to BSA were found in a few sera with particularly high IC levels. These studies suggest that some soluble IC in sera of patients with NBL may contain BSA-like antigens bound to anti-BSA.

MATERIALS AND METHODS

Patients. Patients with disseminated NBL (Stages III, IV, IV-S, and one with Stage II disease with a subsequent metastatic occurrence) were included after informed consent had been received. Diagnosis was based on histological examination of excised tissue. Staging reflected current classification criteria (15). At each visit, patients were also classified as having progressive disease, a >25% increase in previously recognized tumor and/or development of one or more new lesions, or as being in remission, defined as the absence of detectable tumor. Blood samples were collected at intervals which depended on the patients' treatment schedule and need for venipuncture. One to 20 ml of blood were collected at room temperature (22°C) and allowed to retract at 4°C for up to 24 hr. Sera were stored in small aliquots at –70°C. Frozen sera from 13 healthy age- and sex-matched children were kindly provided by Dr. Arthur L. Frank, Baylor College of Medicine.

IC Detection. The fluid-phase C1q binding assay was performed, using EDTA-treated sera (37). The normal range was <6.2% (95% confidence limits) as established by testing 179 adult volunteers (26). Raji cell RIAs (34) were performed in the laboratory of Dr. Noorbibi K. Day, Sloan-Kettering Institute, New York, N. Y. Values for healthy age-matched children were 0 to 16 μg equivalents of aggregated human IgG per ml of 1.4 diluted serum. Variance of triplicate determinations was 4% of the mean (27).

Quantitative Measurement of Anti-BSA, Anti-Casein and Anti-BGG Activities. Anti-BSA was measured by the ammonium sulfate method (23). BSA (Pentex; bovine albumin Fraction V; Miles Laboratories, Kankakee, Ill.) was labeled with 125I by the chloramine-T method (18). More than 97% of the isotope was protein bound as shown by trichloroacetic acid precipitation at a specific activity of 0.4 to 0.8 mCi/mg. Stock solutions of 125I-BSA were diluted to 3 μg/ml for the assay. Duplicate aliquots of sera were diluted initially 1:10 with BBS, pH 8.4, and 50 μl each of the diluted serum and antigen were mixed in test tubes precoated with 5% ovalbumin phosphate-buffered saline. After overnight incubation at 4°C, saturated ammonium sulfate was added to a final concentration of 50%; test tubes were incubated at 4°C for 30 min and centrifuged at 700 × g for 30 min. Precipitates were washed because they have combined with antigens shed by the tumor (19, 32) or because they have formed complexes with anti-idiotypic antibodies or other anti-immunoglobulins (21, 24, 25).
once with 50% ammonium sulfate in BBS. Results were expressed as percentage of antigen precipitated (23). Quantitative measurement of anti-BGG and anti-casein activities were performed in the same manner using polyethylene glycol (M, 6000, average) at final concentrations of 7.5 and 10%, respectively, to precipitate bound antigen (22). BGG (Pentex Fraction II) and γ-casein (ICN-Nutritional Biochemicals, Cleveland, Ohio) were also labeled with 125I to a specific activity of 0.4 to 0.8 mCi/mg. Antigen and serum were mixed in the same proportions and dilutions as in the assay of anti-BSA activity and incubated for 30 min at 37°C and then overnight at 4°C before addition of polyethylene glycol.

Because the quantities of sera available were limited, we were able to test only 29 of the 30 sera with high levels of IC (>16 μg equivalents of heat-aggregated γ-globulin) for antibody to BSA, BGG, and casein.

**Dissociation of IC.** Acidification (pH 3.0) to dissociate IC (17) was performed by adding 980 μl of 0.2 M glycine-HCl buffer, pH 2.8, containing 0.15 M NaCl, to duplicate 50-μl aliquots of serum diluted in BBS. After 30 min at room temperature, 50 μ1 of 125I-BSA diluted to 10 μg/ml were added, and the mixture was incubated for 30 min at 37°C and then overnight at 4°C. The next morning, samples were neutralized (pH 7.5) by adding 20 μl of 3 M Tris base containing 0.15 M NaCl. Samples were then assayed for anti-BSA activity by the routine Farr assay. Nonacidified samples of the same sera were always tested in parallel. BBS was added to the control samples in the same volume as were the acidifying and neutralizing buffers used in serial samples. The mean variation in replicate counts was ±5% (S.D.) on acidified samples ±7% and on nonacidified samples.

**Antisera.** Polyclonal rabbit anti-BSA prepared in this laboratory was affinity purified by adsorption over BSA bound to Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N. J.). Commercially available rabbit anti-BSA was also used (Calbiochem-Behring Corp., La Jolla, Calif.; Lot 009601). Normal rabbit serum (Grand Island Biological Co., Grand Island, N. Y.) served as control.

**Miscellaneous.** Protein concentration was determined by a dye-binding assay (Bio-Rad protein assay; Bio-Rad Laboratories, Richmond, Calif.) using BGG as a standard. IgA levels in sera were measured by single radial diffusion using monospecific antisera (Meloy Laboratories, Springfield, Va.).

**Statistical Tests.** The nonparametric Mann-Whitney U test was used to compare groups of results. The Spearman rank correlation coefficient test was used to correlate results of Raji tests and anti-BSA activity in sera. The Wilcoxon matched signed ranks test was used to compare anti-BSA activity with and without precipitation of the sample (31).

**RESULTS**

**Characteristics of the Patient Population.** Seventeen patients were followed longitudinally. Ten were boys and 7 were girls, with a median age of 4 years (range, 2 months to 14 years). Nine were Stage IV, 4 were Stage IV-S, 3 were Stage III, and 1 was Stage II with later metastasis. Sixteen underwent treatment at this institution, and 1 case was evaluated only. Eleven entered the study at the time of diagnosis; 6 entered while undergoing treatment. The period of study averaged 9 months (range, 1 to 22 months). Presently, 8 are dead and 6 are alive (including 4 with active disease). Three are lost to follow-up. A total of 64 serial samples from the first 12 patients were tested for IC by the Raji cell RIA; 62 of these 64 sera were also tested by the C1q binding test. Sera from these 12 patients and 20 additional samples from 5 more patients were assayed for antibody to BSA and other milk proteins.

All patients had ingested cow's milk before diagnosis. None had a history of cow's milk hypersensitivity, a malabsorption syndrome, or growth measurements below the tenth percentile at the time of diagnosis. None was deficient in serum IgA.

Every patient received chemotherapy as basic treatment, with or without surgery. Nine also received radiation therapy during the study. The control group had a mean age of 3 years (range, 16 months to 13 years).

**IC Determinations.** Sixty-two samples from 10 patients were tested by the C1q binding test. Ten sera were positive (16.1%). Abnormal values were detected in sera from 4 patients; 6 were serial samples from 1 patient. Tumor burden increased on at least 13 occasions, and 3 patients died without attendant changes in C1q binding assay.

Thirty of a total of 64 sera (47%) from 12 patients contained IC according to the Raji cell test (Table 1). Ten of the 12 patients (83%) had IC at some time during follow-up. The quantity of IC, when present, greatly exceeded the quantity detected in previous sequential studies of normal volunteers using this assay (27). On 5 occasions, the Raji IC measurements decreased during intervals when the tumor burden increased. Tumor burden remained unchanged or decreased on 7 occasions when the Raji levels increased. Moreover, there were 5 occasions when tumor burden increased or decreased significantly without an interval change in serum IC level. There were also 4 intervals during which the IC levels decreased, but no change in tumor burden was observed. Thus, even though there were 11 occasions when tumor burden and Raji levels changed in the same direction, measurement of IC by the Raji cell assay did not provide a reliable indicator of change in tumor burden (Table 1). When separated into 2 groups according to sex and then ranked, female patients exhibited significantly higher IC levels than did males (p = 0.01, Mann-Whitney U test).

**Antibodies to BSA and Other Milk Antigens.** In initial experiments to evaluate whether patient sera contained antibodies reactive with cultured NBL cells, Tween 80 lysates of a neuroblastoma cell line, LAN-1 (30), extrinsically labeled with 125I, were used as antigen. The labeled extracts inevitably contained traces of BSA, which was present in the fetal calf serum used to maintain these cells in culture. The BSA also became labeled with 125I along with the tumor cell proteins. When the sera were evaluated for antibody to these tumor cell extracts, it became clear that NBL patient sera preferentially precipitated more 125I-BSA than did sera from controls (data not shown).

To determine if there were similar increases in antibodies to other bovine antigens commonly present in food and to discover the relationship of these antibodies to the soluble IC found in these sera, we quantitated serum antibodies to BSA, BGG, and γ-casein. Anti-BSA activity in the patients' sera exceeded that found in age-matched controls (Chart 1). Taking the initial sample from each patient as a random sampling of those with NBL, the results still showed that they had higher anti-BSA activity (p < 0.05, Mann-Whitney U test). In contrast, there were no significant differences in the levels of anti-BGG and anti-casein antibodies in patient and control sera (Chart 1). Serial samples from the same patient generally had either persistently high or persistently low levels of anti-BSA antibody (Table 2). Six of the 7 females belonged to the group with abnormally high anti-BSA antibody (Table 2) (p < 0.02, Mann-Whitney U test).

The anti-BSA activity of the NBL patient sera correlated significantly with the levels of IC as measured by the Raji cell
Table 1

Serial changes in tumor burden and circulating IC in patients with neuroblastoma

Values of C1q binding test are not shown. Only 10 sera gave abnormal values. These abnormal results were clustered in 4 patients (Patients 2, 4, 5, and 10). Four of these positive samples were in sera from Patient 2.

<table>
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<th>Patient</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>Stage</th>
<th>0 mo.</th>
<th>1 mo.</th>
<th>2 mos.</th>
<th>3 mos.</th>
<th>4 mos.</th>
<th>5 mos.</th>
<th>6 mos.</th>
<th>7 mos.</th>
<th>8 mos.</th>
<th>9-11 mos.</th>
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<td>N</td>
<td>N</td>
<td>R</td>
<td>PD</td>
<td>N</td>
<td>R</td>
<td>N</td>
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<td>M</td>
<td>7</td>
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<td>R</td>
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<td></td>
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<td></td>
<td></td>
<td>Alive (17)</td>
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*TS, tumor status; PD, progressive disease; R, remission; N, <16 µg equivalents heat-aggregated γ-globulin.

Discussion

Nearly one-half the sera tested had IC, as measured by the Raji cell RIA. Of the 10 sera that had abnormal Raji cell values at some point in their course, only 1 had IC detectable by the C1q binding test, changes in the quantity of IC, as measured by either test, did not correlate with changes in tumor burden. Raji cell IC levels, however, correlated significantly with serum anti-BSA activity. The latter correlation was wholly unexpected and was discovered serendipitously as a consequence of attempts to evaluate these patients' sera for antibodies to antigens present on cultured NBL cells.

We initially postulated that they might have developed hypersensitivity to bovine serum protein antigens in their food. Antibodies to bovine albumin and other milk antigens are almost ubiquitous in sera of young children from countries where cow's milk is an important constituent in the diet (5, 7, 14, 20). The decline in serum antibodies to milk antigens after the first 15 months of life, seen in most normal children (20), may reflect the fact that continued oral ingestion of antigens promotes immunological tolerance (2, 10, 35).

A persistent circulating antibody response to food antigens is found in sera of children with IgA deficiency (13). Failure to develop a persistent circulating antibody response to food antigens apparently allows milk proteins to pass into the mesenteric circulation and stimulates a circulating antibody response to these antigens in the gut wall (13). This results in formation of circulating IC that can bind to these antigens in the gut wall and stimulate the formation of circulating IC. Failure to develop a secretory IgA response to food antigens, as seen in patients with selective IgA deficiency (13), may be the reason that some children have a persistent circulating antibody response to food antigens.

In attempting to explain this persistent antibody response to food antigens, we postulated that these children may have a persistent circulating antibody response to food antigens and that this response may be associated with a persistent circulating antibody response to antigens present on cultured NBL cells. In patients with a persistent circulating antibody response to food antigens, this persistent antibody response to food antigens may be associated with a persistent circulating antibody response to antigens present on cultured NBL cells.

In developing this hypothesis, we postulated that the persistent antibody response to food antigens may be associated with a persistent circulating antibody response to antigens present on cultured NBL cells. In patients with a persistent circulating antibody response to food antigens, this persistent antibody response to food antigens may be associated with a persistent circulating antibody response to antigens present on cultured NBL cells.

In patients with a persistent circulating antibody response to food antigens, this persistent antibody response to food antigens may be associated with a persistent circulating antibody response to antigens present on cultured NBL cells. In patients with a persistent circulating antibody response to food antigens, this persistent antibody response to food antigens may be associated with a persistent circulating antibody response to antigens present on cultured NBL cells. In patients with a persistent circulating antibody response to food antigens, this persistent antibody response to food antigens may be associated with a persistent circulating antibody response to antigens present on cultured NBL cells.
Increased gastrointestinal permeability might also result in an abnormal circulating antibody response to bovine serum protein antigens. Gastrointestinal permeability is abnormal in a small percentage of children with NBL either as a result of treatment or because their tumor cells release vasoactive intestinal peptide (12). This, also, was probably not an important cause of the high anti-BSA and IC levels in these children. As with selective IgA deficiency, one would expect that serum antibodies to antigens other than BSA would also be increased if gastrointestinal permeability to food antigens was increased. None of these patients had diarrhea or other abnormalities associated with vasoactive intestinal peptide secretion (12).

We also considered the possibility that the high frequency of positive results in the Raji cell RIA might be a technical artifact related to the increased levels of anti-BSA in these sera. Because Raji cells are grown in media containing fetal calf serum (34), addition of sera containing high levels of anti-BSA to suspensions of Raji cells might cause formation of BSA:anti-
BSA complexes in vitro. It is unlikely, however, that this was an important explanation for the relationship shown in Chart 2. A number of sera that were totally nonreactive in the Raji cell RIA had high anti-BSA levels. If formation of BSA:anti-BSA complexes in vitro had contributed significantly to the detection of IC by the Raji cell assay, one would expect all sera with high anti-BSA levels to give positive tests.

To further examine the relationship between anti-BSA and IC in these sera, we evaluated whether any that contained high IC levels and relatively low anti-BSA activity might contain hidden or blocked antibodies to BSA. The hypothesis underlying these experiments was that such sera might also contain large quantities of anti-BSA, but that these antibodies would be inactivated because they were bound to BSA-like antigens. In 3 cases, acidification of sera in the presence of $^{125}$I-BSA increased the quantity of $^{125}$I-BSA precipitated after acidification and neutralization. Five sera that already had high anti-BSA and relatively low IC levels did not appear to contain "hidden" anti-BSA antibodies when tested prior to acidification. One other serum (Chart 3, serum II-4) with high levels of IC had only a small negligible increase in anti-BSA activity when tested after acidification and neutralization. Five sera that already had high anti-BSA and relatively low IC levels did not appear to contain "hidden" anti-BSA antibodies when tested under these conditions. In some sera, the quantity of $^{125}$I-BSA precipitated after acid treatment was less, suggesting that the acid may have damaged antibodies in these sera. While these results do not prove that all IC in these sera were caused by anti-BSA bound to BSA-like antigens, they suggest that some may have resulted from this antigen-antibody system.

The nature of the BSA-like antigens that promote these responses remains unknown. These antigenic substances could include fragments of BSA derived from food; anti-idiotypes specific for anti-BSA antibodies; or tumor-cell products that cross-react with BSA. In fact, at any time point in the course of the disease, more than one of these may be incorporated in the circulating IC.

In another paper, we show that some NBLs produce proteins that cross-react with BSA (4). Anti-idiotypes have also been recognized as a component of certain naturally occurring IC (24, 25). Finally, Cunningham-Rundles, et al. (13) have pointed out that even in individuals with a normal secretory immune system, small quantities of milk antigens can cross the gastrointestinal barrier. These may form circulating IC in individuals who have high levels of preexisting serum antibody to these antigens.

If food and tumor-derived antigens as well as anti-idiotypes can participate in the IC found in these children's sera, one may understand why the observed fluctuations in IC levels did not correlate with changes in tumor burden. In this hypothetical situation, different ligands would be competing for the same population of antibodies in the circulation at any particular moment. It is unlikely that each of these ligands for anti-BSA would enter the circulation in synchrony. For example, entry of BSA-like antigens into the blood, from either the gut or the tumor, could drive antigen-antibody complexes already in the circulation that contain anti-BSA toward high antigen excess. The circulating IC would become smaller. Perhaps they would become so small that they would be unable to fix sufficient complement to be detected by either the Raji or C1q binding...
tests. Likewise, at times when no BSA-like substances are circulating, the sudden entry of antigens cross-reactive with BSA from the gut or the tumor could result in transient showers of complement-fixing, circulating IC in patients with high serum anti-BSA levels. Under these circumstances, dietary BSA could become a source of both false-positive and false-negative results that would confound attempts to correlate serum IC levels with tumor burden.

Previous studies by Brandeis et al. (6) showed that circulating IC, as detected by the Raji cell RIA, correlated with the stage of disease in NBL. At first glance, our results appear to be at odds with these observations. But since the present study tested only one patient with Stage II and none with Stage I disease, we are not in a position to comment concerning the relationship between stage of disease and incidence of circulating IC in NBL. To the extent that we also find that IC are frequently demonstrable in sera of patients with Stage III or IV disease, our results agree with those of Brandeis et al. (6). The novel insight provided by the present investigation is the correlation of serum IC and anti-BSA activity. It is conceivable that quantities of tumor-associated BSA-like antigens, sufficient to stimulate high levels of anti-BSA, are present only in the later stages of the disease.

Surprisingly, in this very small sample of children with NBL, we observed a statistically significant predominance of serum anti-BSA and circulating IC in females. It may be useful in future studies of larger numbers of patients with NBL to evaluate if this observation can be sustained. However, it is well known that females often make more antibody than males when stimulated with diverse antigens (11, 36). This effect has been attributed to high estrogen levels. Relatively high serum antibody levels in females may actually be associated with a comparative deficiency of cell surface androgen receptors (11). The latter may be particularly important in explaining the superior antibody-forming abilities and relative resistance to infection in prepubescent females.

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