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Affinity chromatography
carcinoembryonic antigen
neuroblastoma tissue
nonspecific cross reacting antigen

Immunologic and Biochemical Studies on the Carcinoembryonic Antigen-Like Substance in Human Neuroblastoma

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Summary

Several authors have observed that the plasma levels of carcinoembryonic antigen (CEA) in patients with neuroblastoma were significantly elevated. The present study was undertaken to investigate the nature of CEA activity in neuroblastoma tissue. This tumor tissue contains a small amount of CEA-like substance reacting with anti-CEA serum which is characterized by γ-globulin electrophoretic mobility, a molecular weight that is approximately equal to that of albumin (4.6S) by gel filtration, and a glycoprotein staining with periodic acid-Schiff (PAS). According to the double immunodiffusion method, this antigen is partially identical to purified CEA of colon carcinoma, and is completely identical to nonspecific crossreacting antigen (NCA). This antigen is, therefore, referred to not as the CEA as described by Gold, but as NCA in neuroblastoma tissue. The elevation of plasma CEA activity in patients with neuroblastoma may be due to the release of NCA from tumor cells, or to the destruction tissues by metastasis, of normal which are rich in NCA, or to a combination of both.

Speculation

The slight but significant elevation of plasma CEA activity in patients with neuroblastoma may be due to the release of NCA from tumor cells, or to the destruction by metastasis of normal tissues, or to a combination of both.

Since the plasma CEA activity assay is of little value for early diagnosis of neuroblastoma, another new immunologic method for early diagnosis in neuroblastoma might be expected from the standpoint of a tumor-specific antigen of neuroblastoma.

In 1965 Gold and Freedman (5) demonstrated the presence of a new specific surface antigen in human adenocarcinoma of the colon. This specific antigen has been further characterized as a glycoprotein soluble in perchloric acid (PCA). Since identical antigens were also present in the fetal gut, liver, and pancreas, this antigen was termed CEA (6). A radioimmunoassay for plasma CEA was established (17) and studies in numerous patients with various diseases were undertaken. Elevated CEA levels have been found in patients with entodermal origin cancers, nonentodermal cancers, and noncancerous conditions such as ulcertive colitis and liver cirrhosis. Several authors reported that the plasma levels of CEA were significantly elevated in patients with neuroblastoma (4, 16, 19).

CEA-like substances were found in normal tissue that reacts with anti-CEA serum. One of the representatives is designated as NCA, which is a PCA-soluble glycoprotein of about 4S and with β-globulin mobility (15, 18).

The objectives of the present investigation were to define the chemical and immunologic nature of the CEA-like substance in neuroblastoma tissue and especially to show the identity or non-identity of neuroblastoma CEA activity with colonic CEA or with NCA. It was found that the active substance in neuroblastoma is clearly distinguished from colonic CEA and is closely related to the NCA by chemical and immunologic criteria.

MATERIALS AND METHODS

TISSUES

Ten neuroblastoma tumors, consisting of seven primary tumors, three hepatic metastases, one primary colon carcinoma, and one primary pancreatic carcinoma were obtained by surgical resection or from autopsy. Normal liver and lung tissues were obtained from individuals who died from trauma. All tissues were kept frozen at −20° until used. The diagnoses of neuroblastoma were confirmed histologically and by the levels of urinary catecholamine metabolites (9). The age of the patients with neuroblastoma ranged from 7 days to 4 years at the time of admission. Their diseases were classified according to the criteria of Evans *et al.* (3).

PLASMA

Venous blood was drawn into tubes containing crystallized EDTA. The tubes were centrifuged within 2 hr after collection. The blood samples of patients with neuroblastoma were taken on admission before treatment. Age-matched blood samples were taken from healthy individuals in our outpatient department.

ANTISERUM

Specific anti-CEA serum, which was prepared by immunizing a horse with purified CEA of colon according to the method described by Krupey *et al.* (10), was kindly provided by Dr. Nishi of the Department of Biochemistry, Hokkaido University School of Medicine. The details of the preparation and properties of antiserum have been described (12, 13). This antiserum was not absorbed with NCA.

EXTRACTION AND PURIFICATION

CEA from colon carcinoma and a CEA-like substance reacting with anti-CEA serum from neuroblastoma tissue were purified according to the method of Nishi and Hirai (Fig. 1) (12, 13). Tumor tissue, suspended in phosphate-buffered saline (PBS), pH 7.2, was homogenized in a Waring blender and mixed with an equal volume of 2 M PCA. After the precipitate was removed by centrifugation, one part of the supernatant, dialyzed against PBS for 24 hr, was used for the quantitative analysis of CEA activity by radioimmunoassay. Another part was neutralized to pH 7.3 with tris-aminomethane powder, and cold ethanol was added to a final volume of 66%. The precipitate was dissolved in PBS, then ethanol was added to a 33% volume. Following centrifugation, cold ethanol was added to the supernatant to a volume of 66%. The precipitate was dissolved in PBS and used for further purifi-

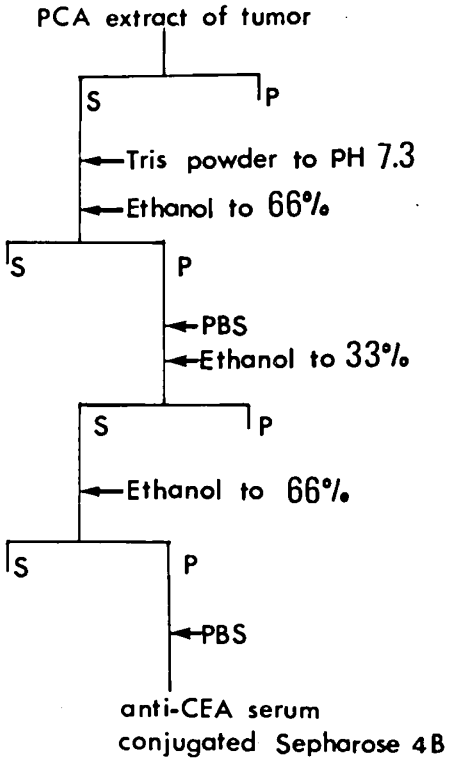

Fig. 1. Procedure for purification of CEA or CEA-like substance (12, 13).

cation of colonic CEA or CEA-like substance in neuroblastoma tissue.

PURIFICATION OF CEA FROM COLON CARCINOMA AND CEA-LIKE SUBSTANCE IN NEUROBLASTOMA TISSUE BY AFFINITY CHROMATOGRAPHY

The γ-globulin fraction of the horse anti-CEA serum was prepared using the conventional 33% saturated ammonium sulfate method. The γ-globulin fraction obtained was then coupled to 6-aminohexyl-Sepharose 4B. The activation of Sepharose 4B was performed according to the method of Cambiaso *et al.* (1). The gel-antibody conjugate was packed in a 2.6 x 20-cm column. The 66% ethanol precipitate of tumor was applied to the column which was subsequently washed with PBS until the effluent had no measurable absorbance at 280 nm. After the elution of CEA activity with 8 M urea, the elute was immediately applied to a Sephadex G25 column (2.6 x 50 cm) to remove urea. This purified material was concentrated by ultrafiltration. The protein concentration was measured with Folin phenol reagent using bovine serum albumin as reference (11).

IMMUNOLOGIC ANALYSIS

Immunodiffusion Techniques. Double immunodiffusion in agar was done according to the method of Ouchterlony (14).

Electrophoresis. Immunoelectrophoresis was performed in 1% agar in barbital buffer (pH 8.3), and $\mu = 0.05$ (7). Disc electrophoresis was done in acrylamide gel according to Davis (2), and the gel was stained by amido black or PAS (20).

Radioimmunoassay. The quantitave CEA activity analysis of plasma and PCA extracts of tumors was determined by use of the zirconium phosphate gel radioimmunoassay method (8), which was provided by Hoffmann-LaRoche Inc. All determinations were carried out in triplicate. The reliability of the CEA assay requires that the SD be less than 0.5 ng/ml for the CEA titer of 0–5 ng/ml, less than 1.0 ng/ml for 5–10 ng/ml, and less than 2.0 ng/ml for 10–20 ng/ml. If the assay exceeded this requirement, then the measurement was repeated.

RESULTS

DEMONSTRATION OF THE CEA-LIKE SUBSTANCE IN NEUROBLASTOMA TISSUES

Anti-CEA serum clearly reacted with PCA extracts of neuroblastoma by double immunodiffusion techniques, whereas the antiserum reacted with neither the pooled normal sera nor PBS extracts of human liver or adrenal gland (Fig. 2).

PLASMA AND TUMOR LEVELS OF CEA ACTIVITY IN PATIENTS WITH NEUROBLASTOMA

By using radioimmunoassay, CEA activity levels of PCA extracts in neuroblastoma tissues were determined and expressed by the quantity of CEA-like substance in starting neuroblastoma tissue. The levels of CEA activity were less than 24.3 ng/g tissue weight in all neuroblastoma tissues. The levels in colon carcinoma and pancreatic carcinoma tissues were, in contrast, more than 4800 ng/g and 3100 ng/g, respectively, and even the CEA levels in normal lung and liver were 336 ng/g and 168 ng/g, respectively. These data indicate that the levels of CEA activity in neuroblastoma tissues were lower than those in other normal and neoplastic tissues examined. Plasma CEA activity levels measured at the time of diagnosis in five patients with neuroblastoma ranged from 2.6–8.4 ng/ml (mean 5.2 ng/ml). These five patients, except Patient 2, were in advanced stages of neuroblastoma. The control group, consisting of 39 healthy children, had plasma CEA values of 1.8 ± 1.5 ng/ml (mean ± SD). The elevation of the plasma CEA activity values in neuroblastoma patients was statistically significant by Student's t test ($P < 0.05$) (Table 1).

NATURE OF CEA-LIKE SUBSTANCE IN NEUROBLASTOMA TISSUES

By using immunoelectrophoresis, about 500 μg protein (11) of concentrated PCA extract of neuroblastoma tissue were applied to the well, and this extract gave a single precipitation line against anti-CEA serum in the γ-globulin region (Fig. 3). To determine the molecular weight of this CEA-like substance, 50 mg protein (11) of PCA extract of neuroblastoma were subjected to gel filtration on a Sephadex G200 column (2.6 x 100 cm). After sufficient concentration by ultrafiltration of the Sephadex effluent, the CEA activity was confirmed to be contained just after the albumin peak by the double immunodiffusion (Fig. 4).

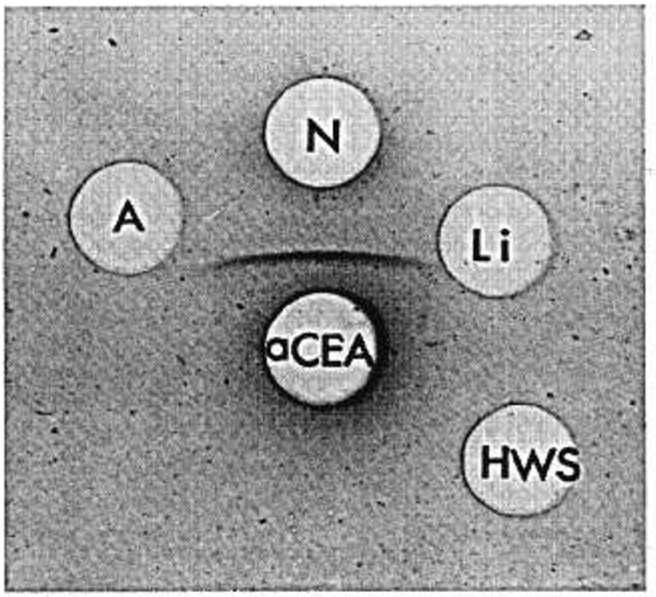

Fig. 2. Demonstration of the CEA-like substance in neuroblastoma tissue. Anti-CEA serum (aCEA) reacted with PCA extract of neuroblastoma tissue (N). HWS, human whole serum; Li, PBS extract of livers; A, PBS extract of adrenal glands.

Table 1. *Plasma and tumor levels of CEA activity by radioimmunoassay in patients with neuroblastoma*

Patient no.	Age	Tumor stage[1]	Tumor site for CEA activity analysis	Tumor levels of CEA activity (ng/g tissue)	Plasma levels of CEA activity[2] (ng/ml)
1	7 days	IVS	Hepatic Metastasis	24.3	8.4
2	14 days	II	Primary	ND[3]	2.8
3	2 months	IV	Primary	4.9	
4	1 yr	IV	Primary	ND	
5	2 yr	III	Hepatic Metastasis	7.9	
6	2 yr	III	Hepatic Metastasis	3.0	
7	3 yr	IV	Primary	0.5	5.3
8	3 yr	IV	Primary	13.4	6.7
9	3 yr	IV	Primary	1.0	
10	3 yr	IV			2.6
11	4 yr	IV	Primary	17.6	

[1] Clinical stage of the disease at the time of admission.
[2] Plasma levels of CEA activity were measured at the time of admission without any treatment.
[3] ND, no detectable amounts.

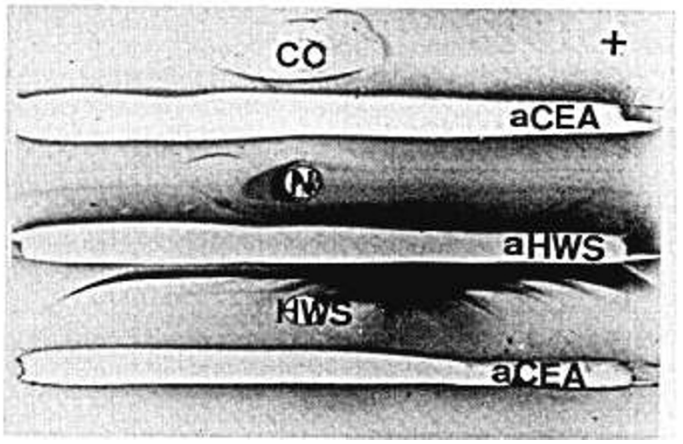

Fig. 3. Immunoelectrophoretic pattern of PCA extract of neuroblastoma (N) and colon carcinoma (CC) against anti-CEA serum (aCEA). A single precipitation line was seen in the γ-globulin region by the PCA extract of neuroblastoma tissue, whereas a single precipitation line was seen in the β-globulin region by the PCA extract of colon carcinoma tissue. HWS, human whole serum; aHWS, antihuman whole serum antiserum.

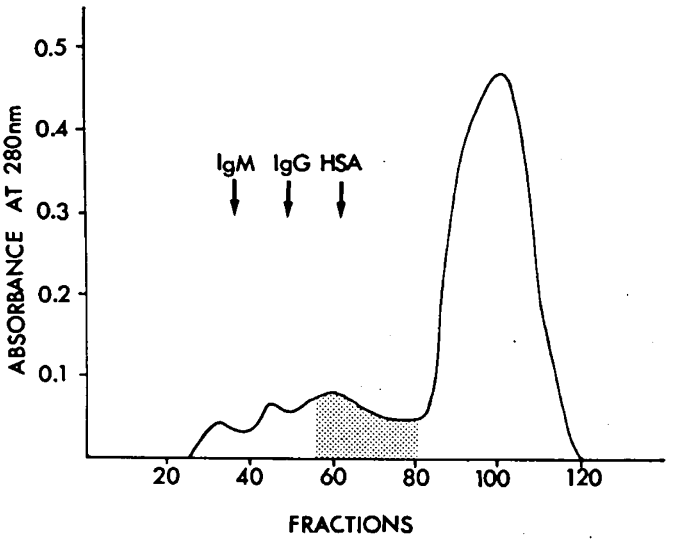

Fig. 4. Sephadex G200 filtration of PCA extract from neuroblastoma. The fraction of CEA activity was detected by the double immunodiffusion technique. The antigenic activity, indicated by the *dotted area*, was eluted just after the albumin peak. The molecular weight of this antigen is estimated to be approximately equal to that of albumin (4.6S).

PURIFICATION OF CEA-LIKE SUBSTANCE IN NEUROBLASTOMA TISSUE BY AFFINITY CHROMATOGRAPHY

The 66% ethanol precipitate of tumor extract was purified by affinity chromatography as described in *Materials and Methods.* Starting from pooled 2.0 kg neuroblastoma tissue, only 100 μg protein (11) of CEA-like substance was obtained. After sufficient concentration by ultrafiltration, 40 μg protein of this purified material was applied to disc electrophoresis. The gel stained for periodate oxidizable sugars revealed a single diffuse band in the γ-globulin region. No staining was detectable on the gel with amido black (Fig. 5).

IDENTIFICATION OF CEA-LIKE SUBSTANCE IN NEUROBLASTOMA TISSUE

The complete identity was observed between the lung PCA extract (NCA) and the neuroblastoma PCA extract as well as the CEA-like substance in neuroblastoma tissue purified by affinity chromatography by double immunodiffusion. Only partial identity was revealed between the purified CEA of colon carcinoma and the CEA-like substance in neuroblastoma tissue purified by affinity chromatography (Fig. 6).

DISCUSSION

Several authors have evaluated the plasma levels of CEA in patients with neuroblastoma using the zirconium phosphate gel radioimmunoassay method. According to Reynoso *et al.* (16), six of seven children with active neuroblastoma had elevated levels of CEA. In three successfully treated children, the CEA levels returned to normal (16). Wang *et al.* (19) reported that 9 of 17 children with neuroblastoma had elevated levels of CEA. Nine of these 17 patients had active disease and 8 of these 9 children with active disease had a high level of CEA. The levels of CEA in children with active neuroblastoma had a tendency to be as high as those in adult patients with colon carcinoma, and correlated well with the patient's clinical status (19). Frens *et al.* (4) assayed the plasma levels of CEA in 14 children with this disease. Eight children had an initial CEA value above 2.5 ng/ml and six of these eight patients had values of 4.0 ng/ml or more. They concluded that slight but definite elevations in plasma CEA can be expected in children with neuroblastoma and the levels might

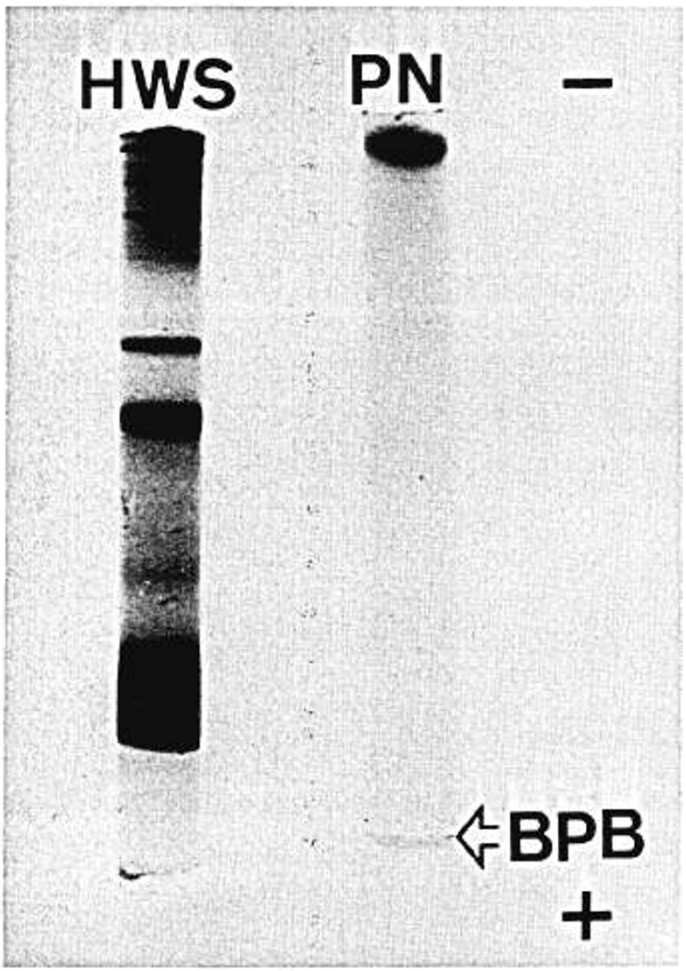

Fig. 5. Disc electrophoresis of the CEA-like substance in neuroblastoma tissue purified by affinity chromatography (PN). The gel showed a single diffuse band in the γ-globulin region with PAS stain. BPB, bromophenol blue tracking; HWS, human whole serum.

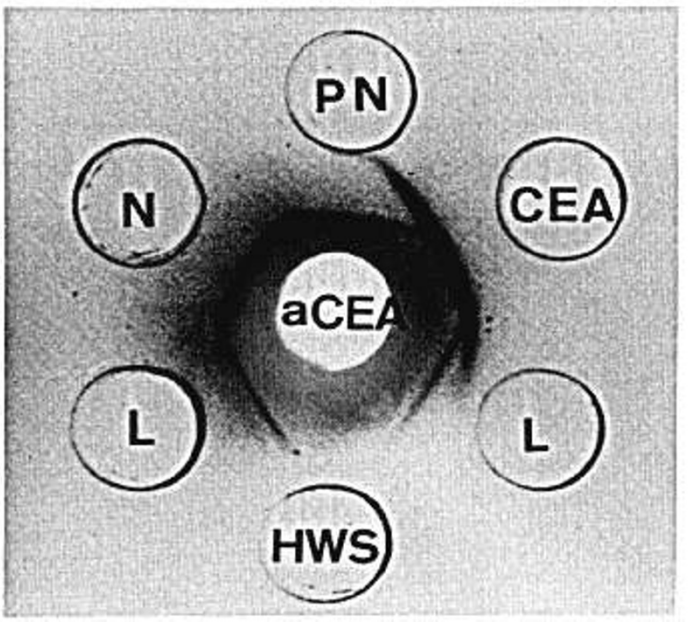

Fig. 6. Demonstration of the partial identity between the CEA-like substance in neuroblastoma tissue purified by affinity chromatography (PN) and the CEA of colon carcinoma purified by affinity chromatography (CEA). Complete identity between the purified CEA-like substance in neuroblastoma tissue and the extract (NCA) of lung (L) was observed. N, PCA extract of neuroblastoma; aCEA, anti-CEA serum; HWS, human whole serum.

be of value in following the disease course (4). These authors did not demonstrate whether the CEA was specific for neuroblastoma tissue, or whether these cancer cells actually released the antigen.

There are some substances in normal tissue which react with anti-CEA serum. One of the representatives is designated as NCA. NCA is a PCA-soluble glycoprotein which is eluted just after the albumin (4.6S) peak by gel filtration on Sephadex G200. Therefore, NCA estimated to be about 4S is smaller than the CEA of human colon carcinoma which is about 8S in molecular weight. This glycoprotein can be extracted in significant amounts from normal lung and other normal tissues. In electrophoresis NCA has a β-globulin mobility, which is a little different from that of CEA (15, 18).

Our comparative study of the CEA activity content of colon carcinoma, pancreatic carcinoma, neuroblastoma, and normal lung and liver tissues demonstrated that neuroblastoma tissue contained a small amount of CEA-like substance, which reacted with anti-CEA serum. We have shown that this CEA-like substance in neuroblastoma tissue is characterized by γ-globulin electrophoretic mobility by immunoelectrophoresis, is approximately 4S by gel filtration, and is a glycoprotein in nature as revealed by staining with PAS by disc electrophoresis. This antigen is partially identical to purified CEA of colon carcinoma, and is completely identical to NCA of normal lung by double immunodiffusion. From these data obtained, it was concluded that the antigenic substance in neuroblastoma tissue is clearly different from that of colonic CEA. Both antigenic materials are different in molecular weight and electrophoretic mobility, and the antigenicity of both are only partially identical. By far, the CEA-like substance in neuroblastoma tissue is very similar to the NCA of the lung, especially in molecular weight and antigenicity, whereas the electrophoretic mobility of both substances are slightly different. From these data, the CEA-like substance in neuroblastoma tissue may be referred to as NCA in neuroblastoma tissue. Therefore, the level of CEA activity in neuroblastoma tissue measured by radioimmunoassay is to be interpreted as detection of a NCA, and it is not the CEA as described by Gold.

The plasma levels of CEA activity in our five patients with neuroblastoma ranged from 2.6 to 8.4 ng/ml (mean 5.2 ng/ml) on admission. The mean value is significantly higher in comparison with the control group, and indicates a trend toward elevated levels in the patients with advanced stages of this disease as demonstrated by Wang *et al.* (19) and Frens *et al.* (4). Serial studies of plasma CEA activity levels may be useful in monitoring the response to therapy in patients with elevated plasma CEA activity on admission. The plasma levels of CEA activity is, however, not usually elevated until the tumor is widely disseminated. Therefore, we consider the plasma level of CEA activity less useful in the early diagnosis of neuroblastoma.

From our data it is possible to state that the elevation of plasma CEA activity in patients with neuroblastoma may not demonstrate the presence of CEA but that of NCA. This plasma NCA may be due to the release of NCA in tumor tissues, or to the destruction by metastasis of normal tissues, which are good sources of NCA, or to a combination of both. In any case, the specificity of plasma CEA activity in patients with neuroblastoma awaits further clarification in the future. To demonstrate the specificity of plasma CEA activity in patients with neuroblastoma, the specific anti-CEA serum, which does not react with NCA, is needed. However, absorption studies on the anti-CEA serum by NCA of lung using affinity chromatography proved to be unsuccessful with us, because of the common determinants on the molecules of both CEA and NCA antigens. For the clinical interpretation of the CEA assay, the nature of anti-CEA serum should be always taken into account for the evaluation of the results of plasma CEA activity in neuroblastoma patients.

CONCLUSION

We concluded from our data that: 1) neuroblastoma tissue contains a small amount of CEA-like substance reacting with anti-CEA serum which is characterized as NCA; 2) slight but significantly elevated plasma levels of CEA activity in patients with neuroblastoma might be caused by either the release of NCA from tumor tissue, or by the destruction by metastasis of normal tissues which include significant amounts of NCA, or by a combination of both; and 3) for the CEA assay, the specificity of anti-CEA serum should be taken into account for the evaluation of its plasma elevation.

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