Circulating Immune Complexes in Sera of Children with Neuroblastoma

CORRELATION WITH STAGE OF DISEASE

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ABSTRACT The presence of circulating immune complexes (ICS) in freshly drawn sera of 67 children with neuroblastoma was studied by the Raji cell radioimmunoassay of Theofilopoulos et al. (*J. Clin. Invest.* 57: 169–182), with particular emphasis on the correlation of levels of ICS with stage of disease and changes attributable to treatment. There was a close correlation between amount of complexes and stage of disease and treatment. Levels of ICS increased as the stage of the disease advanced, and were significantly higher ($P < 0.005$) in stage IV than in all other stages combined. When patients with stage IV disease were subdivided into "before," "during," and "after" treatment groups, there was a significant decrease in ICS levels as treatment progressed. Studies of complement and complement components did not give such a clear relationship. A significant decrease of hemolytic C1 values was found in patients with "active disease" compared to normal age-matched controls. Some high C3 levels, determined immunochemically, were associated with low hemolytic levels of C3, which were attributed to C3 cleavage detected by immunoelectrophoresis. Based on our survival data, ICS, which were significantly different in 20 patients now deceased when compared to those of other patients, are very valuable in the prognosis of neuroblastoma.

INTRODUCTION

Neuroblastoma is a tumor of much interest to those involved in the immunology of neoplastic diseases. This interest stems from both the clinical evidence that suggests a role for host immune response against this tumor and laboratory evidence for a host immune reaction against tumor associated antigens (1). Neuroblastoma has the highest incidence of spontaneous regression

of any human neoplasm, primarily in children <1 yr of age (1–3). Sometimes neuroblastoma differentiates spontaneously, or after therapy, into a benign form, i.e., ganglioneuroma (4, 5).

Neuroblastoma was one of the first human tumors to be studied immunologically in vitro (6–8). Humoral antibodies to tumor antigens capable of killing neoplastic cells in vitro in the presence of complement were described by Hellström et al. (6, 9, 10) and Tamerius et al. (11). Using a colony inhibition technique, Hellström and his associates (6, 9, 10) showed that lymphocytes from patients with neuroblastoma were found to inhibit colony formation in vitro by the patient's own tumor cells and by tumor cells from other patients with the same disease. On the other hand, serum from patients with progressively growing neuroblastoma protected neuroblastoma cells from cytotoxic immune lymphocytes and did not inhibit colony formation.

This serum effect was first believed to be mediated by "blocking antibodies" (9). Further observations suggested that blocking factors are actually under a variety of circumstances antigen-antibody complexes (12–16).

In animal studies, Oldstone (17) showed that in neuroblastoma-bearing mice a deposition of soluble immune complexes can be demonstrated in the kidneys.

In this study we have investigated and correlated levels of circulating immune complexes (ICS)[1] with complement (C) components in sera of children with different stages of neuroblastoma.

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[1] *Abbreviations used in this paper*: AHG, aggregated human gammaglobulin; C, complement; $EAC1^{gp}$, sensitized sheep erythrocytes with guinea pig C1; $EAC4^{hu}$, sensitized sheep erythrocytes with human C4; $EAC1^{gp}4^{hu}$, sensitized sheep erythrocytes with guinea pig C1 and human C4; $EAC1^{gp}-7^{hu}$, sensitized sheep erythrocytes with guinea pig C1 and human C4–C7; ICS, immune complexes; NED, no evidence of disease; TCH50, total hemolytic complement.

METHODS

Patient population. The diagnosis was based on the following criteria: (*a*) clinical presentation (abdominal mass, severe diarrhea, cachexia, orbital hematoma) (18); (*b*) characteristic histopathology of biopsy (19); (*c*) 24 h urinary excretion of catecholamines over normal age-matched levels (20).

The patients were classified according to a scheme of staging, based on the extent of dissemination at the time of diagnosis (21) ranging from stage 1, when the tumor is confined to the tissue or organ of origin to stage IV, when distant metastasis in bones, soft tissue, liver, and skin are present. Stage IVs includes patients of stage I and II but with distant metastasis to skin, liver, or soft tissue and not to the bones. For the staging, all patients had a bone marrow biopsy and liver and bone scan. Nine patients had thoracic neuroblastoma, three with involvement of the spinal canal. In 26 patients we found lytic bone lesions. In this retrospective study, we used 123 serum samples of 55 patients of the Memorial Sloan-Kettering Cancer Center, and 12 patients of the Children's Hospital of the University of Heidelberg, Germany. As controls for all determinations, we used sera obtained from healthy age-matched children. They were free of infections and had no malignancies.

Distribution of neuroblastoma patients studied. The distribution of clinical stages of the 67 patients is presented in Table I. C levels and ICS were determined in 123 sera of the 67 patients. These sera were subdivided as follows: sera of patients with no evidence of disease (NED) ($n = 66$), with active disease ($n = 57$), before therapy (surgery and [or] chemotherapy) ($n = 12$), during treatment ($n = 85$), and after cessation of therapy ($n = 26$). There were no differences in age between the samples from patients with active disease and those without evidence of disease.

Patient sera. Blood samples (5–10 ml without anticoagulants) were drawn under sterile conditions, allowed to clot at room temperature for 1 h, clarified by centrifugation, fractionated, and stored at −70°C. The serum samples, in this retrospective study, were taken before therapy at time intervals of at least 3 wk in serial follow-up of patients and at irregular time intervals after cessation of therapy.

Measurement of total hemolytic C (TCH50) and C components. Buffers for C assays were prepared as described (22). Assays of TCH50 and C components were carried out as before (22) with functionally pure C components C1–C9 and intermediate cells EAC1^{gp}, EAC4^{hu}, EAC1^{gp}4^{hu} and EAC1^{gp}–7^{hu} cells. Functional factor B was assayed with glutathione-treated cells and purified cobra venom factor according to the described methods (23). Cobra venom factor *Naja naja* was purified according to the methods described earlier (24). In the second step of purification, Sephadex G-100 (Pharmacia Fine Chemicals, Div. of Pharmacia Inc. N. J.) was used. The purified preparation gave a single band on disk electrophoresis and was free of phospholipase activity. C1q and C3 were determined immunochemically by the Mancini et al. (25) technique using monospecific antisera against these components (26). Immunoelectrophoretic analysis was carried out by the described methods (27).

Raji cell radioimmunoassay. Raji cell radioimmunoassay for the determination of ICS was carried out according to the method of Theofilopoulos et al. (28). Values of 15 sera, obtained from healthy age-matched children, ranged from 0–16 μg equivalent aggregated human gamma globulin per milliliter in a 1:4 diluted serum. For the sake of simplicity results of levels of circulating immune complexes are expressed as microgram per milliliter. The Raji cells were obtained from Dr. T. Takahashi, Sloan-Kettering Institute, and cultured in Eagle's minimum essential medium. Cell viability was determined by trypan blue exclusion. The cells used were always after 48–72 h passage in tissue culture.

Preparations of aggregated human gamma globulin (AHG). Purified human immunoglobulin (Ig)G from Cohn fraction II is freed from aggregates by centrifugation at 40,000 g for 90 min. The protein concentration of the supernate was determined by the Lowry et al. method (29). AHG was prepared by heating purified IgG in a waterbath at 63°C for 30 min, and the protein content of the soluble AHG was again determined.

Radioiodination of rabbit anti-human IgG. Radioiodination of rabbit anti-human IgG with ^{125}I (New England Nuclear, Boston, Mass.) was performed using the chloramine T method according to McConahey and Dixon (30). The labeled anti-human IgG was extensively dialyzed against phosphate-buffered saline, pH 7.2, for 36 h. The specific activity of the ^{125}I-rabbit anti-human IgG (0.3 mg/ml) was 0.05–0.2 μCi μg/protein.

Biostatistical methods. The Kruskal-Wallis test was used to test for the differences of ICS levels among the various stages of disease and among the treatment groups. The Welch two-sided t test was used to test the differences between the mean values of the complement levels in the patients' sera with the normal control of each (31).

RESULTS

Levels of TCH50 and C components. Table II presents the mean, standard deviation, and sample size of each of the serum complement component determinations of the patients in the active disease group, the NED group, and the normal control of sera obtained from 15 healthy age-matched children. All values of the hemolytic titers C1–C9 and factor B are expressed as the percentage of pooled normal serum obtained from the healthy children. Each value represents the first serum sample of each patient. The only significant difference between the mean values of the active disease and NED groups was in the hemolytic C1 titer (*P*

TABLE I
Distribution of Patients with Neuroblastoma

	Total patients	Total sera	Activity of disease		Relation to therapy		
			NED	Active disease	Before	During	After
	n	*n*					
Stage I	6	8	6	2	2	1	5
Stage II	8	10	6	4	4	3	3
Stage III	14	28	27	1	1	15	12
Stage IV	37	75	25	50	5	66	4
Stage IVs	2	2	2	—	—	—	2
Total	67	123	66	57	12	85	26

Distribution of the 67 patients with neuroblastoma according to the clinical stages I–IVs at the time of diagnosis. The 123 serum samples were subdivided into samples from patients with active disease or no evidence of disease and related to treatment.

TABLE II
Complement Component Levels in Neuroblastoma

Hemolytic	Active disease		No evidence of disease		Normal control	
	n	mean±SD	n	mean±SD	mean±SD	Range
TCH50	5	134.6±86.4	13	102.2±40.8	100±14	86–114
C1	8	59.0±38.7‡	13	139.6±65.8	100±33	67–133
C4	9	119.4±87.1	11	91.5±100.0	100±41	59–141
C2	9	143.0±99.9	14	132.6±51.8	100±37	62–137
C3‖	4	70.2±33.1	10	55.1±36.7§	100±20	80–120
C5	1	30	9	78.8±61.8	100±24	76–124
C6	1	111	4	126.6±42.7	100±18	82–118
C7	4	120.8±22.8	11	116.1±55.2	100±26	74–126
C8¶	3	77.0±28.2	8	66.5±35.3	100±30	70–130
C9	3	124.7±69.2	8	108.6±53.1	100±31	69–131
Factor B	19	92.8±42.7	16	83.9±24.2	100±25	75–125
Immunochemical						
C1q	20	22.0±8.2	20	28.7±28.2	20.7±4.8	17.9–23.5
C3	20	128.7±36.1	19	112.7±32.2	107±23	84–130

Levels of TCH50 and C components. Mean, standard deviation, and sample size of the TCH50 and the hemolytically and immunochemically determined complement components in the active disease and NED groups are compared to those of 15 healthy age-matched children and the significance indicated by P values. All values of the hemolytic titers are expressed as percent of normal serum obtained from the 15 healthy children. Each value represents the first sample from each patient.
* n = number of serum samples.
‡ Active disease, lower than control, $P < 0.025$.
§ NED, lower than control, $P < 0.005$.
‖ Active disease and NED combined lower than control, $P < 0.001$.
¶ Active disease and NED combined lower than control, $P < 0.002$.

< 0.002); however, they were on opposite sides of the normal mean. Pooling the active disease and NED groups, the means of the patients' values were not significantly different from the normal controls except for C3 and C8 which were both lower. Because of the large number of comparisons made, the large variability obtained in each test, and the fact that only a small number of determinations were available for some tests, this analysis is meant to indicate the direction of change in C component levels between the two groups. There were no significant correlations between any pair of C components or between any C component and ICS levels. Low C3 hemolytic values were associated with high C3 levels determined immunochemically (Table II) in sera of several patients. Further analysis of these five sera, with low hemolytic C3 values by immunoelectrophoresis using monospecific antiserum to C3, indicated that C3 had been cleaved by >50% (not shown). This cleavage was not due to storage of serum because serum obtained from freshly drawn blood gave identical results in each instance.

Levels of circulating ICS in neuroblastoma patients.
With the Raji cell radioimmune assay (28) we determined the levels of ICS of 61 patients with different stages of neuroblastoma before, during, and after therapy. Table III presents the mean, median, and range of the ICS levels for each group of neuroblastoma patients subdivided by the stage of the disease and the course of treatment. All normal control values were under 16 μg/ml. We used only the values of the first determination of each patient so that each patient is represented only once in the analysis. Stage IVs cases were not included in subsequent analyses. 12 patients were studied before therapy, 32 patients during therapy with or without evidence of tumor, and 15 patients after successful treatment and cessation of therapy. As shown in Table III, an association between the stage of the disease and ICS levels was obtained.

The ICS levels are higher in the more advanced stages of disease and in these stages they are higher before treatment than during or after treatment. The increase of ICS levels with respect to disease stages is more evident within the before treatment group; the medians for stages I–IV are: 16, 28, 160, and 1,100 μg/ml, respectively. The decrease of ICS levels over the course of treatment is most evident in stage IV patients; the medians for before, during, and after treatment are 1,100, 51 (combining the results of the two during therapy groups) and 16 μg/ml, respectively.

Within the NED group no significant differences

TABLE III
Levels of Circulating Immune Complexes in Neuroblastoma*

	Active disease								No evidence of disease								Total			
	Before therapy				During therapy				During therapy				After therapy							
	Mean	Me-dian	Max		Mean	Me-dian	Max		Mean	Me-dian	Max		Mean	Me-dian	Max		Mean	Me-dian	Max	
				n ‡				n				n				n				n
Stage I	16	16	16	2	—	—	—		16	16	16	1	16	16	16	3	16	16	16	6
Stage II	40	28	88	4	—	—	—		16	16	16	3	16	16	16	1	29	16	88	8
Stage III	160	160	160	1	—	—	—		24	17	48	4	30	16	128	8	38	16	160	13
Stage IV	1,013	1,110	1,800	5	159	58	1,600	20	22	16	40	4	24	16	40	3	263	52	1,800	32
Stage IVs	—	—	—		—	—	—		—	—	—		16	16	16	2	16	16	16	2
Stages I–IV	452	64	1,800	12	159	58	1,600	20	21	16	48	12	25	16	128	15	156	16	1,800	59

Levels of circulating immune complexes in neuroblastoma patients using the Raji cell radioimmunoassay. Mean, median, and maximal value of 61 patients with different stages of disease before, during, and after therapy further subdivided by active disease or no evidence of disease. Only the first sample of each patient is used. The sensitivity of the Raji cell radioimmunoassay is 16 μg equivalent AHG/ml of a 1:4 diluted serum, therefore in the calculation 16 is used for those reported as under 16 μg/ml. Stage IVs cases were not included in subsequent analysis. Levels expressed as microgram equivalent AHG per milliliter. The minimum of every group is 16 μg/ml.
* First sample of each patient.
‡ n = sample size.

between ICS levels could be detected in the during treatment and after treatment groups, and the differences among the stages of the disease were not significant.

The differences between the stages of disease can be seen in Figs. 1 and 2. In Fig. 1 the cumulative frequencies are shown on log-normal probability paper. For any given ICS level on the abscissa, the corresponding value on the ordinate as determined by the curve gives the proportion of ICS determinations that is less than or equal to that level. As shown, the distributions shift to higher levels with advanced stages, particularly with stage IV. Fig. 2 shows the individual
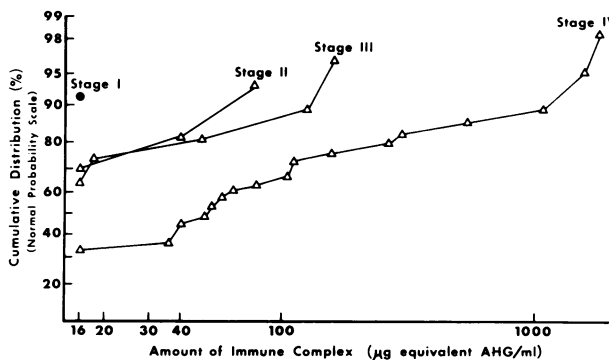

FIGURE 1 Cumulative distribution (percentage) of patients with different stages of neuroblastoma in relation to the amount of ICS. The data are plotted on log normal probability paper. The 50% point is the median of each group. The distribution shifts to higher levels with advanced stages of the disease.

determinations for stages I–IV. It can be seen that the range of the levels increases sharply with the stage of disease. A nonparametric Kruskal-Wallis test for differences in the frequency distributions showed significant differences among the stages of the disease ($P < 0.01$). However, this is a result of the higher values of ICS in stage IV, which are significantly higher than all of the stages I–III combined ($P < 0.005$). Although there is evidence of a steady rise with each stage of the disease the differences between stages I–III are not significantly different, possibly because of the small sample sizes.

Comparison of ICS levels with the course of treatment. If the ICS levels of the neuroblastoma patients are subdivided by the course of treatment, there is a significant difference among the three groups ($P < 0.05$). In 12 patients before treatment was initiated the median of ICS levels was 64, in 32 patients during treatment with or without evidence of disease the median was 27, and in 15 patients after cessation of treatment, the median decreased to 16 μg/ml (Table III).

Within the stage IV group, the differences among before, during, and after treatment groups are significant ($P < 0.05$) (Fig. 3). A two-way analysis of variance (31) was also carried out on the log-transformed ICS values, with the stages of the disease and the disease status as the two factors. The conclusions are similar to the results above.

Serial data of ICS in neuroblastoma. 18 patients with repeated observations over periods of 1–24 mo were studied. These are 10 patients with NED and 8 with active disease. However, most of the NED
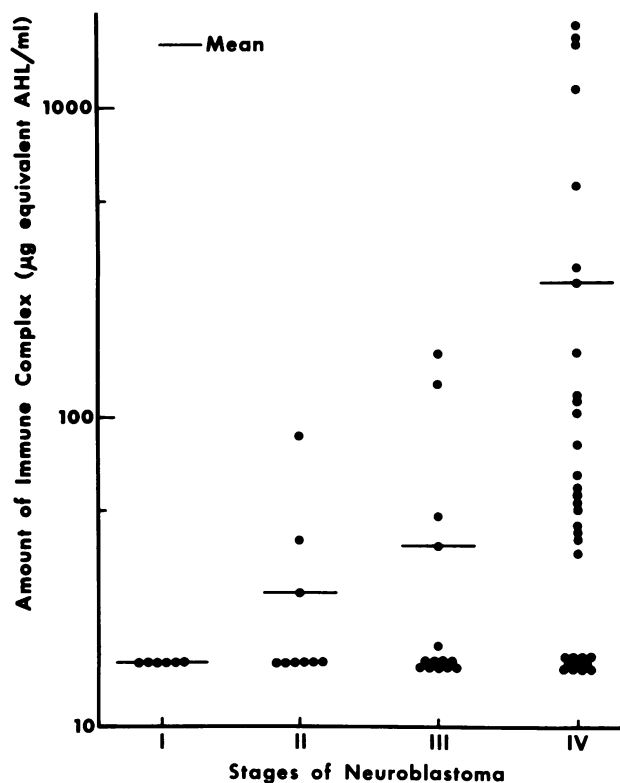
FIGURE 2   ICS levels (μg equivalent AHG/ml serum) in different stages of neuroblastoma. Each point represents the first sample of each patient. There is a close correlation with advanced stages of neuroblastoma and the mean of ICS levels.

patients only have ICS measurements after treatment, and these values in general all remain 16 μg/ml in repeated measurements of up to 7 mo. Only three NED patients showed elevated ICS one time in the several analyses, and all three values are under 50 μg/ml. In Fig. 4, patient R. M. was used as an illustration as representative of this group of patients. Of the eight active disease patients, only four with measurements over 1 mo were presented (H. D., R. L. and T. W. died during treatment; B. B. changed from active disease to NED). The other four patients had all of their determinations within 1 mo and did not show much variation, so they were not included in Fig. 4.

ICS levels and survival.   At least 8 mo after diagnosis all 29 patients in stages I–IVs survived up to the last follow-up. Of the 32 stage IV patients, 11 were surviving as long as the study was carried out. As a result of the retrospective nature of the data, the ICS measurements were not made at fixed intervals, so they do not lend themselves directly to survival analysis. However, restricting attention only to those 24 stage IV patients who had ICS measurements during treatment with at least 3 mo of therapy, it was found that no correlation existed between the ICS values and the

time interval between the start of the therapy and the ICS determinations. Dividing the patients into those with elevated ICS values (>16 μg/ml) and those without elevated values (≤16 μg/ml), no difference in the interval between the start of therapy and the ICS determinations was found. Analysis on the survival time was carried out after the ICS determinations. The survival time for those 13 patients with elevated ICS values was significantly shorter than for the 11 patients without elevated values (P < 0.01, using both the log rank test and Cox regression analysis) (31). Due to the irregular timing of determinations, we wish to mention these results only as a suggestion for further studies.

DISCUSSION

Recent studies in our laboratories have shown that >50% of cancer sera have circulating ICS, and that
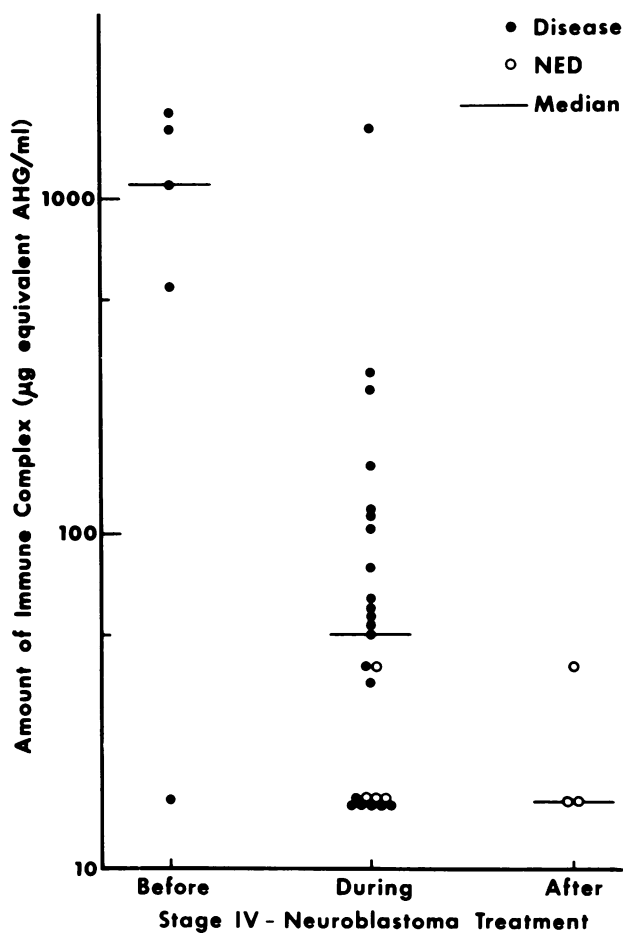

FIGURE 3   ICS levels (μg equivalent AGH/ml) in stage IV neuroblastoma before, during, and after treatment. (●) represent patients with active disease. (○) represent patients with no evidence of disease. The median values of immune complexes are significantly elevated before treatment, significantly lower during treatment, and within normal limits after treatment.
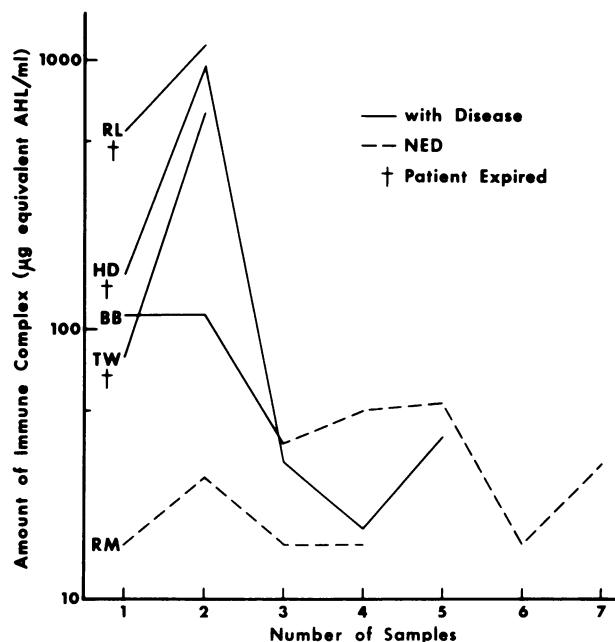
FIGURE 4 ICS levels (μg equivalent AHG/ml) of some patients over time and course of treatment. Serial studies show a fluctuation of ICS with period of time. In two patients (H.D., B.B.) the levels decreased during the time of follow-up. One patient (B.B.) changed from an active disease state to no evidence of disease.

over 10% of these sera are hypocomplementemic. In one report of a detailed study of serum of a patient with untreated chronic lymphocytic leukemia associated with hypocomplementemia and cryoglobulinemia, the immune complexes were identified and isolated, and it was found that they were made up of IgM antilymphocyte antibody and lymphocyte membrane antigens (32). Using the ^{125}I-C1q deviation test, sera from patients with various malignancies, melanoma, breast, head and neck, gynecological, lung, colon, and rectal cancer were studied, and it was found that many patients had ICS in their serum and the levels were sometimes as high as those detected in patients with systemic lupus erythematosus (33). By sucrose density gradient ultracentrifugation of some sera studied, the ICS were shown to be ≅19S or greater. With the Limulus test (34), we demonstrated that DNA and endotoxin, substances known to interfere in the C1q deviation test, were negligible in sera of 50 cancer patients with high levels of ICS.

The above studies were carried out with adult cancer sera by the ^{125}I-C1q deviation test, and the levels of ICS determined were not correlated with the extent or stages of cancer or treatment.

In this study we investigated sera of patients with neuroblastoma using the Raji cell radioimmunoassay (28), with particular emphasis on the correlation of

levels of ICS with stage of disease and changes attributable to treatment. Patients were classified according to a scheme based on the extent of dissemination of the neuroblastoma at the time of diagnosis, ranging from stage I when the tumor is confined to the organ or tissue of origin to stage IV with distant metastasis in bones, distant lymph nodes, marrow, or soft tissues. We investigated 123 serum samples from 67 patients before, during, and after therapy and showed that there was a close correlation between the levels of complexes, stage of disease and treatment. The levels increased with the stage of disease and decreased during and after treatment. The increase of levels with respect to different stages was most evident in the before treatment group. Significant differences among the four stages were evident ($P < 0.01$). In particular, this was a result of the higher values of ICS in stage IV which was significantly higher than all other stages combined ($P < 0.005$). Patients with more localized disease had less chemotherapy, whereas patients with stage IV were treated more intensively. Despite this very intensive chemotherapy, that is also immunosuppressive, these patients had the highest levels of circulating immune complexes. The differences in chemotherapy in the various stages therefore did not affect significantly the levels of immune complexes. When the levels of ICS were subdivided by the course of treatment, there was a significant difference among the three groups before, during, and after treatment ($P < 0.05$). Within the no evidence of disease group, the difference of levels of ICS between during treatment and after treatment was not significant, and the differences across the stages were also not significant. In contrast, within the stage IV group the differences among before, during, and after treatment groups were significant ($P < 0.05$). Further, when it was possible to obtain serum samples serially, advancing disease and treatment were associated with changes in levels for ICS. For example, in two patients' sera where five or more determinations were done initially and during treatment, ICS levels decreased progressively during treatment.

Studies of C and C components did not give such a clear relationship. Although a significant decrease compared to normal hemolytic levels of C1 ($P < 0.025$) were found in the active disease group and C3 ($P < 0.005$) was low in patients without evidence of tumor, the remaining C components were found to fall in the normal range or not to be significantly different from those of the normal controls. This could be due to the large variability in each test and to the small number of samples available for analysis. The influence of acute-phase reactivity on certain C components (35) tends to elevate levels of these components thus masking the changes of pathological C component use. This phenomenon may provide a likely explanation of our findings of normal or even elevated values for most com-

ponents despite the presence of ICS in the circulation. Of interest, are our observations of some high C3 levels determined immunochemically that were associated with low hemolytic levels of C3 of freshly drawn neuroblastoma sera. Analysis of three such sera by immunoelectrophoresis showed that this discrepancy was attributable to cleavage of C3. Quantitative studies of split products of C3 and factor B might be a more sensitive method to study C activation as shown by Nydegger et al. (36), and such studies should be carried out in children with neuroblastoma and other forms of cancer. Recently, Gerson et al. (37) have shown that C3 levels determined immunochemically are elevated in neuroblastoma patients when the disease is active. These investigators, however, did not study C3 by hemolytic analysis.

The presence of immune complexes in various malignancies established by different techniques has also been reported by other investigators and in some studies fixation of immune complexes in the glomeruli of patients with leukemia, lymphoma, Hodgkin's disease (38, 39), colon carcinoma (40), lung carcinoma (41), African Burkitt's lymphoma (42), melanoma (43, 44), and other malignancies (43, 44, 45) has been found. Circulating immune-complex-like substances have been reported in patients with melanoma (43, 46–49), breast cancer (50), lung cancer, colon cancer (48–50), leukemias (51, 52), Hodgkin's disease (53–55), and non-Hodgkin's lymphomas (56). In a few studies, the relationship of the amount of ICS to stage of disease has been reported (43, 48, 52, 53). Although Hellström et al. (10) used the colony inhibition technique and Jose and Seshardi (15) used a counterelectrophoresis method for detecting complexes in patients with human neuroblastoma, no detailed studies as those discussed here correlating levels of ICS with stage of neuroblastoma and treatment have been reported.

In unpublished preliminary observations, we have also noted that levels of ICS are significantly greater in advanced disease than in the earlier stages, in childhood Hodgkin's disease, adult colon-rectal disease, and breast cancer.

Most important, of course, is to determine the composition of these ICS and further to characterize them by physical, chemical studies. However, based on our survival data, the ICS levels may be of significant value in prognosis of neuroblastoma. Further, the determination of ICS may serve to complement contemporary methods for staging and determining the efficacy of treatment in children with this disease.

ACKNOWLEDGMENTS

REFERENCES

1. Bodian, M. 1963. Neuroblastoma: an evaluation of its natural history and the effects of therapy with particular reference to treatment by massive doses of vitamin B12. Arch. Dis. Child. 38: 606–610.
2. Beckwith, J. B., and E. V. Perrin. 1963. In situ neuroblastomas: a contribution to the natural history of neural crest tumors. Am. J. Pathol. 43: 1089–1104.
3. Beckwith, J. B., and R. F. Martin. 1968. Observations of the histopathology of neuroblastomas. J. Pediatr. Surg. 3: 106–110.
4. MacMillan, R. W., W. B. Blanc, and T. V. Santulli. 1976. Maturation of neuroblastoma to ganglioneuroma in lymph nodes. J. Pediatr. Surg. 11: 461–462.
5. Helson, L. 1975. Management of disseminated neuroblastoma. Ca-A Cancer Journal for Clinicians. 25: 264–277.
6. Hellström, J. E., K. E. Hellström, G. E. Pierce, and A. H. Bill, 1968. Demonstration of cell-bound and humoral immunity against neuroblastoma cells. Proc. Natl. Acad. Sci. U. S. A. 60: 1231–1238.
7. Bill, A. H., and A. Morgan. 1970. Evidence for immune reactions to neuroblastoma and future possibilities for investigation. J. Pediatr. Surg. 5: 111–116.
8. Bill, A. H. 1971. Immune aspects of neuroblastoma current information. Am. J. Surg. 122: 142–146.
9. Hellström, J., K. E. Hellström, C. A. Evans, G. H. Heppner, G. E. Pierce, and J. P. S. Yang. 1969. Serum-mediated protection of neoplastic cells from inhibition by lymphocytes immune to their tumor-specific antigens. Proc. Natl. Acad. Sci. U. S. A. 62: 362–368.
10. Hellström, J., K. E. Hellström, A. H. Bill, G. E. Pierce, and P. S. Yang. 1970. Studies on cellular immunity to human neuroblastoma cells. Int. J. Cancer. 6: 172–188.
11. Tamerius, J. D., I. Hellström. 1974. In vitro demonstration of cytotoxic antibodies to Moloney sarcoma cells J. Immunol. 112: 1987–1996.
12. Sjögren, H. O., I. Hellström, S. C. Bansal and K. E. Hellström. 1971. Suggestive evidence that the "blocking antibodies" of tumor-bearing individuals may be antigen-antibody complexes. Proc. Natl. Acad. Sci. U. S. A. 68: 1372–1375.
13. Baldwin, R. W., M. R. Price, and R. A. Robins. 1972. Blocking of lymphocyte-mediated cytotoxicity for rat hepatoma cells by tumor specific antigen-antibody complexes. Nat. New Biol. 238: 185–187.
14. Baldwin, R. W., M. R. Price, and R. A. Robins. 1973. Inhibition of hepatoma-immune lymph node cell cytoxicity by tumor-bearer serum and solubilized hepatoma antigen. Int. J. Cancer. 11: 527–535.
15. Jose, D. G., and R. Seshadri. 1974. Circulating immune complexes in human neuroblastoma: direct assay and role in blocking specific cellular immunity. Int. J. Cancer. 13: 824–838.
16. Jose, D. G., and F. Skvaril. 1974. Serum inhibitors of cellular immunity in human neuroblastoma. IgG subclass of blocking activity. Int. J. Cancer 13: 173–178.
17. Oldstone, M. B. A. 1975. Immune complexes in cancer.

Demonstration of complexes in mice bearing neuroblastoma. *J. Natl. Cancer Inst.* **54:** 223–228.

18. Helson, L., and D. Namerow. 1972. Clinical observations on diagnosis of neuroblastoma. *Clin. Bull.* **2:** 43–46.

19. Harkin, J. C., and R. J. Reed. 1969. Tumors of the peripheral nervous system. *In* Atlas of Tumor Pathology. Armed Forces Institute of Pathology, Washington, D. C. **3:** 221–252.

20. Helson, L., M. Fleisher, V. Bethune, M. L. Murphy, and M. K. Schwartz. 1972. Urinary cysthathionine, catecholamine and metabolities in patients with neuroblastoma. *Clin. Chem.* **18:** 613–615.

21. Evans, A. E., G. J. D'Angio, and J. Randolph. 1971. Proposed staging for children with neuroblastoma. *Cancer.* **27:** 374–378.

22. Mayer, M. M. 1961. Complement and complement fixation. *In* Kabat and Mayer: Experimental Immunochemistry. 2nd edition. Charles C. Thomas, Publisher, Springfield, Ill. 133–240.

23. Götze, O., and H. J. Müller-Eberhard. 1972. Paroxysmal nocturnal hemoglobinuria. Hemolysis initiated by the C3 activator system. *N. Engl. J. Med.* **286:** 180–184.

24. Ballow, M., and C. G. Cochrane. 1969. Two anticomplementary factors in cobra venom. Hemolysis of guinea pig erythrocytes by one of them. *J. Immunol.* **103:** 944–952.

25. Mancini, J. A., O. Carbonara, and J. F. Hermanns. 1967. Immunochemical quantitation of antigen by single radial immunodiffusion. *Immunochemistry.* **2:** 235–242.

26. Ballow, M., J. E. Shira, S. Y. Yang, and N. K. Day. 1975. Complete absence of the third component of complement in man. *J. Clin. Invest.* **56:** 703–710.

27. Day, N. K., H. Geiger, R. McLean, J. Resnick, A. Michael, and R. A. Good. 1973. The association of respiratory infection, recurrent hematuria and focal glomerulonephritis with activation of the complement system in the cold. *J. Clin. Invest.* **52:** 1698–1706.

28. Theofilopoulos, A. N., C. B. Wilson, and F. J. Dixon. 1976. The Raji cell radioimmune assay for detecting of immune complexes in human sera. *J. Clin. Invest.* **57:** 169–182.

29. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin Phenol reagent. *J. Biol. Chem.* **193:** 265–275.

30. McConahey, P. J., and F. J. Dixon. 1966. A method of trace iodination of proteins for immunologic studies. *Intl. Arch. Allergy Appl. Immunol.* **29:** 185–193.

31. Sokal, R. R., and F. J. Rohlf. 1969. Biometry. W. H. Freeman and Co., San Francisco. 299–342.

32. Day, N. K., J. B. Winfield, T. Gee, R. Winchester, H. Teshima, and H. G. Kunkel. 1976. Evidence for immune complexes involving anti-lymphocyte antibodies associated with hypocomplementaemia in chronic lymphocytic leukemia (CLL). *Clin. Exp. Immunol.* **26:** 189–195.

33. Teshima, H., H. G. Kunkel, and N. K. Day. 1976. C1q binding substances in cancer and complement deficient sera. *J. Immunol.* **116:** 1752. (Abstr.)

34. Levin, J., P. A. Tomasulo, and R. S. Oser. 1970. Detection of endotoxin in human blood and demonstration of an inhibitor. *J. Lab. Clin. Med.* **75:** 903–911.

35. Schutte, M., R. DiCamelli, P. Murphy, M. Sadove, and H. Gewurz. 1974. C3 proactivator (C3PA) as an acute-phase reactant. *Clin. Exp. Immunol.* **18:** 251–256.

36. Nydegger, U. E., R. H. Zubler, R. Gabay, G. Joliat, C. H. Karagevreskis, P. H. Lambert, and P. A. Miescher. 1977. Circulating complement breakdown products in patients with rheumatoid arthritis. Correlation between plasma C3d, circulating immune complexes and clinical activity. *J. Clin. Invest.* **59:** 862–868.

37. Gerson, J., A. E. Evans, and F. S. Rosen. 1977. The prognostic value of acute phase reactants in patients with neuroblastoma. *Cancer.* **40:** 1655–1658.

38. Sutherland, J. C., and M. R. Mardiney. 1973. Immune complex disease in the kidneys of lymphoma-leukemia patients. The presence of an oncornavirus-related antigen. *J. Natl. Cancer. Inst.* **50:** 633–644.

39. Sutherland, J. C., R. V. Markham, H. E. Ramsey, and M. R. Mariney. 1974. Subclinical immune complex nephritis in patients with Hodgkin's disease. *Cancer Res.* **34:** 1179–1181.

40. Constanza, M. E., F. Pinn, R. S. Schwartz, and L. Nathanson. 1973. Carcinoembryonic antigen-antibody complexes in a patient with colonic carcinoma and nephrotic syndrome. *N. Engl. J. Med.* **289:** 520–523.

41. Loughridge, L. W., and M. G. Lewis. 1971. Nephrotic syndrome in malignant disease of non-renal origin. *Lancet.* **1:** 256–258.

42. Oldstone, M. B. A., A. N. Theofilopoulos, G. Klein, and P. Gunven. 1975. Immune complexes associated with neoplasia. Presence of Epstein-Barr virus antigen-antibody complexes in Burkitt's lymphoma. *Intervirology.* **4:** 292–302.

43. Jerry, L. M., M. G. Lewis, and P. Cano. 1976. Anergy, anti-antibodies and immune complex disease: a syndrome of disordered immune regulation in human cancer. *In* Immunocancerology in Solid Tumors. M. Martin and L. Dionne, editors. Stratton Intercontinental Medical Book Corp., New York. 63–80.

44. Weksler, M. E., T. Carey, N. K. Day, M. Susin, R. Sherman, and C. Becker. 1974. Nephrotic syndrome in malignant melanoma: demonstration of melanoma antigen-antibody complexes in the kidney. 7th Annual Meeting of the American Society of Nephrology 7: 99. (Abstr.)

45. Kaplan, B. S., J. Klassen, and M. H. Gault. 1976. Glomerular injury in patients with neoplasia. *Annu. Rev. Med.* **27:** 117–125.

46. Ozawa, T., R. Pluss, J. Lacher, E. Boedecker, S. Guggenheim. W. Hammond, and R. McIntosh. 1975. Endogenous immune complex nephropathy associated with malignancy. I. Studies on the nature and immunopathogenic significance of glomerular bound antigen and antibody, isolation and characterization of tumor specific antigen and antibody and circulating immune complexes. *Q. J. Med.* **44:** 523–541.

47. Heppner, G. H., L. Stolbach, M. Byrne, F. J. Cummings, E. McDonough, and D. Calabresi. 1973. Cell mediated and serum blocking reactivity to tumor antigens in patients with malignant melanoma. *Int. J. Cancer* **11:** 245–260.

48. Rossen, R. D., M. A. Reisberg, E. M. Hersh, and J. U. Gutterman. 1977. The C1q binding test for soluble immune complexes: clinical correlations obtained in patients with cancer. *J. Natl. Cancer Inst.* **58:** 1205–1215.

49. Teshima, H., H. Wanebo, C. Pinsky, and N. K. Day. 1977. Circulating immune complexes detected by ^{125}I-C1q deviation test in sera of cancer patients. *J. Clin. Invest.* **59:** 1134–1142.

50. Hoffken, K., I. D. Meredith, R. A. Robins, R. W. Baldwin, C. J. Davies, and R. W. Blamey. 1977. Circulating immune complexes in patients with breast cancer. *Br. Med. J.* **2:** 218–226.

51. Carpentier, N. A., R. H. Zubler, G. T. Lange, P. H. Lambert, and P. A. Miescher. 1976. Complexes immuns circulants dans les leucémies humaines. *Schweiz. Med. Wochenschr.* **106:** 1363–1364.

52. Carpentier, N. A., G. T. Lange, D. M. Fiere, G. J. Fournie, P. H. Lambert, and P. A. Miescher. 1977. Clinical relevance of circulating immune complexes in human leukemia. Association in acute leukemia of the presence of immune complexes with unfavorable prognosis. *J. Clin. Invest.* **60**: 874–884.

53. Amlot, P. L., J. M. Slaney, and B. D. Williams. 1976. Circulating immune complexes and symptoms in Hodgkin's disease. *Lancet.* **I**: 449–451.

54. Kavai, M., E. Berenyi, E. Palkovi, and G. Szegedi. 1976. Immune complexes in Hodgkin's disease. *Lancet.* **I**: 1249.

55. Long, J. C., C. L. Hall, C. A. Brown, C. Stamatos, S. A. Witzman, and K. Carey. 1977. Binding of soluble immune complexes in serum of patients with Hodgkin's disease to tissue cultures derived from the tumor. *N. Engl. J. Med.* **297**: 295–301.

56. Heimer, R., and G. Klein. 1976. Circulating immune complexes in sera of patients with Burkitt's lymphoma and nasopharyngeal carcinoma. *Int. J. Cancer.* **18**: 310–316.