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Biochemistry

Isolation and characterization of the normal crossreacting antigen: Homology of its NH_2-terminal amino acid sequence with that of carcinoembryonic antigen

(immunoadsorbents/radioimmunoassay/affinity chromatography)

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ABSTRACT **The normal antigen, NCA, which crossreacts with the carcinoembryonic antigen, CEA, was purified from normal spleen tissue by an immunochemical purification method using insolubilized antibodies to either CEA or NCA. The highly purified NCA obtained was extensively characterized by immunological tests. The molecular weight of NCA determined by chromatography on Sephadex G-200 was approximately 100,000. The total amount of carbohydrate in NCA was 30%, compared to 60% in CEA. NCA and CEA also differed in sugar composition. The amino acid composition of NCA was nearly identical to that of CEA, except for the apparent presence of methionine in NCA but not in CEA. The sequence of the first 26 NH_2-terminal amino acids in NCA was identical to that of CEA except at position 21, where alanine was found in NCA instead of valine in CEA.**

Carcinoembryonic antigen (CEA) is a high molecular weight glycoprotein produced by various tumors (1, 2). Radioimmunoassay of circulating CEA is used to monitor the treatment of various types of cancer. Antigens immunologically indistinguishable from CEA or crossreacting with it have been found in a variety of normal tissues and body fluids (3–6). The normal crossreactive antigens may contribute to the lack of cancer specificity of several CEA assays in current use (2, 7, 8).

One of the normal antigens, isolated from normal colon washings, is virtually identical to CEA both immunologically and chemically [6; Shively, J. E., Todd, C. W., Go, V. L. & Egan, M. L. (1978) *Cancer Res.*, in press]. Other normal CEA-related antigens, such as the normal crossreacting antigens, NCA (3, 4, 9) and NCA-2 (10), CELIA (5), and the biliary glycoprotein BGP1 (11) differ from CEA both immunologically and in some physical characteristics, such as molecular weight.

Since an understanding of the chemical basis of the antigenic differences between CEA and the crossreacting antigens might be helpful in developing reagents with higher cancer specificity, we undertook this study on the chemical properties of NCA.

CEA is a product of epithelial cells (12), and NCA is present in granulocytes and histiocytes (13, 14). Both CEA and NCA are present in extracts of tumors of the gastrointestinal tract in high amounts. NCA in such tumor extracts is, however, difficult to physically separate from low molecular weight CEA, which is also present (15). We, therefore, chose to isolate and characterize NCA from normal tissue, notably normal spleen, which does not contain epithelial tissue or CEA.

We report here that highly purified NCA from nontumorous tissue has striking similarities to CEA in the protein portion of the molecule but distinct differences in its degree of glycosylation.

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MATERIALS AND METHODS

Tissue. Human spleens were obtained at autopsy from individuals without malignancy. The tissues were homogenized in water, and perchloric acid was added to 0.6 M final concentration. After centrifugation, the supernatant was neutralized, dialyzed, and lyophilized. Normal human serum was similarly treated with perchloric acid.

Antisera. Antisera were prepared in sheep, goats, and rabbits by intramuscular injections of antigen emulsified in Freund's complete adjuvant on day 0, 14, and 28, and then at monthly intervals. The animals were first bled on day 35 and then 1 week after each injection. The 35-day sera were used for preparation of immunoadsorbents, and sera obtained from later bleedings for immunoassays. The following antisera were used in this study. Rabbit and sheep anti-CEA were obtained by immunization with 200 μg per injection of purified CEA, and goat anti-NCA with 100 μg per injection of purified NCA. Rabbit anti-tumor(perchlorate) was obtained by immunization with 10 mg per injection of perchloric acid-soluble material from a pool of 10 colonic tumors, rabbit anti-spleen(perchlorate) with 5 mg per injection of acid-soluble material from normal spleen, and rabbit anti-normal serum(perchlorate) with 5 mg per injection of acid-soluble material extracted from normal serum.

Immunoadsorbents. Immunoadsorbents were prepared by coupling the immunoglobulin fraction, obtained by 18% Na_2SO_4 precipitation, to cyanogen bromide-activated Sepharose (16).

Immunoassays. Immunodiffusion was performed with 0.6% agarose (Bio-Rad Laboratories, Richmond, CA) in phosphate-buffered saline, pH 7.2. Antigen-binding activity of antisera was measured by double-antibody radioimmunoassay with antigens labeled with ^{125}I by means of chloramine T. NCA was quantitated by the inhibition of binding of radiolabeled NCA by the goat anti-NCA.

Purification of NCA. NCA was prepared by two methods. Preparation I was obtained with an anti-CEA immunoadsorbent. Two grams of spleen perchloric acid extract in phosphate-buffered saline was incubated with sheep anti-CEA immunoadsorbent prepared from 60 ml of antiserum. After washing, bound protein was eluted by 8 M urea in 50 mM Tris·HCl (pH 7.0), dialyzed against water, and lyophilized. Ten batches were combined and purified on concanavalin A–Sepharose (Pharmacia, Piscataway, NJ). NCA eluted from the column with 1 M α-methyl-D-mannopyranoside (Sigma Chemical Co., St. Louis, MO) was treated with rabbit anti-sheep immunoglobulin linked to Sepharose to remove traces of sheep

Abbreviations: NCA, normal crossreacting antigen; CEA, carcinoembryonic antigen.

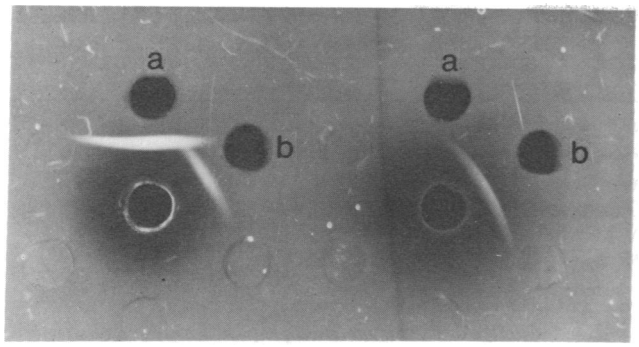

FIG. 1. Immunodiffusion in agarose. Sheep anti-CEA (*Left*) and goat anti-NCA (*Right*) were tested against CEA (a), 1 mg/ml, and NCA (b), 0.2 mg/ml and 1 mg/ml, respectively.

immunoglobulin leaked from the immunoadsorbent and with rabbit anti-spleen(perchlorate) adsorbent to remove a contaminating protein immunologically unrelated to CEA and NCA. The preparation was further purified on a column (1.5 × 100 cm) of Sephadex G-200. Peak fractions were pooled, dialyzed, and lyophilized. A portion of this preparation was used for immunization of a goat. Preparation II was prepared by a similar procedure except that NCA from spleen perchloric acid extract was absorbed to and eluted from an immunoadsorbent prepared from 50 ml of goat anti-NCA. Four batches were prepared. Treatment with the anti-spleen(perchlorate) adsorbent was omitted.

Amino Acid Analysis. The analysis was performed by a modification of the method of Liu and Chang (17). Duplicate samples (100 μg) were hydrolyzed under reduced pressure in heavy-walled ignition tubes at 110° for 48 hr in duplicate with 0.5 ml of 3 M *p*-toluenesulfonic acid containing 0.2% 3-(2-aminoethyl)indole. The hydrolysates were analyzed on a Beckman 121H amino acid analyzer. The basic amino acids and amino sugars were eluted from a 20 × 0.9 cm column of PA35 (Beckman) in sodium citrate buffer, pH 5.26 (0.4 M sodium). The acidic and neutral amino acids were eluted from a 56 × 0.9 cm column of AA15 (Beckman) with sodium citrate buffers, pH 3.10 (0.16 M sodium), pH 3.60 (0.20 M sodium), and pH 4.20 (0.20 M sodium).

Carbohydrate Analyses. Duplicate samples (3–10 μg) were methanolyzed under nitrogen at 80° for 24 hr with 0.5 ml of 1.5 M methanolic HCl, evaporated to dryness under reduced pressure over KOH, and treated with trifluoroacetic anhydride at 145° for 10 min (18). The trifluoroacetyl derivatives of the methylglycosides were separated on a 0.5 mm × 7.0 m packed capillary column (coated in situ on Chromosorb 100–150 mesh with a 1% solution of OV-210, Applied Science Laboratories, Inc., Bellefonte, PA). The temperature was programmed from 120° to 210°. Quantitation was performed by a Hewlett Packard 5709 electron capture detector coupled to an Autolab System IV peak integrator.

Determination of Sequence. Amino acid sequence was determined on a Beckman model 890C sequencer using a dimethylallylamine program (Beckman 102974). The phenylthiohydantoin derivative of aminoisobutyric acid (4.5 nmol) was added to each Edman degradation cycle as an internal standard. Thiazolinone amino acid derivatives were converted to phenylthiohydantoin derivatives in 20% aqueous trifluoroacetic acid at 55° (19). The phenylthiohydantoin derivatives were separated and quantitated by gas chromatography according to Pisano and Bronzert (20) on a Hewlett Packard 5701A gas chromatograph and by high-pressure liquid chromatography on a Waters Associates liquid chromatograph, both equipped with an Autolab System IV peak integrator. The liquid chromatography was linearly programmed from 0 to 35% component B over 30 min at 2 ml/min, where component A was 0.1 M sodium acetate (pH 7.6)/acetonitrile 95:5 (vol/vol) and component B was 100% acetonitrile. The separation of phenylthiohydantoin amino acids by high-pressure liquid chromatography was similar to that obtained by Zimmerman *et al.* (21) and Downing and Mann (22). In addition, the thin-layer chromatographic procedure of Summers *et al.* (23) was used with 2,5-bis-2-(5-tert-butylbenzoxazolyl)thiophene (Packard) as the fluor. Reagents, solvents, and sperm whale apomyoglobin obtained from Beckman were used throughout this work.

RESULTS

Purification of NCA. About 2 g of perchloric acid extract was obtained per kg of spleen tissue, with an average NCA content of 0.5% as measured by radioimmunoassay. After the immunoadsorbent step with either anti-CEA or anti-NCA adsorbents, the NCA content was approximately 40% by weight. The material was contaminated by immunoglobulin leaking from the immunoadsorbents during the urea elution step. Most of the contaminating gamma globulin was removed by affinity chromatography on concanavalin A–Sepharose and the rest by treatment with anti-gamma globulin–Sepharose. At this stage, NCA preparation I, but not II, was found to contain small amounts of another protein present in large amounts in the original extract. This was removed by absorption with anti-spleen(perchlorate) coupled to Sepharose. Pooled fractions from gel filtration on Sephadex G-200 gave 2.5 mg and 2.3 mg for preparation I and II, respectively. Within experimental error, preparations I and II gave identical results in immunological and chemical analysis.

Immunochemical Properties of Purified NCA. Purified NCA, when tested in concentrations between 0.1 and 2 mg/ml, gave a single identical precipitin line in immunodiffusion with rabbit and sheep anti-CEA, goat anti-NCA, and rabbit anti-tumor(perchlorate). In agreement with results obtained from this (24) and other laboratories (3, 4, 10), the NCA precipitation line fused with the CEA precipitation line, using anti-CEA, showing the partial identity of NCA with CEA (Fig. 1). Purified NCA did not react with monkey anti-CEA sera, in agreement with earlier results that such antisera are specific for CEA and do not crossreact with NCA (24).

Tested at up to 5 mg/ml, purified NCA did not react in immunodiffusion with anti-normal human serum, anti-sheep or goat gamma globulin, anti-concanavalin A, or with antisera against perchloric acid extracts of spleen or human serum. The serum produced in a goat by immunization with purified NCA strongly precipitated NCA (Fig. 1) and only NCA from spleen perchloric acid extracts and did not react with normal human serum. Goat anti-NCA did not precipitate CEA in immunodiffusion (Fig. 1) but bound 90% of ^{125}I-labeled CEA in radioimmunoassay (Fig. 2). Anti-CEA and anti-NCA both bound 90–95% of radiolabeled NCA. The antiserum prepared against perchloric acid extracts of spleen also bound ^{125}I-labeled NCA in radioimmunoassay (Fig. 2). None of the other control antisera [anti-normal human serum, anti-normal human serum(perchlorate), anti-sheep gamma globulin, and anti-concanavalin A] showed detectable binding of NCA in radioimmunoassay.

Fig. 3 shows the elution behavior of iodinated CEA and NCA run separately on a column of Sephadex G-200 that was calibrated with blue dextran, IgG, and serum albumin. The ap-

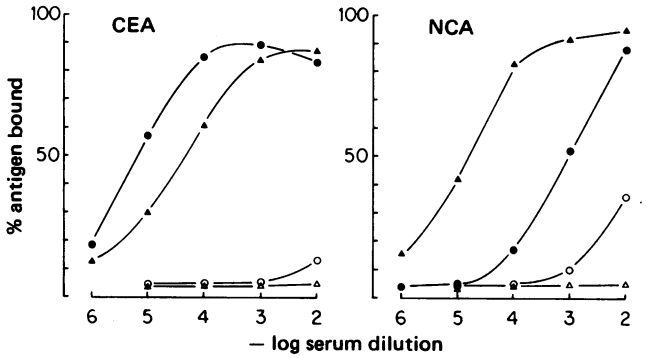

FIG. 2. Titration of sheep anti-CEA (●), goat anti-NCA (▲), rabbit anti-spleen(perchlorate) (○), and rabbit anti-serum(perchlorate) (△) against CEA and NCA in radioimmunoassay.

proximate molecular weights are 200,000 and 100,000 for CEA and NCA, respectively.

Carbohydrate and Amino Acid Analysis of NCA. Table 1 shows the carbohydrate composition of NCA. NCA contained less fucose and galactose and considerably more mannose than CEA (25). *N*-Acetylglucosamine was the only hexosamine present in NCA, as determined by amino acid and carbohydrate analyses. From the sugar composition and the amount of *N*-acetylglucosamine obtained by amino acid analysis, the total carbohydrate content of NCA was estimated to be 30%. This is approximately half of that reported for CEA.

The amino acid composition of NCA was very similar to that of CEA (Table 2). Aspartic acid (including asparagine) was the most abundant amino acid in NCA, as in CEA, and relatively high levels or serine, glutamic acid, proline, and threonine were present in both proteins. Small but reproducible amounts of methionine were found in NCA. The data for NCA shown in Table 2 are in excellent agreement with recent data by Hammarström *et al.* (15), but differ slightly from those of Turberville *et al.* (26) and Newman *et al.* (27).

NH_2-Terminal Sequence. The NH_2-terminal amino acid sequence of NCA shown in Table 3 is identical to that obtained from CEA with one exception. Alanine was unambiguously identified at position 21 for NCA, but valine is consistently found at position 21 for CEA isolated from many different tumor preparations (28, 29, 31).

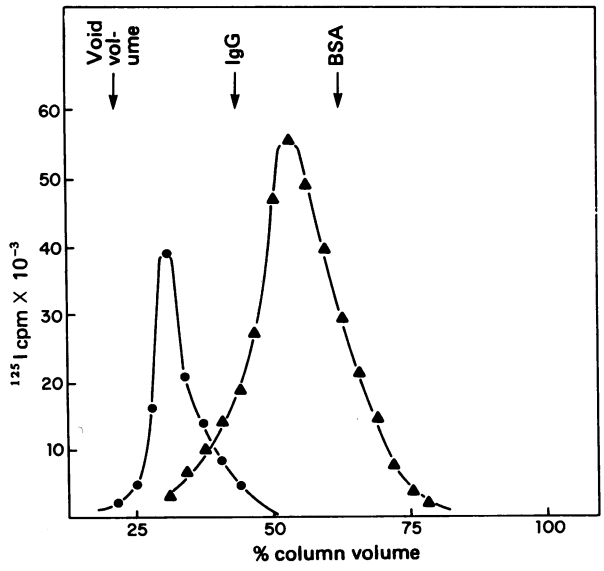

FIG. 3. Gel filtration on Sephadex G-200 of ^{125}I-labeled NCA (▲) and CEA (●). The elution positions of blue dextran, IgG, and serum albumin (BSA) are indicated by arrows.

Table 1. Carbohydrate composition of NCA and CEA expressed as mol % (residues per 100 residues)

Sugar	NCA*	CEA†
Sialic acid	4.3 (2.5–5.8)	2.1 (0.1–5.0)
Fucose	11.0 (8.3–12.2)	18.1 (14.5–20.9)
Mannose	46.2 (37.8–50.6)	17.2 (15.2–19.2)
Galactose	12.3 (12.1–12.8)	24.7 (21.8–27.1)
N-Acetylglucosamine	25.3 (22.1–31.4)	34.0 (30.9–35.8)
N-Acetylgalactosamine	0	3.8 (2.3–7.6)

* Mean and range of results on three preparations. A third preparation, available in amounts sufficient only for immunochemical and carbohydrate analyses, was prepared as described for NCA preparation I.

† Mean and range of five preparations according to Egan *et al.* (25).

DISCUSSION

We have isolated and characterized NCA from nontumorous tissue and compared it to CEA immunologically and with respect to amino acid and carbohydrate composition and NH_2-terminal amino acid sequence. NCA is a minor component among the proteins of normal spleen tissue. Even after perchloric acid extraction, used as an efficient way to enrich the glycoproteins including NCA, it comprised only about 0.5% of the weight of such an extract. Purification of NCA to homogeneity was made possible by the immunochemical procedures developed by Vuento *et al.* (32). These procedures should be useful in the isolation of other CEA-related antigens present in minute amounts in various tissues and body fluids.

Immunochemical characterization of purified NCA confirmed its well-known crossreactivity with CEA (3, 4, 10). Antiserum produced against purified NCA, while crossreacting with CEA, had predominantly antibodies of the noncrossreacting type. Extensive immunological differences thus exist between NCA and CEA.

Table 2. Amino acid composition of NCA and CEA*

Amino acid	mol/100 mol NCA†	mol/100 mol CEA*
Tyr	5.1 (4.3–5.6)	5.0 (4.8–5.3)
Phe	2.3 (1.7–2.8)	2.7 (2.5–2.9)
Trp	2.0 (1.5–2.4)	1.2 (1.0–1.5)
Lys	3.2 (3.1–3.3)	2.9 (2.6–3.5)
His	1.3 (1.2–1.4)	1.9 (1.7–2.1)
Arg	3.3 (2.9–3.9)	3.7 (3.4–3.9)
Asp	13.4 (13.0–13.7)	14.9 (13.9–16.0)
Thr	7.9 (7.7–8.5)	8.4 (8.2–8.7)
Ser	9.5 (8.9–10.0)	9.9 (9.8–10.0)
Glu	11.8 (11.6–11.9)	9.8 (9.7–9.9)
Pro	7.0 (5.2–8.1)	8.1 (7.6–8.6)
Gly	6.9 (6.6–7.3)	5.4 (5.2–5.6)
Ala	6.1 (5.9–6.4)	5.6 (5.5–5.7)
½ Cys	1.4 (0.8–1.9)	1.3 (1.3–1.4)
Val	6.2 (5.9–6.9)	6.4 (6.2–6.6)
Met	0.5 (0.3–0.9)	0.0
Ile	3.7 (3.5–4.0)	4.1 (4.0–4.3)
Leu	8.4 (8.3–8.5)	8.5 (8.1–8.8)

* Mean and range of three preparations of CEA from Egan *et al.* (6).

† Mean and range of three determinations.

Table 3. NH_2-terminal sequence analysis of NCA*

Position	GC†	HPLC‡	TLC§	NCA sequence	CEA sequence¶
1	—	Lys	Lys	Lys	Lys
2	Leu	Leu	Leu/Ile	Leu	Leu
3	Thr	Thr	Thr	Thr	Thr
4	Ile	Ile	Leu/Ile	Ile	Ile
5	—	Glu	Glu	Glu	Glu
6	Ser	Ser	Ser	Ser	Ser
7	Thr	Thr	Thr	Thr	Thr
8	Pro	Pro	Pro	Pro	Pro
9	Phe	Phe	Phe	Phe	Phe
10	—	Asn	Asn	Asn	Asn
11	Val	Val	Val	Val	Val
12	Ala	Ala	Ala	Ala	Ala
13	—	Glu	Glu	Glu	Glu
14	Gly	Gly	Gly	Gly	Gly
15	—	Lys	Lys	Lys	Lys
16	—	Glu	Glu	Glu	Glu
17	Val	Val	Val	Val	Val
18	Leu	Leu	Leu/Ile	Leu	Leu
19	Leu	Leu	Leu/Ile	Leu	Leu
20	Leu	Leu	Leu/Ile	Leu	Leu
21	Ala	Ala	Ala	Ala	Val
22	—	His	His	His‖	His
23	—	Asn	Asn	Asn	Asn
24	Leu	Leu	Leu/Ile	Leu	Leu
25	—	Glu	Glu	Glu	Glu
26	(Leu/Ile)	(Leu)	(Leu/Ile)	(Leu)	Leu

* Results obtained for duplicate runs on a Beckman 890C sequencer.

† Gas chromatographic analysis according to Pisano and Bronzert (20). The pair Leu/Ile were distinguished by comparison of gas chromatography runs before and after trimethylsilylation.

‡ High-pressure liquid chromatographic analysis.

§ Thin-layer chromatographic analysis according to Summers *et al.* (23).

¶ Results obtained for CEA (28, 29) and repeatedly confirmed in this laboratory.

‖ His was confirmed by a positive spot test performed according to Easley (30).

It is known that the antigenic determinants specific for CEA reside in the protein portion of the molecule and are conformationally dependent (33, 34). The crossreacting determinants in CEA and NCA also seem to be protein in nature but to be less conformationally dependent. This is based on the observation that these determinants are not affected by either periodate oxidation, which destroys sugars containing vicinal hydroxyl groups, or unfolding of the molecules by complete reduction and alkylation (unpublished data). We do not know yet the nature of the antigenic determinants specific for NCA.

The apparent molecular size of NCA obtained by the procedure used was approximately 100,000, as judged by its behavior on gel filtration on Sephadex G-200 (Fig. 3). This was higher than what has been reported earlier. Mach and Pusztaszeri (4) found that NCA eluted slightly after serum albumin on gel filtration, and Engvall *et al.* (35) reported a similar elution pattern for immunopurified NCA. However, in earlier studies, Engvall *et al.* (35) found two peaks of NCA activity on gel filtration of monkey spleen extract. Since urea/formic acid was used to elute CEA (32) and NCA (24, 35) from anti-CEA adsorbents and since it is known that CEA is acid-labile (36), and perhaps NCA is also, the lower molecular weight material may have been a degradation product of the higher molecular weight peak. The milder conditions now used for making perchloric acid extracts and for eluting NCA from anti-CEA and anti-NCA immunoadsorbents gave no evidence for lower molecular weight NCA.

The carbohydrate portions of CEA and NCA show extensive differences. The amount of carbohydrate is approximately 60% by weight for CEA and only 30% for NCA. The carbohydrate compositions were also different, NCA containing relatively less fucose and galactose and much more mannose than CEA.

The amino acid composition of NCA was strikingly similar to that of CEA. NCA contains methionine, an amino acid that is not present in CEA. Although we believe that methionine is part of the NCA polypeptide, the possibility that the methionine is present in a contaminating protein cannot presently be excluded.

The most significant result of our studies was the extensive homology shown between the NH_2-terminal sequences of CEA and NCA. Of the 26 amino acid residues whose sequence was determined in NCA, only one was different from those in CEA. The replacement of alanine at position 21 in NCA for valine at the same position in CEA represents the effect of a single base mutation. It may be that the gene coding for CEA/NCA has undergone a very recent duplication. This could explain why CEA has not been identified in any nonhuman species. The only nonhuman antigen with a proven relationship to CEA and NCA is an NCA-like antigen found in Cynomolgus monkeys (35).

In view of the extensive antigenic differences, as judged by immunodiffusion, between NCA and CEA, and the differences in the amount and composition of carbohydrate, it was rather surprising that their NH_2-terminal amino acid sequences differed only in 1 out of 26 residues. It is unlikely that this difference would be due to an allotypic variant, because the sequences of several CEA samples have been determined in various laboratories without any evidence of alanine in position 21. Similarly, the two NCA preparations, in spite of the fact that they came from pools of spleens, showed only alanine, with no valine at position 21.

The extensive NH_2-terminal amino acid sequence homology shown in these studies to exist between CEA and NCA provides a chemical basis for their observed antigenic relatedness.

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