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Comparison of Calbindin D-28k and S-100 Protein B in Neuroblastoma as Determined by Enzyme Immunoassay

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Levels of two calcium-binding proteins, calbindin D-28k (calbindin-D) and S-100 protein B (S-100b), were measured by immunoassay in solid tumors obtained surgically from pediatric patients. Mean concentrations of calbindin-D and S-100b in 73 neuroblastomas (23 ganglioneuroblastomas and 50 neuroblastomas) were 10- or 25-fold higher, respectively, than those in other types of solid tumors in pediatric patients (n=15). The mean tumor concentration of calbindin-D in patients with neuroblastoma (n=73) was 25.1 ng/mg (range 0.20 to 317.0 ng/mg soluble protein, SE=6.26); that of S-100b was 278.3 ng/mg (range 0.93 to 2521 ng/mg soluble protein, SE=71.7). The mean concentration of calbindin-D (4.4 ng/mg soluble protein) was significantly ($P<0.05$) lower in stage IV, the most advanced stage. The mean concentration of S-100b (74.0 ng/mg soluble protein) was lower in patients with undifferentiated neuroblastomas ($P<0.01$). Tumor levels of the two calcium-binding proteins were not correlated in patients with neuroblastoma, but each was strongly correlated with outcome in patients with neuroblastoma. The evidence suggests that measurements of the calcium-binding proteins calbindin-D and S-100b would be useful for evaluating the prognosis of patients with neuroblastoma.

Key words: Calbindin-D — S-100 protein — Neuroblastoma — Enzyme immunoassay — Differentiation

Neuroblastoma, the most common solid malignant tumor of childhood, is derived from the neural crest, and generally retains some ability to produce catecholamine precursors. This tumor sometimes shows a morphologic differentiation to neuronal or schwannian cells either spontaneously or following treatment. Shimada *et al.*[1] reported that the degree of ganglionic and schwannian differentiation was related to the prognosis of this disease. Calbindin D-28k (calbindin-D) and S-100 protein B (S-100b) are calcium-binding proteins that are found mainly in the nervous system, according to immunohistochemical evaluation.[2,3] These proteins are thought to act as intracellular calcium buffers,[4,5] and to play key roles in nerve cell function.[6] Kurobe *et al.*[7] found that levels of calbindin-D in rat brain gradually increased and reached adult levels by 4–5 weeks of age. Nakashima *et al.*[8] and Misugi *et al.*,[9] using immunohistochemical methods, reported that S-100b is a good indicator of schwannian tissues in neuroblastomas. We conducted the present study to determine whether tissue levels of calbindin-D and/or S-100b may indicate the differentiation of neuroblastomas and help to establish the prognosis. Calbindin-D and S-100b were measured in tumor tissue obtained from patients with neuroblastoma.

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MATERIALS AND METHODS

Tissues Tumor tissues and adrenal glands were obtained intraoperatively from 94 Japanese patients. There were 48 males and 46 females aged 5 days to 13 years (mean 2.4 years). Diagnoses included neuroblastoma (50), ganglioneuroblastoma (23), ganglioneuroma (6), rhabdomyosarcoma (6), Wilms' tumor (5), adrenocortical tumor (2), hepatoblastoma (1), and yolk sac tumor (1). All samples were rapidly frozen and stored at −80°C until assayed. Patients with neuroblastoma or ganglioneuroblastoma were classified into five categories, I, 24; II, 10; III, 16; IV, 16; IVs, 7, according to the pretreatment staging procedures of Evans *et al.*[10] Eight of 16 patients with stage III neuroblastoma and 12 of 16 patients with stage IV neuroblastoma received intensive preoperative chemotherapy with cyclophosphamide (CPA), vincristine (VCR), pirarubicin (THP-ADM), and cisplatin. Tumor tissues from these patients were obtained within 6 months after the initiation of chemotherapy. Postoperatively, 28 of 41 patients with neuroblastoma classified as stages I, II and IVs received adjuvant chemotherapy with CPA and VCR for 3 to 12 months. Nine patients in stage III without disease recurrence received chemotherapy postoperatively for 6 to 24 months. Fifteen of 24 patients with distant metastases or

recurrences received a bone marrow transplant after achieving a complete clinical remission. Survival time was determined from the start of treatment. The diagnosis was confirmed histologically in each case.[11]

Preparation of tissue extracts Tissues were homogenized at 0°C in 10 volumes of 10 m*M* Tris-HCl (pH 7.5) containing 1 m*M* EDTA, using a Polytron-type homogenizer. Each homogenate was centrifuged at 4°C at 125,000*g* for 40 min, and the supernatant was used for assay of calbindin-D and S-100b. Each extract was diluted 100- to 5000-fold with buffer; 0.5-ml aliquots of the diluted sample were subjected to immunoassay in duplicate.

Preparation of calbindin-D and S-100b and their antibodies Human calbindin-D was purified from human kidney as described by Hitchman *et al.*[12] with modifications. Human S-100b was purified from human brain as described by Kimura *et al.*[13] Antibodies were raised in rabbits with human calbindin-D or S-100b as an immunogen and purified as described previously.[14, 15]

Enzyme immunoassay Calbindin-D was assayed by a sandwich-type immunoassay for human calbindin-D as described by Zhu *et al.*[14] The system consists of a solid phase (polystyrene ball) with immobilized purified antibodies to human calbindin-D and the same antibodies labeled with β-D-galactosidase from *Escherichia coli*. Purified human calbindin-D was used as the standard for immunoassay of calbindin-D. Results are expressed as ng of human calbindin-D per mg of soluble protein.

S-100b was also assayed by using an enzyme immunoassay system similar to that used for calbindin-D.

Protein concentrations in crude extracts were determined by a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Richmond, Calif.). This kit applies the principle of protein-dye binding[16] and uses bovine serum albumin as a standard.

Statistics The Mann-Whitney-Wilcoxon (2-sample) rank sum test, a nonparametric method, was used for the analysis of the separation between two groups. Spearman's coefficient test, a nonparametric method, was used for the analysis of the correlation coefficient between tissue calbindin-D and S-100b in neuroblastomas. The event-time distributions were estimated by the product limit method of Kaplan and Meier. Differences between event-time distributions were tested for statistical significance by using the generalized Wilcoxon *t* test.

RESULTS

Tumor concentrations of calcium-binding protein Table I shows the mean concentrations of each calcium-binding protein in solid tumors and in adrenal gland obtained from pediatric patients. Mean concentrations of calbindin-D and S-100b in the tumors obtained from 73 patients with neuroblastoma (ganglioneuroblastoma or neuroblastoma) were 10- or 25-fold higher, respectively, than those (calbindin-D, range 0.13 to 11.48; S-100b, range 0.51 to 26.0 ng/mg of soluble protein) in other solid tumors obtained from pediatric patients. In the neural crest tumors, ganglioneuroma, a benign tumor similar to neuroblastoma, contained a lower level of calbindin-D and a higher level of S-100b than those of neuroblastomas ($P<0.01$). The mean concentration of calbindin-D was 25.1 ng/mg of soluble protein (range 0.20 to 317.0, SE=6.26) and that of S-100b was 278.3 ng/mg of soluble protein (range 0.93 to 2521, SE=71.7) in the patients with neuroblastoma. These levels were also higher than those in normal adrenal gland from which neuroblastomas mostly originate.

Concentrations of calbindin-D and S-100b in neuroblastomas Fig. 1 shows the mean concentrations of calbindin-D and S-100b in neuroblastomas. A significantly higher level of S-100b was found in ganglioneuroblastoma than in neuroblastoma ($P<0.01$). Fourteen of the 23 ganglioneuroblastomas contained more than 100 ng of S-100b per mg of soluble protein. However, only 5 of the 50 neuroblastomas contained S-100b in amounts greater than 100 ng/mg soluble protein. Levels of calbindin-D were unrelated to the pathological differentiation of neuroblastoma.

Table I. Concentrations of Two Calcium-binding Proteins in Solid Tumors and Adrenal Gland in Children

	No.	Calbindin-D (SE)[a]	S-100 protein B (SE)[a]
Neural crest tumors	79	23.3 (5.91)[b]	304.0 (65.3)[b]
Neuroblastoma	73	25.1 (6.26)	278.3 (71.7)
Ganglioneuroma	6	1.4 (0.29)	993.3 (201.2)
Other pediatric tumors	15	2.6 (0.65)	11.2 (4.4)
Adrenal gland	6	6.0 (0.63)	46.9 (12.7)

a) Neuroblastoma versus other pediatric tumors=$P<0.01$, neuroblastoma versus ganglioneuroma =$P<0.01$.

b) Given as ng/mg soluble protein.

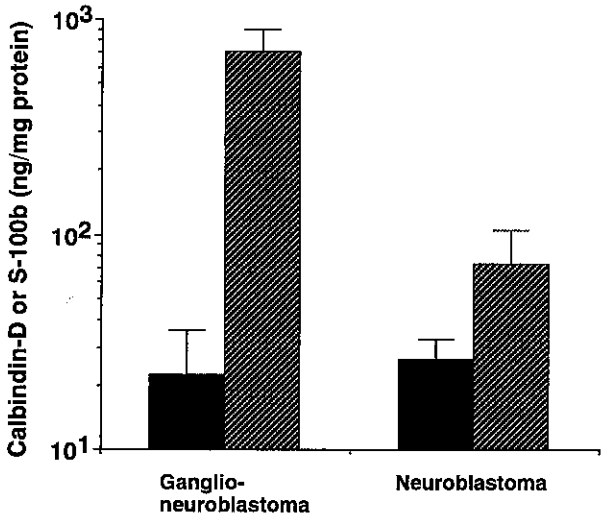

Fig. 1. Comparison of mean concentrations of calbindin-D and S-100b in neuroblastomas classified by pathological diagnosis. Error bars represent standard errors. A significantly higher level of S-100b was found in ganglioneuroblastoma than in neuroblastoma ($P<0.01$). The level of calbindin-D did not differ significantly between neuroblastomas and ganglioneuroblastomas. Black bar, calbindin-D; slashed bar, S-100b.

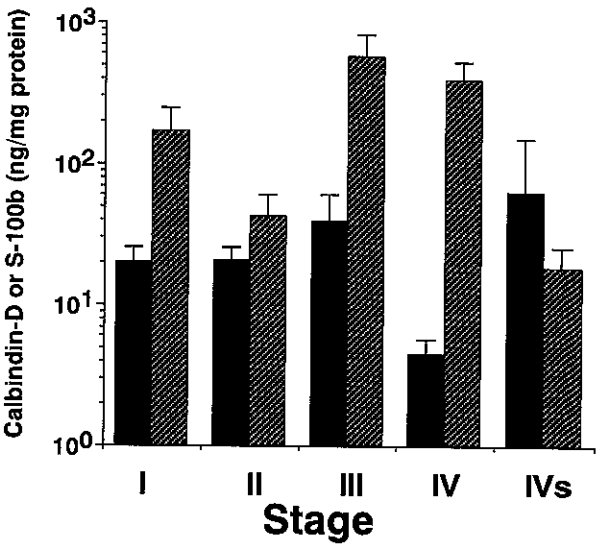

Fig. 2. Comparison of mean concentrations of calbindin-D and S-100b in neuroblastomas of different stages. Error bars represent standard errors. The mean value of calbindin-D in stage IV was the lowest among the 5 stages (IV versus I=$P<0.01$, IV versus II=$P<0.01$, IV versus III=not significant (NS), IV versus IVs=(NS). Black bar, calbindin-D; slashed bar, S-100b.

Table II. Concentrations of Calbindin-D in 73 Neuroblastomas

Stage	No.	Not administered chemotherapy[a] (mean±SE)	No.	Administered chemotherapy[b] (mean±SE)
I	24	20.3±5.2[c]	0	— —
II	10	20.3±5.7	0	— —
III	8	50.5±38.5	8	27.7±20.7[c]
IV	4	3.6±2.0	12	4.7±1.7
IVs	4	90.3±65.0	3	25.7±15.9
Total	50	29.6±8.5	23	15.4±7.5

a) Stage IV versus I=$P<0.05$, IV versus II=$P<0.01$.
b) Difference not significant.
c) Given as ng/mg soluble protein.

Concentrations of calcium-binding proteins and clinical stage of neuroblastoma Fig. 2 shows the mean concentrations of each calcium-binding protein in the different stages of neuroblastoma. The mean value of calbindin-D in stage IV, the lowest of the 5 stages, was 4.4 ng/mg soluble protein ($P<0.05$). The level of S-100b did not differ significantly among the 5 stages. Since a majority of patients with advanced neuroblastoma (i.e., stages III and IV) received preoperative chemotherapy, we examined the effects of chemotherapy on tumor concentrations of calcium-binding proteins (Tables II amd III). The level of calbindin-D was unaffected by chemotherapy. The mean concentration of calbindin-D (±SE) in neuroblastomas from patients (stages III and IV) who received chemotherapy (n=20) was 13.9 (±8.4) ng/mg of soluble protein, which is not significantly different from the concentration (34.9±26.0 ng/mg soluble protein) in neuroblastomas from patients (stages III and IV) not receiving chemotherapy (n=12) ($P=0.631$). The mean value of calbindin-D in stage IV was unchanged, remaining the lowest among the 5 clinical stages. However, the level of S-100b was higher in the group administered chemotherapy versus that not administered chemotherapy. Especially in stage IV, the mean tumor concen-

Table III. Concentrations of S-100b in 73 Neuroblastomas

Stage	No.	Not administered chemotherapy[a] (mean±SE)	No.	Administered chemotherapy[b] (mean±SE)
I	24	174.0±83.9[c]	0	— —
II	10	42.5±18.0	0	— —
III	8	393.0±355.0	8	748.0±362.0[c]
IV[d]	4	6.5±0.4	12	524.0±182.0
IVs	4	28.6±7.1	3	3.1±1.5
Total	50	157.0±61.7	23	535.0±163.0

a) Stage IV versus I=$P<0.01$, IV versus II=$P<0.0$, IV versus III=$P<0.055$, IV versus IVs=$P<0.01$.
b) Stage IV versus IVs=$P<0.05$.
c) Given as ng/mg soluble protein.
d) Significant difference between group not administered chemotherapy versus that administered chemotherapy ($P<0.05$).

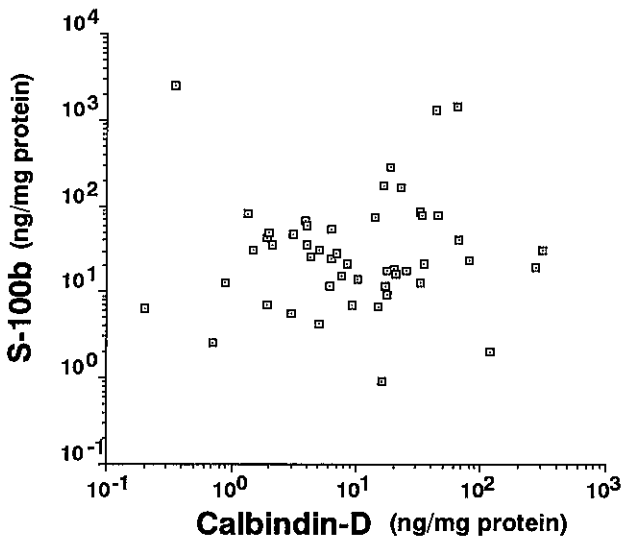

Fig. 3. Lack of correlation between tissue levels of calbindin-D and S-100b in 50 neuroblastomas in patients not administered chemotherapy ($r=0.154$, Spearman).

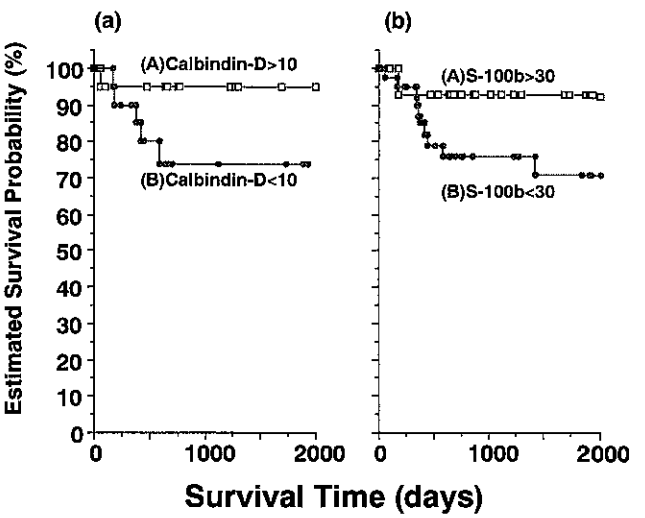

Fig. 4. Correlation between the tumor levels of calcium-binding proteins and overall survival rate of children with neuroblastomas, calculated by the Kaplan-Meier method.

tration of S-100b in patients not administered chemotherapy was 6.5 ng/mg of soluble protein, which is significantly lower than the level of 524.0 ng/mg of soluble protein observed in the group administered chemotherapy ($P<0.05$).

Correlation between tissue calbindin-D and S-100b We evaluated the correlation between tissue calbindin-D and S-100b in neuroblastomas from 50 patients who did not receive chemotherapy (Fig. 3). No significant correlation was evident ($r=0.154$).

Correlation between calcium-binding proteins and neuroblastoma patients' survival The correlations between the concentration of calbindin-D and of S-100b and cumulative survival of patients with neuroblastoma were determined by the Kaplan-Meier method. Patients were evaluated according to whether their tumor concentration of calbindin-D was greater or less than 10 ng/mg of soluble protein (Fig. 4a) and that of S-100b was greater or less than 30 ng/mg of soluble protein (Fig. 4b). Calbindin-D of >10 ng/mg of soluble protein or S-100b >30 ng/mg of soluble protein were each associated with a better survival, but the finding was not statistically significant. The cumulative survival of patients with respect to calbindin-D and S-100b levels is plotted in Fig. 5. In this figure, neuroblastoma patients were divided more clearly into two groups, two having a good prognosis and one (calbindin-D <10 ng and S-100b <30 ng) having a poor prognosis.

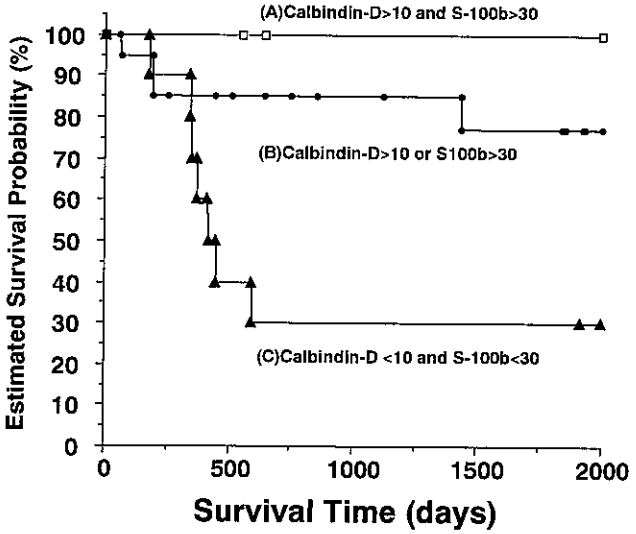

Fig. 5. Correlation between the tumor levels of calcium-binding proteins and overall survival rate of children with neuroblastomas, calculated by the Kaplan-Meier method. Of the three groups, patients with tissue calbindin-D concentrations <10 ng and tissue S-100b concentrations <30 ng had significantly shorter survival ($P<0.01$).

DISCUSSION

The present study demonstrated that neuroblastomas contained higher levels of calbindin-D and S-100b than other solid tumors did. The mean concentration of S-100b in the neuroblastomas was 278.3 ng/mg of soluble protein, approximately one-tenth of that reported in rat cerebellum.[17] The mean concentration of calbindin-D in the neuroblastomas was 25.1 ng/mg of soluble protein, which is 1/300 of that reported for rat cerebellum.[7] In neural crest tumors, the calbindin-D level was higher in the malignant tumors (neuroblastoma and ganglioneuroblastoma) than in the most differentiated benign tumors (ganglioneuroma). Mean concentrations of calbindin-D were similar in both ganglioneuroblastomas and neuroblastomas. The concentration of calbindin-D in neuroblastoma differed from that of S-100b in two ways: (1) the concentration of S-100b increased with the morphological differentiation of neuroblastoma, and (2) the concentration of S-100b increased following chemotherapy.

Calbindin-D and S-100b are both localized mainly in nervous tissues, and are related to the differentiation of the central nervous system (CNS).[2-4,18] S-100b has been detected in primary tumors of the human nervous systems by enzyme immunoassay; immunoreactivities for S-100b increase with the morphological differentiation of neuroblastoma, and its presence in the supportive stroma is correlated with the outcome for such patients.[19-21] Immunohistochemical studies have shown that S-100 protein-positive cells in neuroblastomas are schwann cells or their precursors.[20,21] Calbindin-D was originally discovered in avian intestine[22] and is also present in the kidney and in the CNS. Kurobe *et al.*[7] showed that the concentration of calbindin-D in the rat CNS increased during maturation. Jande *et al.*,[2] who studied immunohistochemically the localization of calbindin-D in brain, found this protein in various cells throughout the brain, but only in neuronal, not in glial, endothelial or ependymal cells. Tsokos *et al.*[23] suggested that primitive neuroblastic cells can differentiate into neuronal, schwannian and melanocytic cells. Our results indicate that the level of calbindin-D is related to prognosis, but not to the pathological differentiation of neuroblastoma. The presence of glial stroma is one of the main factors to decide the degree of differentiation. The level of calbindin-D may reflect the neuronal differentiation of neuroblastoma specifically. Patients with stage IV neuroblastoma who received no chemotherapy consistently showed significantly lower levels of calbindin-D and of S-100b as compared with tumors of other stages ($P<0.01$). These results suggested that stage IV neuroblastoma is the least differentiated tumor both to glial and neuronal cells. Age of patient at diagnosis is as important a factor as disease stage. The prognosis in patients younger than 1 year old is better than in older patients, even in patients with advanced neuroblastoma. However, calcium-binding protein concentrations were not associated with the patients' age (Table IV). In our series only two cases, stage III and stage IV, demonstrated greater than 10-fold amplification of the N-*myc* oncogene. We could not clarify the relationship between calcium-binding protein concentration and N-*myc* amplification in neuroblastomas.

Table IV. Concentrations of Two Calcium-binding Proteins in 50 Neuroblastomas from Patients Not Receiving Chemotherapy

Age of patient	No.	Calbindin-D (SE)	S-100 Protein B (SE)
<1 year old	35	30.7 (8.5)[a]	87.9 (47.1)[a]
$\geqq 1$ year old	15	16.9 (11.0)	44.2 (17.9)

a) Given as ng/mg soluble protein.

In conclusion, the concentration of calbindin-D in neuroblastoma seemed to be related to neuronal differentiation and that of S-100b to glial differentiation. The calcium-binding proteins calbindin-D and S-100b may be useful as predictors of the prognosis of patients with neuroblastoma.

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REFERENCES

1) Shimada, H., Chatten, J., Newton, W. A., Sachs, N., Hamoudi, A. B., Chiba, T., Marsden, H. B. and Misugi, K. Histopathologic factors in neuroblastic tumors: definition of subtypes of ganglioneuroblastoma and age-linked classification of neuroblastomas. *J. Natl. Cancer Inst.*, **73,** 406–416 (1984).
2) Jande, S. S., Maler, L. and Lawson, D. E. M. Immunohistochemical mapping of vitamin D-dependent calcium-binding protein in brain. *Nature,* **294,** 765–767 (1981).
3) Zomzely-Neurath, C. E. and Walker, W. A. Nervous system-specific proteins; 14-3-2 protein, neuron-specific enolase, and S-100 protein. *In* "Proteins of the Nervous System," ed. R. A. Bradshaw and D. M. Schneider, pp. 1–57 (1980). Raven Press, New York.
4) Baimridge, K. G. and Miller, J. J. Immunohistochemical localization of calcium-binding protein in the cerebellum, hippocampal formation and olfactory bulb of the rat. *Brain Res.*, **245,** 223–229 (1982).
5) Baudier, J. and Cole, R. D. Interactions between the microtubule-associated proteins and S100b regulate phosphorylation by the Ca^{2+}/calmodulin-dependent protein kinase II. *J. Biol. Chem.*, **263,** 5876–5883 (1988).
6) Iacopino, A. M. and Christakos, S. Specific reduction of calcium-binding protein (28-kilodalton calbindin-D) gene expression in aging and neurodegenerative diseases. *Proc. Natl. Acad. Sci. USA,* **87,** 4078–4082 (1990).
7) Kurobe, K., Inaguma, Y., Shinohara, H., Semba, R., Inaguma, T. and Kato, K. Developmental and age-dependent changes of 28-kDa calbindin-D in the central nervous tissue determined with a sensitive immunoassay method. *J. Neurochem.*, **58,** 128–134 (1992).
8) Nakashima, T., Kameya, T., Watanabe, S., Hirota, T., Sato, Y. and Shimosato, Y. An immunoperoxidase study of S-100 protein distribution in normal and neoplastic tissues. *Am. J. Surg. Pathol.*, **6,** 715–727 (1982).
9) Misugi, K., Aoki, I., Kikyo, S., Tsunoda, A. and Nakajima, T. Immunohistochemical study of neuroblastoma and related tumours with anti-S-100 protein antibody. *Pediatr. Pathol.*, **3,** 217–226 (1985).
10) Evans, D., D'Angio, J. and Randolph, G. A proposed staging for children with neuroblastoma. *Cancer,* **27,** 374–378 (1971).
11) Willis, R. A. "Pathology of the Tumours," 2nd Ed., pp. 843–871 (1953). Butterworths, London.
12) Hitchman, A. J. W., Kerr, M. K. and Harrison, J. E. The purification of pig vitamin D-induced intestinal calcium binding protein. *Arch. Biochem. Biophys.*, **155,** 221–222 (1973).
13) Kimura, S., Kato, K., Semba, R. and Isobe, T. Regional distribution of S-100ao($\alpha\alpha$), S-100a($\alpha\beta$) and S-100b($\beta\beta$) in the bovine central nervous tissue determined with a sensitive enzyme immunoassay system. *Neurochem. Int.*, **6,** 513–518 (1984).
14) Zhu, Y., Takahashi, M., Miyake, K. and Kato, K. Sensitive enzyme immunoassay for human 28 kDa calbindin-D. *Clin. Chim. Acta,* **201,** 183–192 (1991).
15) Kato, K., Suzuki, F., Kurobe, N., Okajima, K., Ogasawara, N., Nagaya, M. and Yamanaka, T. Enhancement of S-100β protein in blood of patients with Down's syndrome. *J. Mol. Neurosci.*, **2,** 109–113 (1990).
16) Bradford, M. M. A. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72,** 248–254 (1980).
17) Suzuki, F., Nakajima, T. and Kato, K. Peripheral distribution of nervous system-specific S-100 protein in rat. *J. Biochem.*, **92,** 835–838 (1982).
18) Garcia-Seruga, L. M., Baetens, J. R., Norman, A. W. and Orci, I. Immunohistochemical mapping of calcium-binding protein immunoreactivity in the rat central nervous system. *Brain Res.*, **296,** 75–86 (1984).
19) Ishiguro, Y., Kato, K., Ito, T. and Nagaya, M. Determination of three enolase isozymes and S-100 protein in various tumors in children. *Cancer Res.*, **43,** 6080–6084 (1983).
20) Shimada, H., Aoyama, C., Chiba, T. and Newton, W. A., Jr. Prognostic subgroups for undifferentiated neuroblastoma. Immunohistochemical study with anti-S-100 protein antibody. *Hum. Pathol.*, **16,** 471–476 (1985).
21) Nagoshi, M., Tsuneyoshi, M. and Enjoji, M. S-100 positive undifferentiated neuroblastomas with a special reference to the tumor stroma related to favorable prognosis. *Pathol. Res. Pract.*, **188,** 273–283 (1992).
22) Wasserman, R. H. and Taylor A. N. Vitamin D_3-induced calcium-binding protein in chick intestinal mucosa. *Science,* **152,** 791–793 (1966).
23) Tsokos, M., Ross, R. A. and Triche, T. J. Neuronal, schwannian and melanocytic differentiation of human neuroblastoma cells *in vitro. In* "Advances in Neuroblastoma Research," ed. A. E. Evans, G. J. D'Angio and R. C. Seeger, pp. 55–68 (1984). Alan R. Liss, New York.