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Characterization of Carcinoembryonic Antigen-related Antigens in Normal Adult Feces

Yuji Matsuoka,[1,4] Yoshino Matsuo,[1] Kokichi Sugano,[2,3] Hisanao Ohkura,[2] Motomu Kuroki[1] and Masahide Kuroki[1]

[1]*First Department of Biochemistry, School of Medicine, Fukuoka University, 7-45-1 Nanakuma, Jonan-ku, Fukuoka 814-01,* [2]*Clinical Laboratory Division, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104 and* [3]*Department of Surgery, Hino Municipal Hospital, 6-1-1 Toyoda, Hino-shi, Tokyo 191*

About 50–70 mg in total of carcinoembryonic antigen (CEA) (or CEA-related antigens) was detected in normal adult feces evacuated during one day (200–250 g). Ten percent or less of the antigen was found to be in soluble form in fresh feces (naturally solubilized antigen), while 90% or more was still in membrane-bound form which was releasable with phosphatidylinositol-specific phospholipase C (PI-PLC-solubilized antigen). The naturally solubilized and PI-PLC-solubilized antigens are antigenically different from each other and similar to normal fecal antigen (NFA)-2 and CEA, respectively, suggesting that "CEA-distinctive" antigenicity detected so far in CEA from cancerous tissues is not due to the difference between antigens in normal and malignant tissues but is probably due to the presence of the glycosylinositolphosphate moiety at the carboxyl-terminus of the antigen molecule. Thus, "CEA-distinctive" antigenicity is by no means cancer-specific, but this antigenicity seems to be critical for the clinical significance of CEA as a tumor marker, because an assay system (Kit II) which is able to distinguish CEA from NFA-2 revealed much improved features in cancer diagnosis as reported recently.

Key words: Carcinoembryonic antigen — Normal fetal antigen — Glycolipid anchor — Phosphatidylinositol-specific phospholipase C — CEA-distinctive antigenicity

Carcinoembryonic antigen (CEA),[5] a highly glycosylated glycoprotein with a molecular mass of approximately 180 kDa,[1,2] is a well-established tumor marker. CEA has been thought to be a typical example of oncofetal antigens or derepressed embryonic gene expression caused by malignant transformation of epithelial cells.[2] We found recently, however, that normal colon mucosae produce CEA quite actively, as cancerous tissues do, when maintained in an organ culture, although fresh normal mucosae contained a very small quantity of CEA, unlike cancerous tissues.[3] This suggests that the normal cell product may be rapidly released into the lumen of the digestive tract and turn into normal fecal antigens (NFAs), which were identified in normal adult feces as antigens very closely related to CEA.[4,5] In contrast, the abnormal cellular architecture of cancerous tissues may lead to a disturbance of the releasing mechanism of the antigen from mucosa into the lumen, resulting in an accumulation of CEA in the cells and its outflow into the circulation. Thus, elucidation of the releasing mechanism of CEA (or CEA-related antigens) from normal mucosa seems to be indispensable for understanding the cancer specificity of CEA.

After recent elucidation of the primary structure of CEA peptide by cloning of the corresponding cDNA,[6] we found that CEA is anchored to the cell membrane through a glycosylphosphatidylinositol (GPI) which is cleavable with PI-PLC,[7] and that the antigen produced by normal colon mucosa is also anchored to the cell membrane through the same GPI.[3] This allows us to analyze the membrane-anchoring form of the antigens on the basis of susceptibility to PI-PLC treatment.

In the present study, we characterized features of the CEA-related antigens in normal adult feces and found that the majority of the antigens in feces exists still in membrane-bound form, which is releasable by PI-PLC treatment, and that the antigen newly solubilized with PI-PLC is different antigenically from the antigen originally present in soluble form in fresh feces.

MATERIALS AND METHODS

Antigen preparations Fresh feces from normal adults were suspended in an extraction buffer (0.01 *M* phosphate-buffered saline pH 7.0 containing 10 μg each of

[4] To whom correspondence should be addressed.

[5] Abbreviations: CEA, carcinoembryonic antigen; kDa, kilodalton; NFA, normal fecal antigen; GPI, glycosylphosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; RIA, radioimmunoassay; MAb, monoclonal antibody; NCA, nonspecific cross-reacting antigen; NFCA, normal fecal cross-reacting antigen; GIP, glycosylinositolphosphate.

pepstatin A, chymostatin, leupeptin and antipain per ml, and 0.1% NaN_3), then fractionated by stepwise centrifugations. Fractionated samples were immediately frozen in acetone-dry ice, and stored at −25°C until use.

Enzyme and inhibitors Highly purified PI-PLC was purchased from Funakoshi Pharmaceutical Co. (Tokyo). Its specific activity was 9.6 units per ml of enzyme solution in glycerol. Protease inhibitors, pepstatin A, chymostatin, leupeptin and antipain were purchased from Protein Research Foundation (Osaka).

Reference antigens NFA-2 and CEA were highly purified from normal adult feces and colonic cancerous tissues, respectively, and used as reference antigens. Purification procedures and chemical and antigenic properties of these antigens were described previously.[5, 8]

Antibodies and RIA Properties of MAbs used in this study and the antigenic structure of CEA analyzed by these MAbs were described elsewhere.[9, 10] In brief, the antigenic structure of CEA was divided into 5 parts, the NCA-common part recognized by MAbs of Group I, the NFCA-common part recognized by MAbs of Group II, the NFA-1-common part recognized by MAbs of Group III, the heterogeneous part of carbohydrate nature recognized by MAbs of Group IV and the CEA-distinctive part recognized by MAbs of Group V. F4-82 (Group II) and F3-30 (Group III) reacted strongly with both CEA

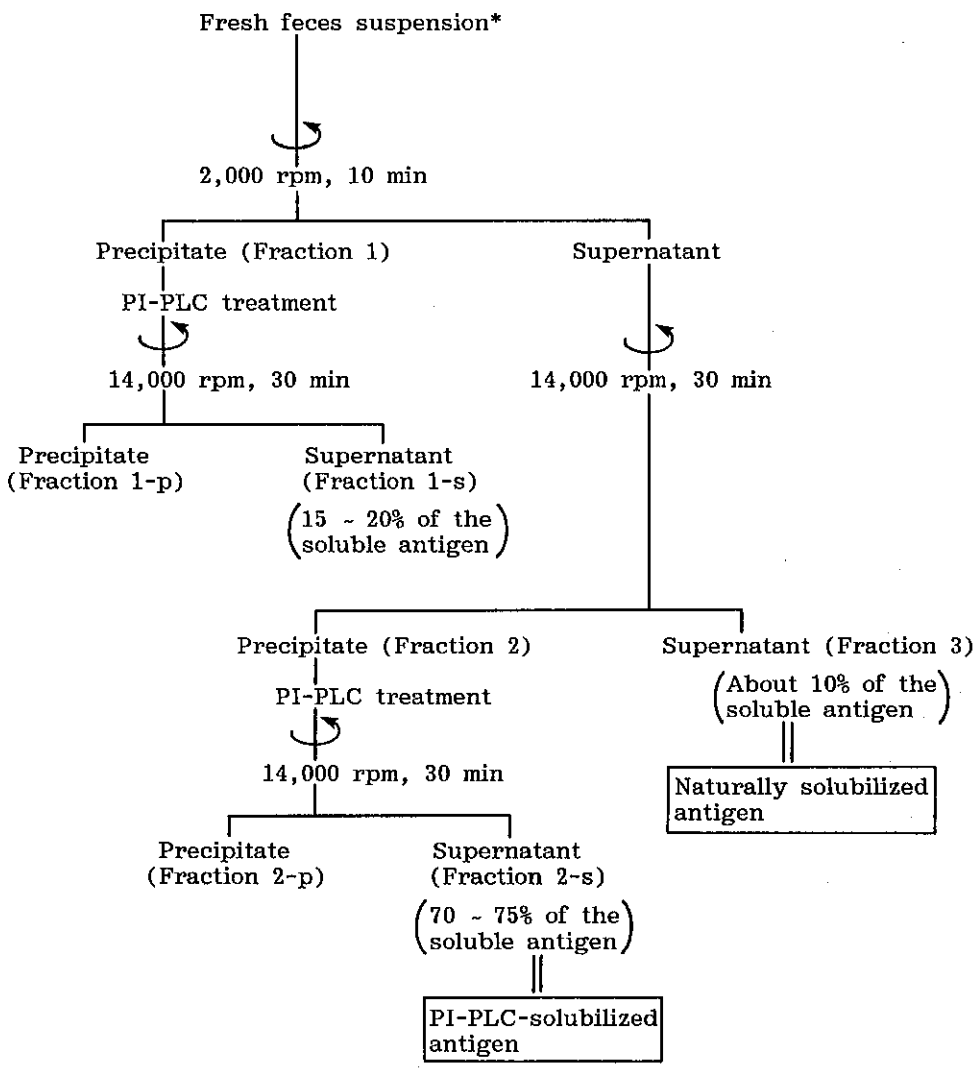

Fig. 1. Fractionation of fresh feces and antigen distribution. For detailed procedures, see the text. * About 30–50 mg (100%) of soluble antigen was detected in the feces evacuated in one day (200–250 g), after PI-PLC treatment.

and NFA-2, and F33-104 (Group V) reacted strongly with CEA but weakly with NFA-2.

Quantitation of antigens was carried out by using two solid-phase sandwich-type RIA systems. A polyclonal system (Kit I) showed a reactivity with NFA-2 almost equivalent to that with CEAs,[11] and a monoclonal system (Kit II) in which MAbs F4-82 and F3-30 were used as the catcher and F33-104 labeled with ^{125}I was used as the tracer showed a very homogeneous reactivity with various preparations of CEA purified from tumor tissues but a weak reactivity with NFA-2 (approximately 1/5–1/10 of that with CEAs).[10] For both assay systems, a preparation of CEA (TY) was used as the standard antigen for calibration.

To estimate the reactivity of antigen preparations with MAb F3-30 or F33-104, each MAb was immobilized on the surface of polystyrene beads as the catcher, and the same polyclonal anti-CEA antibody as used for Kit I was used for the tracer labeled with ^{125}I.

Gel filtration Antigens were chromatographed on a column of Sepharose 6B as reported previously.[8]

RESULTS

Fractionation of fresh feces and antigen distribution Ten to 20 g of fresh feces from normal adults was suspended in 10 volumes of extraction buffer. After the removal of massive food remnants by gauze-filtration, the suspension was fractionated by stepwise centrifugations as indicated in Fig. 1. Each precipitate was resuspended in the same volume of the buffer as that of the supernatant. Aliquots of each fraction were incubated with (0.14 units/ml) or without PI-PLC for 2 h at 37°C, then centrifuged at 14,000 rpm for 30 min. The antigen concentration in each fraction was estimated by using Kit I. Although some fluctuations were observed, about 30–50 mg of antigen in total was detected as soluble antigen in feces evacuated during one day (200–250 g of feces) after treatment with PI-PLC. Since unsolubilized antigen amounting to more than one-fourth of the solubilized antigen remained in the precipitate after the PI-PLC treatment, the total antigen amount in the feces seemed to be 50–70 mg. As indicated in Fig. 1, 15–20% of the total soluble antigen was detected in Fraction 1-s, 70–75% in Fraction 2-s, and about 10% in Fraction 3, which was originally soluble in the fresh feces and was designated as naturally solubilized antigen. By repeated washings before PI-PLC treatment, most of the antigen was removed from Fraction 1 and refractionated into Fraction 2, indicating that the antigen in Fraction 1 was essentially the same as that in Fraction 2. Consequently, 90% or more of the antigen in fresh feces seems to be still in membrane-bound form and only 10% or less is in soluble form.

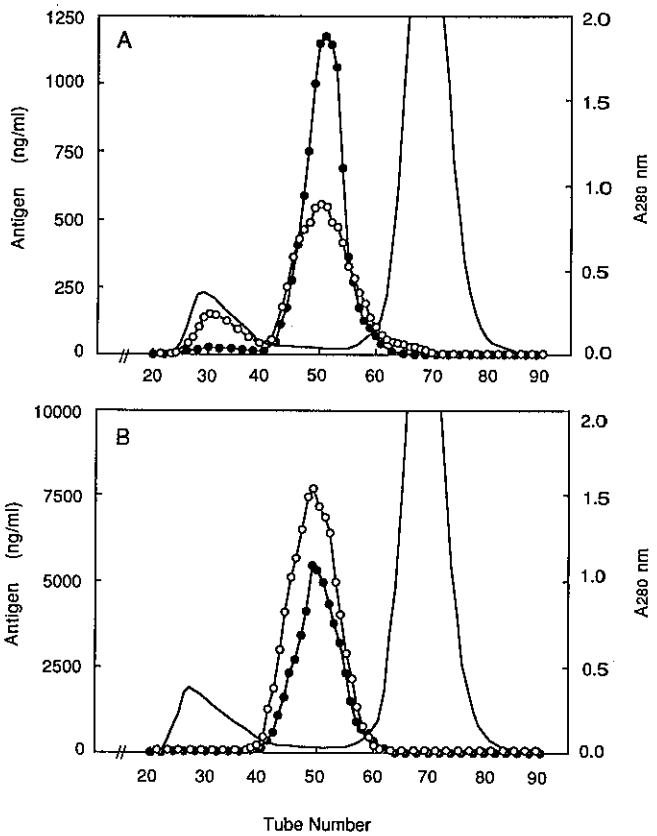

Fig. 2. Gel filtration profiles of naturally solubilized (A) and PI-PLC-solubilized (B) antigens from a Sepharose 6B column. An aliquot of 1.5 ml of Fraction 3 (A) or Fraction 2-s (B) was applied to a column (1.4×70 cm) of Sepharose 6B which had been equilibrated with 0.05 *M* phosphate-buffered saline pH 5.2 containing 0.01% NaN_3, then eluted with the same buffer at a flow rate of 3 ml/h. The eluate was collected in fractions of 1.5 ml each. Protein concentration of the eluate was traced by measuring the absorbance at 280 nm ($A_{280\ nm}$) (—), and antigen concentration was estimated by using Kits I (●) and II (○).

Gel filtration profiles of naturally solubilized and PI-PLC-solubilized antigens The elution profiles of the naturally solubilized antigen in Fraction 3 and the PI-PLC-solubilized antigen in Fraction 2-s were compared. The concentration of eluted antigen was estimated by using both Kits I and II. As shown in Fig. 2, Fractions 3 and 2-s showed an identical protein elution pattern ($A_{280\ nm}$). Although Fraction 2-s contained about 5–10 times more antigen than Fraction 3, no difference was observed in the elution positions of these antigens, suggesting no apparent difference in molecular size between them. The naturally solubilized antigen in Fraction 3 revealed a stronger reactivity with Kit I than with Kit II, mimicking the reactivity of NFA-2 purified from

Table I. Reactivities of Antigens in Naturally or PI-PLC-solubilized Form and in Membrane-bound Form with MAbs of Groups III and V

Antigen preparations	Reactivities of 100 ng antigen[a] with		
	Group III[b] (F3-30)	Group V[b] (F33-104)	Kit II[c]
		cpm	
Naturally solubilized (Fraction 3)	23,000	2,700	4,200
PI-PLC solubilized (Fraction 2-s)	34,000	10,000	13,600
Membrane-bound (Fraction 2, before PI-PLC)	29,000	11,200	9,700
Membrane-bound (Fraction 2-p, after PI-PLC)	24,000	7,800	8,900
NFA-2	13,200	1,200	3,000
CEA(TY)[d]	19,800	8,200	6,800
CEA(DB)[d]	20,000	6,700	8,300

a) Reactivities were normalized for 100 ng of antigens estimated by the polyclonal Kit I.
b) MAbs were used for solid-phase antibody and the same polyclonal antibody as in Kit I was used for the tracer.
c) In Kit II, F4-82 (Group II) and F3-30 (Group III) were used for the catcher and F33-104 (Group V) for the tracer.
d) Initials of individuals from whom CEA was obtained are indicated in parentheses.

normal feces, whereas the PI-PLC-solubilized antigen showed a stronger reactivity with Kit II than with Kit I, mimicking the reactivity of CEA purified from cancerous tissues. Since Fraction 2-s probably contained some naturally solubilized antigen, a more distinct reactivity difference between Kits I and II might be seen with a putative pure fraction of PI-PLC-solubilized antigen.

Reactivities of naturally solubilized and PI-PLC-solubilized antigens with MAbs Since it has been though that the difference of reactivity between Kits I and II depends mainly on the reactivity of Group V MAb (F33-104), which is able to discriminate CEA from NFA-2, the reactivities of the antigens with MAbs of Group III (F3-30) and Group V (F33-104) were examined. As can be seen in Table I, MAb F3-30 revealed a reactivity with the naturally solubilized antigen comparable to that with the PI-PLC-solubilized antigen, but MAb F33-104 and Kit II showed a much weaker reaction with the former antigen than that with the latter. Similar differences were also observed between NFA-2 and CEAs. These results indicate that the naturally solubilized and PI-PLC-solubilized antigens are antigenically different from each other and similar to NFA-2 and CEA, respectively.

Reactivity of antigen in membrane-bound form with solid-phase RIA It is likely that the reactivity with solid-phase antibodies of antigens on such large particles as are precipitable by centrifugation at 14,000 rpm for 30 min may be much weaker than that of free soluble antigens. As shown in Table II, the reactivity of Fraction 2 increased greatly after the PI-PLC treatment. Provided that the membrane-bound antigen in both Fractions 2 and 2-p had the same antigenic reactivity, the reactivity of antigen equivalent to 200 ng (350 ng − 150 ng) in membrane-bound form increased to 520 ng equivalent in soluble form after PI-PLC treatment. Thus, the antigen in membrane-bound form showed a much weaker reactivity than that of the PI-PLC-solubilized antigen. As can be seen in Table I, however, the antigen in membrane-bound form in both Fractions 2 and 2-p showed a much stronger reactivity with Group V MAb or Kit II than that of the naturally solubilized antigen or NFA-2.

Table II. Reactivities of Antigens in Membrane-bound or in Soluble Form with Solid-phase RIA

Antigen preparations[a]	Reactivity[b] with Kit I (Equivalent antigen in ng)
Fraction 2	350
Fraction 2 treated with PI-PLC	650
Fraction 2-s	520
Fraction 2-p	150

a) See Fig. 1.
b) An aliquot of Fraction 2 with antigenic reactivity equivalent to 350 ng of antigen was treated with PI-PLC, then its reactivity changes were followed. See the text.

DISCUSSION

It is readily conceivable that many factors such as age, eating or drinking habits and physiological conditions of the digestive organs may influence the total amounts of

CEA-related antigens in the feces evacuated during one day. The amounts of 50–70 mg reported here are not small, and strongly support our recent finding that normal colon mucosae produce CEA quite actively, as cancerous tissues do.[3] Nevertheless, the contents of CEA in normal mucosae are reported to be always very low.[3, 12, 13] These findings strongly suggest that the product of normal mucosa may be rapidly released from the mucosa into the lumen of the digestive tract. The findings reported here, however, indicate that normal mucosa releases the antigen, at least the majority of the antigen, not in soluble form but in membrane-bound form, probably in association with extrusion of epithelial cells from the apical area of crypts of colon mucosa.[14] The biological activity of CEA as cell adhesion molecule, reported recently,[15, 16] might be related to the mechanism of the epithelial cell extrusion. To elucidate the biological significance of the active production of CEA by normal colon mucosa, it will be necessary to investigate the physiology of colon mucosa in relation to CEA gene expression, such as the processes of proliferation and differentiation of colonic epithelial cells from the stem cells, the mechanism of epithelial cell migration from the bottom to the apex of the crypt, and the mechanism of cell extrusion from the apical area.

As reported previously,[5] we have identified three molecular species of NFA in normal adult feces; NFA-1, NFCA and NFA-2 with molecular weights of approximately 20 kDa, 90 kDa and 170 kDa, respectively. The former two species seemed to be derived from the latter by proteolytic degradation. NFA-2 has physicochemical and antigenic properties very similar to those of CEA purified from cancerous tissues, but NFA-2 and CEA are antigenically distinguishable with specifically prepared anti-CEA antibodies[17, 18] or with MAbs of Group V,[10] so that the antigenicity recognized by Group V MAbs has been thought to be specific for CEA in cancerous tissues and has been designated as "CEA-distinctive." Since it was confirmed in this study that the antigen in membrane-bound form in normal feces is not extractable by perchloric acid treatment (data not shown), which has been used for purification of NFAs,[5] it seems certain that the three molecular species of NFA (NFA-1, NFCA and NFA-2) identified and characterized so far were derived from Fraction 3 containing the naturally solubilized antigen defined in this study. As shown in Fig. 2 and Table I, the naturally solubilized and PI-PLC-solubilized antigens revealed antigenic reactivities mimicking those of NFA-2 and CEA, respectively, indicating that "CEA-distinctive" antigenicity does not represent the difference between antigens from normal mucosa and from cancerous tissues but represents the antigenic difference between the naturally solubilized and PI-PLC-solubilized antigens. Thus, the concept of "CEA-distinctive" antigenicity must now be amended, though we have used this term throughout the present paper for the sake of consistency with our previous papers.

As reported recently,[7] CEA is anchored to the cell membrane via a GPI which consists of at least ethanolamine, glycan, glucosamine, inositol and phosphatidic acid. Since PI-PLC cleaves the ester linkage between the phosphate and the α-hydroxyl group of diacylglycerol, the antigen newly solubilized with PI-PLC should possess the remaining part of GPI, that is, a glycosylinositolphosphate (GIP). In contrast, as a result of some enzymatic reaction in the digestive tract, the naturally solubilized antigen (including NFA-2) may have lost the glycolipid domain or additionally some more carboxyl-terminal portion of the CEA peptide. Thus, "CEA-distinctive" antigenicity is probably due to the presence of a GIP moiety, although it is not clear at present whether this GIP moiety itself constitutes the epitope for "CEA-distinctive" antigenicity or whether this epitope arises from some indirect effect of the presence of the GIP moiety on the CEA antigenicity.

We established recently a monoclonal solid-phase RIA (Kit II) using Groups II, III and V MAbs, which is able to distinguish CEA from NFA-2, and found that Kit II showed superior cancer specificity, sensitivity and accuracy in clinical trials as compared with a polyclonal RIA (Kit I) which is not able to discriminate CEA from NFA-2.[19] This implies that, although "CEA-distinctive" antigenicity seems to be by no means cancer-specific as mentioned above, recognition of this antigenicity in patients' sera is critical for cancer diagnosis. In cancer patients, the CEA molecules possessing this GIP moiety may be released into the circulation from cancerous tissues by the action of some enzyme presently unknown but probably similar to PI-PLC. Actually, a variable but considerable amount of CEA which is reactive with Group V MAbs was found to be released into the medium from cultured cells of all the established CEA-producing tumor cell lines so far tested (unpublished results), and all the preparations of CEA purified from cancerous tissues are reactive with Group V MAbs, indicating the presence of a GIP moiety on these CEA molecules.[10] Furthermore, the reactivity of GIP-related epitope with Group V MAbs seems not to be hindered much by attachment of CEA to the cell membrane, because the reactivities of Fractions 2 and 2-p (membrane-bound form) with Group V MAb were relatively strong, although their reactivities themselves were weaker than those of antigens in PI-PLC-solubilized form (Tables I and II). To understand the cancer specificity of CEA as a tumor marker, we must elucidate the release mechanism of CEA from cancerous tissues into the circulation in relation to the detailed structure of "CEA-distinctive" or GIP-related antigenicity.

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