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Epitopes Predominantly Retained on the Carcinoembryonic Antigen Molecules in Plasma of Patients with Malignant Tumors but Not on Those in Plasma of Normal Individuals

Masahide Kuroki,[1] Masaaki Murakami,[1] Mamie Wakisaka,[1] Anna Krop-Watorek,[2] Shinzo Oikawa,[2] Hiroshi Nakazato,[2] Goro Kosaki[3] and Yuji Matsuoka[1,4]

[1]*First Department of Biochemistry, School of Medicine, Fukuoka University, 7-45-1 Nanakuma, Jonan-ku, Fukuoka 814-01,* [2]*Suntory Institute for Biomedical Research, 1-1-1 Wakayamadai, Shimamoto-cho, Mishima-gun, Osaka 618 and* [3]*Tokyo Metropolitan Komagome Hospital, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 170*

We have previously reported that a group of monoclonal antibodies (MAbs) to carcinoembryonic antigen (CEA), designated Group F MAbs, are able to discriminate CEA in tumor tissues from normal fecal antigen-2, a soluble form CEA-counterpart in normal adult feces, and that the protein epitopes recognized by them are present on the domain A3-B3 of the CEA molecule. In this study, we further investigated the molecular localization of the epitopes recognized by the Group F MAbs using three new recombinant CEA proteins with restricted domain structures expressed in Chinese hamster ovary cells, and found that the epitopes for the Group F MAbs are present on domain B3 close to the anchoring device of the CEA molecule. The epitopes for the Group F MAbs were retained on the CEA molecules in the plasma of patients with malignant tumors and on the CEA molecules spontaneously released into the culture media from established tumor cell lines. However, a large part of the CEA molecules in the plasma of normal individuals were found to lack the epitopes for the Group F MAbs. These results provide a basis for the improved cancer diagnosis by using our CEA assay system utilizing a Group F MAb, and indicate the potential clinical utility of the Group F MAbs.

Key words: Carcinoembryonic antigen — Epitope — Monoclonal antibody — CEA-distinctive antigenicity — Plasma CEA

Carcinoembryonic antigen (CEA[5]), a highly glycosylated protein with a molecular mass of 180 kDa, is one of the most useful human tumor markers.[1] There are several areas in which MAbs to CEA are being used in the management of patients with malignant tumors. These include blood assays to monitor tumor burden,[2,3] immunohistochemical analyses of tissue samples to detect tumor cells,[4,5] tumor imaging with radiolabeled MAbs,[6,7] and MAb-guided therapy.[8,9] We have reported a group of MAbs to CEA,[10] which had been previously defined as Group 5 on the basis of cross-reactivity with CEA-related antigens[11] and were able to distinguish CEA in tumor tissues from NFA-2, a soluble form CEA-counterpart in normal adult feces,[11,12] so that the antigenicity recognized by them had been called "CEA-distinctive."[10,11] One of the Group 5 MAbs, F33-104, has been classified into epitope group Gold 1 with a high degree of CEA specificity in the International Workshop on anti-CEA MAbs.[13] The Group 5 MAbs have recently been shown to recognize the protein epitopes on domain A3-B3 of the CEA molecule using the recombinant CEA proteins expressed in CHO cells and hence redesignated Group F.[14]

Several recent studies, however, demonstrated that normal colon mucosae produce CEA quite actively[15–17] and that more than 90% of the CEA-related antigen in normal adult feces is still in membrane-bound form which is solubilizable with PI-PLC, and about 10% or less of the antigen is in soluble form from which NFA-2 is derived.[12,18] It has also been clarified that, unlike NFA-2, the PI-PLC-solubilized antigen from normal adult feces reacted well with the Group F MAbs, indicating that the "CEA-distinctive" antigenicity is not due to the difference between antigens in normal and cancerous tissues but is probably due to the structural difference between the naturally soluble antigen and the membrane-bound, PI-PLC solubilized antigen.[18]

However, a new monoclonal RIA,[10] which has recently been established with three MAbs including a Group

[4] To whom correspondence should be addressed.

[5] Abbreviations: CEA, carcinoembryonic antigen; kDa, kilodalton; MAb, monoclonal antibody; NFA-2, normal fecal antigen-2; CHO, Chinese hamster ovary; PI-PLC, phosphatidylinositol-specific phospholipase C; RIA, radioimmunoassay; NCA, nonspecific cross-reacting antigen; PCR, polymerase chain reaction; FBS, fetal bovine serum; PBS, 0.15 *M* Dulbecco's phosphate-buffered saline free from Ca^{2+} and Mg^{2+}, pH 7.6; EIA, enzyme immunoassay; BBS, 0.01 *M* borate-buffered saline, pH 8.0; SDS, sodium dodecyl sulfate; GPI, glycosyl-phosphatidylinositol.

F MAb and is able to discriminate CEA from NFA-2, showed improved cancer specificity, sensitivity and accuracy in clinical trials as compared with a polyclonal RIA which is not able to distinguish CEA from NFA-2.[19] This implies that, although the "CEA-distinctive" antigenicity seems to be by no means cancer-specific as mentioned above, the recognition of this antigenicity in patients' plasma is critical for cancer diagnosis with CEA.[14, 18, 20] This, in turn, led us to the idea that there might be a structural difference essential for reactivity with the Group F MAbs between the CEA molecules in blood of cancer patients and those in blood of normal individuals.

In the present study, we mapped in more detail the epitopes for the Group F MAbs on the CEA domain structure using three new recombinant CEA proteins with restricted domain structures, investigated if the CEA molecules in plasma of patients with malignant tumors or of normal individuals retain the epitopes for the Group F MAbs, and tested if the CEA molecules spontaneously released into the culture media from CEA-producing tumor cell lines possess the epitopes for the Group F MAbs.

MATERIALS AND METHODS

Antibodies Polyclonal anti-CEA antisera were prepared in BALB/c mice or goats as described previously and specifically purified with CEA-Sepharose 4B. [12, 21] An affinity-purified goat anti-CEA antibody was conjugated to CNBr-activated Sepharose 4B (10 mg/g dry gel) and used as an immunoadsorbent.[21] Another affinity-purified goat anti-CEA antibody was further adsorbed with NCA-Sepharose 4B[21] and used as the tracer in sandwich-type solid-phase EIAs or RIAs. Four anti-CEA MAbs used in this study were prepared and purified as described elsewhere.[11, 22, 23] The epitopes recognized by the MAbs were mapped in terms of the domain structure of the CEA molecule as described elsewhere.[14] In brief, F4-82 (Group C) recognized an epitope present on domain N of the CEA molecule, F3-30 (Group E) reacted with domains A2-B2, and F33-104 and F36-96 (both Group F) recognized different epitopes on domains A3-B3, respectively. None of the four MAbs was reactive with NCA-50 or NCA-90.[11] F4-82 and F3-30 reacted strongly with both CEA and NFA-2, and F33-104 and F36-96 both reacted strongly with CEA but weakly with NFA-2.[10, 11] A purified myeloma protein MOPC 21 (IgG1) was used as a negative control (Organon Technika, West Chester, PA).

Reference antigens NFA-2 and CEA were highly purified from nomal adult feces and colonic cancerous tissues, respectively.[12, 24]

Recombinant CEA proteins The schematic structures of three new recombinant CEA proteins used in this study are shown in Fig. 1 in comparison with that of CEA-whole. pdKCR-dhfr-CEA-whole was described previously.[25]

pdKCR-dhfr-CEA-N-III-M was constructed as follows. pdKCR-dhfr-CEA-whole was digested with *Pst* I, then the resultant 6.1 kb and 2.3 kb fragments were recovered and re-ligated to yield pdKCR-dhfr-CEA-N-III-M.

pdKCR-dhfr-CEA-N-M was constructed as follows. pCEA14[26] was digested with *Eco*R I and *Eae* I, then 494 bp *Eco*R I-*Eae* I and 775 bp *Eae* I-*Eco*R I fragments were recovered and ligated into the *Eco*R I site of pUC13 using T4 DNA ligase. Then 1269 bp *Eco*R I fragment was isolated and cloned into the *Eco*R I site of pdKCR-dhfr to yield pdKCR-dhfr-CEA-N-M.

pdKCR-dhfr-CEA-N-B3 was constructed as follows. pdKCR-dhfr-CEA-N-III-M was digested with *Eco*R I, then the resultant 1.8 kb fragment was cloned into the *Eco*R I site of pUC13 to yield pUC13-CEA-N-III-M, which was used as a template for PCR. The fragment encoding the 5′-untranslated region, signal peptide and N domain of CEA was amplified by PCR using a DNA Thermal Cycler (Perkin-Elmer Cetus, Emeryville, CA). PCR was carried out in 100 μl using reagents in the GeneAmpTM DNA Amplification Reagent Kit (Perkin-Elmer Cetus) in a standard reaction mixture according to the instruction manual. The oligonucleotides 5′-GTT-TTCCCAGTCACGAC-3′ (M13 primer M4, Takara Shuzo Co., Ltd., Tokyo) [5′-primer] and 5′-CGGG-TATACCCGGAACTGGCCAGTTG-3′ [3′-primer] were used for the amplification of 5′-untranslated region, signal peptide, and N domain. The amplification was carried out by heating at 94°C for 1 min, followed by annealing at 55°C for 2 min and extension at 72°C for 3 min. This cycle was repeated for 30 cycles followed by a 10-min final extension at 72°C. The PCR product (520 bp) was purified on a 2% agarose gel, blunt-ended with T4 DNA polymerase and then digested with *Eco*R I. The amplification of B3 domain was carried out essentially under the same conditions using 5′-TATGGGCCGG-ACACCCCCATCATTTC-3′ as a 5′ primer and 5′-GATCTAGACTATGCAGAGACTGTGATGC-3′, which contain a stop codon and the *Xba* I site, as a 3′ primer. The PCR product (270 bp) was purified on a 2% agarose gel, blunt-ended with T4 DNA polymerase and digested with *Xba* I. The 520 bp and 270 bp fragments were ligated and introduced into the *Eco*RI and *Xba* I sites of pUC19 to yield pUC19-CEA-N-B3, then the *Hin*d III site was replaced by the *Eco*R I site as described previously[14] and the product was digested with *Eco*R I. The 790 bp *Eco*R I fragment was recovered and introduced into the *Eco*R I site of pdKCR-dhfr to yield

pdKCR-dhfr-CEA-N-B3. The DNA sequence of pUC19-CEA-N-B3 determined by the chain termination method[27] revealed that there was a deletion of 15 nucleotides (nucleotides 401–415; Phe^{104}-Pro^{108})[28] and one base change (C→T) (nucleotide 1917; Ala^{609}→Val^{609}).[28]

Transfection and isolation of $dhfr^+$ transformants were performed as described previously.[25] $CHOdhfr^+$ transformants were cultured in a selection medium, α-MEM lacking ribonucleosides and deoxyribonucleosides (Gibco, Grand Island. NY) supplemented with 10% dialyzed FBS, 100 units of penicillin/ml and 100 μg of streptomycin/ml.

Preparation of crude extracts of the recombinant CEA proteins Adherent cells transfected with pdKCR-dhfr-CEA-whole, -CEA-N-III-M, or -CEA-N-M were removed from about 90% confluent culture flasks with PBS containing 0.25% trypsin and 10 m*M* EDTA. About 3×10^7 cells were suspended in 5 ml of PBS containing protease inhibitors (chymostatin, pepstatin A, leupeptin and antipain, finally 5 μg each/ml, and 100 μM *p*-amidinophenylmethanesulfonyl fluoride) and incubated with PI-PLC (finally 0.2 unit/ml) from *Bacillus thuringiensis* (Funakoshi, Tokyo) for 2 h at 37°C. The cell suspensions were centrifuged at 22,800*g* for 30 min and the resultant supernatant was assayed by EIAs (see below). Adherent cells transfected with pdKCR-dhfr-CEA-N-B3, which did not contain domain M (see Fig. 1), were also removed from about 90% confluent culture flasks as described above. About 1×10^7 cells were suspended in 1 ml of 0.1% Nonidet P-40 in PBS, sonicated (Heat Systems-Ultrasonics, Plainview, NY) on ice for 10 s at scale 8, and centrifuged at 22,800*g* for 30 min. The resultant supernatant was assayed by EIAs (see below).

Protein determination The protein concentrations of the crude extracts were determined by the bicinchoninic acid protein assay method (Pierce, Rockford, IL).[29]

Purification of the antigens in malignant or normal plasma The plasma (M1 to M10) of 10 patients with various carcinomas was collected and stored at −25°C until use. The pooled plasma samples (N1 to N6) from normal blood donors, which were collected from 4 to 6 individuals, respectively, were obtained from Fukuoka Red Cross Blood Center, Chikushino, Japan. The antigens in each plasma reactive with goat anti-CEA antibody were separately adsorbed on and eluted from the corresponding immunoadsorbent as previously described.[21]

The antigens produced by tumor cell lines Four CEA-producing human tumor cell lines were maintained in RPMI-1640 medium supplemented with 10% FBS.[30] The cells and culture supernatant of each human CEA-producing tumor cell line in the stationary phase of growth were also collected. About 1.0×10^7 cells were treated with PI-PLC as mentioned above, and the resultant supernatant as well as the culture supernatant was used for estimation of CEA by RIAs (see below).

EIAs The reactivity of MAbs with the recombinant CEA proteins was estimated by sandwich-type solid-phase EIAs using microtiter plates coated with a given MAb and a biotinylated polyclonal goat anti-CEA antibody as the tracer.[31] Briefly, 100 μl of each MAb at a concentration of 10 μg/ml in BBS was dried overnight at 37°C in each well of 96-well polyvinyl chloride plates (Dynatech, Alexandria,VA), and nonspecific protein absorption was blocked as described previously.[14] Fifty μl of recombinant proteins diluted with 1% BSA in BBS was added to each well and the plates were incubated for 1 h at 37°C. After washing, the plates were successively incubated with 100 μl of the biotinylated goat anti-CEA antibody (1 μg/ml), with 100 μl of horseradish peroxidase-streptavidin (50 ng/ml), and then with 4% *o*-phenylenediamine and 0.006% H_2O_2.[14]

RIAs Quantitation of the antigens from plasma or cultured tumor cell lines was carried out by using a polyclonal sandwich-type solid-phase RIA.[19] A reference CEA was used as the standard antigen for calibration.

To estimate the reactivity of the antigens from plasma or cultured tumor cell lines with a given MAb, each MAb was immobilized on beads at a concentration of 10 μg/ml, and an affinity-purified goat anti-CEA antibody absorbed with NCA was used as the tracer.

Western blotting analyses Antigens were analyzed by SDS-polyacrylamide gel electrophoresis (4–20% linear gradient) and transferred to a hydrophobic Durapore filter as previously described.[32] After blocking, the blots were incubated with each MAb at a concentration of 1 μg/ml, and successively incubated at room temperature with biotinylated horse anti-mouse IgG (H+L), with horseradish peroxidase-streptavidin D, and then with 0.03% 3,3-diaminobenzidine and 0.01% H_2O_2.[14]

RESULTS

Reactivity of the MAbs with the recombinant proteins For detailed mapping of the epitopes for the Group F MAbs, we reconstructed three new cDNAs encoding the derivatives of CEA in a eukaryotic expression vector, pdKCR-dhfr. They were designated CEA-N-III-M, CEA-N-B3, and CEA-N-M (Fig. 1). Reactivities of the Group F MAbs with these recombinant proteins including CEA-whole were tested by solid-phase EIAs. The results of the titrations are shown in Fig. 2. A polyclonal BALB/c mouse anti-CEA antibody reacted with all four recombinant CEA proteins tested, whereas a control mouse myeloma protein, MOPC 21, did not react with any of the recombinant CEA proteins tested. A Group C MAb, F4-82, which recognized an epitope present on

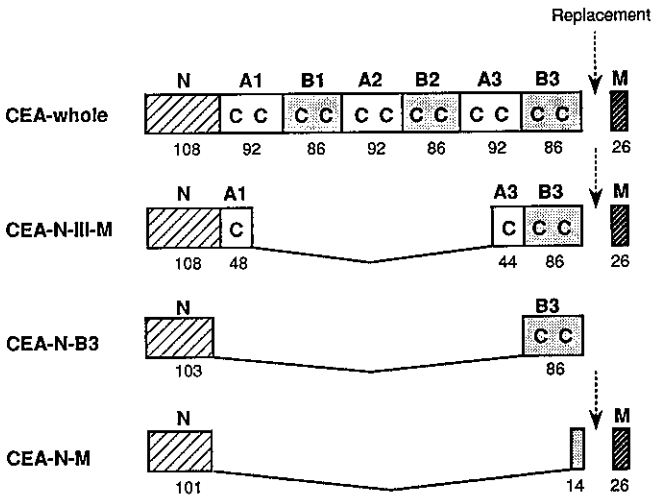

Fig. 1. The abbreviated names and schematic structures of four recombinant CEA proteins used in this study. Different domains are indicated by letters above blocks and different shading, whereby homologous regions have the same shading. N, N-terminal (IgV-like) domain; A1-A3 and B1-B3, IgC-like domains; M, hydrophobic M-domain replaced by GPI anchor. C, cysteine residue. Amino acid numbers reconstructed in this study are presented below the blocks.

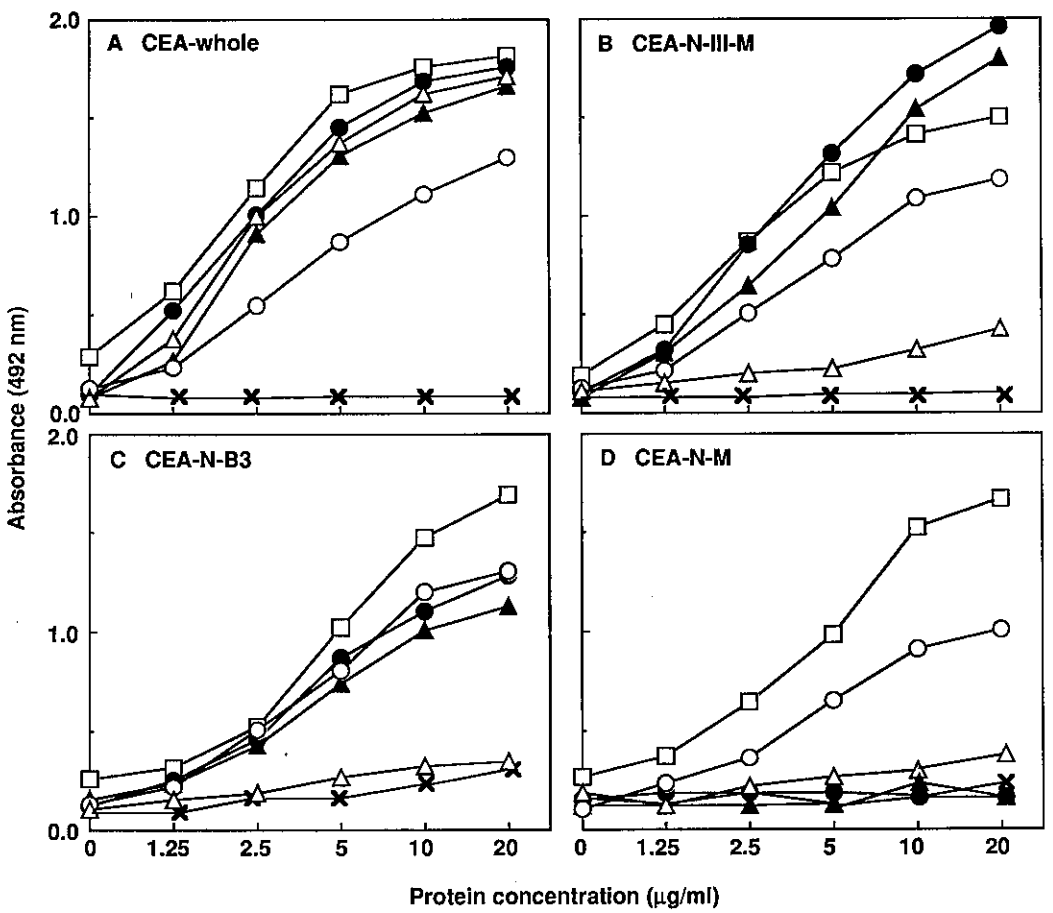

Fig. 2. Reactivity of MAbs of Groups C, E and F with four recombinant CEA proteins in sandwich-type solid-phase EIAs. The recombinant proteins captured on the wells coated with a given MAb or polyclonal antibody were detected by a biotinylated polyclonal goat anti-CEA antibody as described in "Materials and Methods." Antibodies immobilized on wells: F4-82 (Group C) (○); F3-30 (Group E) (△); F33-104 (Group F) (●); F36-96 (Group F) (▲); an affinity-purified BALB/c mouse anti-CEA antibody (□); and MOPC 21 (×). The protein concentrations of the crude extracts of the recombinant proteins were determined by the bicinchoninic acid protein assay method.

Table I. Reactivity of the Antigens Purified from Normal or Malignant Plasma with MAbs of Groups C, E and F

Plasma No.	Diagnosis	Original volume (ml)	Original CEA value (ng/ml)[a]	Final volume (ml)[b]	Final CEA value (ng/ml)[a]	Reactivity (cpm)[c] of 100 ng antigen with		
						Group C MAb (F4-82)[d]	Group E MAb (F3-30)[d]	Group F MAb (F33-104)[d]
M1	colon cancer	3.5	7,800	0.4	39,000	28,100	24,800	38,400
M2	colon cancer	5.0	12,800	0.4	100,000	27,800	25,600	45,600
M3	colon cancer	4.2	10,000	0.5	55,500	29,000	23,900	42,900
M4	colon cancer	7.0	13,200	0.5	122,000	28,900	24,600	38,500
M5	gastric cancer	7.0	9,000	0.5	84,800	26,900	24,900	45,000
M6	gastric cancer	7.2	19,400	0.5	218,000	27,400	24,200	41,500
M7	lung cancer	4.1	8,500	0.5	43,600	26,800	23,500	45,800
M8	lung cancer	6.0	3,200	0.5	25,500	28,500	25,900	40,700
M9	pancreatic cancer	2.3	288	0.4	12,500	27,300	22,700	39,900
M10	breast cancer	6.2	104	0.4	11,800	28,600	23,400	43,800
N1	Normal	1,600	2.3	0.8	2,700	27,700	25,100	2,100
N2	Normal	700	2.1	1.6	600	29,100	22,600	2,200
N3	Normal	1,000	1.9	1.2	1,080	27,900	24,100	1,900
N4	Normal	1,800	2.3	1.0	2,520	26,300	25,700	2,300
N5	Normal	500	2.1	1.7	366	28,300	23,800	2,400
N6	Normal	2,600	2.2	0.5	7,400	26,800	24,900	2,900
Reference CEA[e]		—	—	—	—	28,500	28,000	48,500
Soluble form NFA-2[e]		—	—	—	—	26,300	24,700	2,100

a) CEA values determined by a sandwich-type solid-phase RIA using polyclonal goat anti-CEA antibodies, which do not cross-react with NCAs.
b) Volumes of the antigen preparations purified and concentrated from plasma with goat anti-CEA-antibody immunoadsorbent as described in "Materials and Methods."
c) Reactivity was normalized for 100 ng of antigens estimated by the sandwich-type solid-phase RIA using polyclonal goat anti-CEA antibodies as described in *a*).
d) Each MAb was used as the solid-phase antibody and one and the same polyclonal goat anti-CEA antibody absorbed with NCA was used as the tracer.
e) Purified Preparations were tested in this experiment.

domain N of the CEA molecule, reacted with all four recombinant CEA proteins, while a Group E MAb, F3-30 (Group E), which recognized an epitope on domains A2-B2, reacted only with CEA-whole. Two Group F MAbs, F33-104 and F36-96, were reactive with CEA-whole, CEA-N-III-M, and CEA-N-B3 but not with CEA-N-M, although the reactivity with CEA-N-B3 was slightly lower than those with the two former. These results indicate that the epitopes recognized by the Group F MAbs are present on domain B3.

Reactivity of the MAbs with the antigens from malignant or normal plasma To examine the reactivity of the Group F MAbs with the CEA molecules in the circulation, the antigens reactive with a polyclonal goat anti-CEA antibody were purified from the plasma of 10 cancer patients with elevated CEA levels and from 6 pooled plasma samples of normal individuals. A summary of plasma used and the reactivity of the purified antigens with MAbs of Groups C, E and F obtained by solid-phase RIAs is shown in Table I. The antigens from all 10 malignant plasma samples reacted with a Group F MAb F33-104 as well as a Group C MAb F4-82 or a Group E MAb F3-30. On the other hand, the reactivity of the antigens from all 6 normal plasma samples with F33-104 is about one-tenth of those with F4-82 or F3-30. As previously described, a reference CEA purified from tumor tissues reacted well with a Group F MAb F33-104, but the soluble form NFA-2 showed a much weaker reaction with this MAb although these 2 antigens both showed strong reactivity with F4-82 or F3-30 (Table I). These results indicate that the antigens in all malignant plasma samples tested have the epitopes for the Group F MAbs, while, like the soluble form NFA-2, a large part of the antigens in normal plasma do not possess the epitopes for the Group F MAbs.

Western blotting analysis of the antigens from malignant or normal sera The reactivity of the MAbs with the antigens in plasma was also tested by Western blotting analysis. Fig. 3 shows the Western blotting profile of the antigens purified from plasma of 4 patients with colon

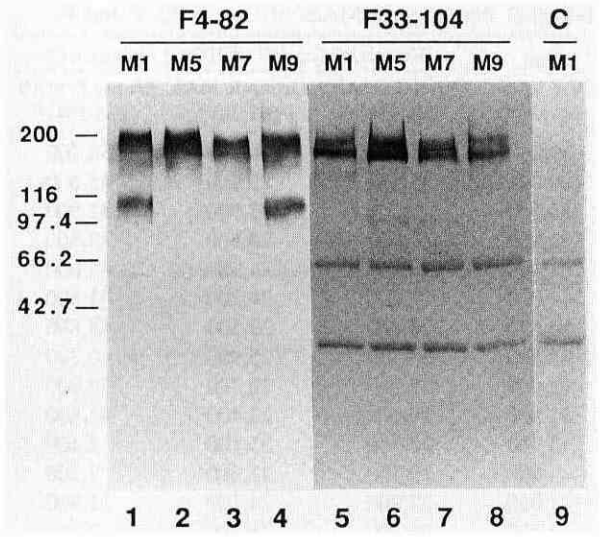

Fig. 3. Western blotting analysis (4–20% polyacrylamide gel) of CEA from the plasma of patients with malignant tumors. About 200 ng of purified antigen was added to each lane. After electrophoresis, the proteins were transferred to a hydrophobic Durapore filter, and immunostained with a Group C MAb (F4-82) or a Group F MAb (F33-104) or a control MAb (C: MOPC 21). For descriptions of M1, M5, M7, and M9, see Table I. Vertical scales, mol. wt. markers ($\times 10^{-3}$).

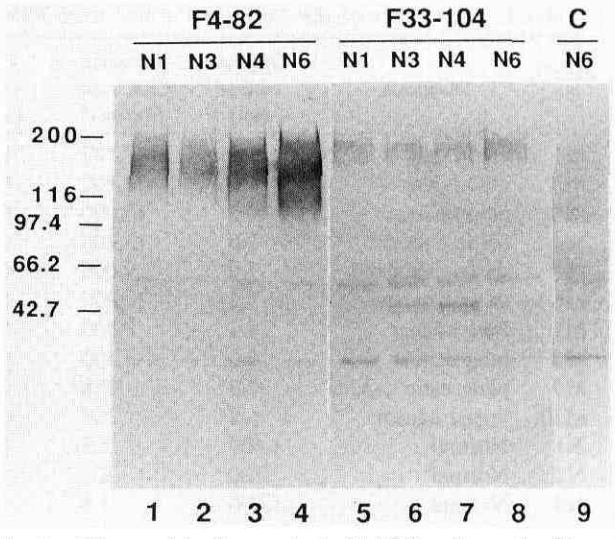

Fig. 4. Western blotting analysis (4–20% polyacrylamide gel) of CEA from the plasma of normal individuals. The antigen preparations (N1, N3, N4, and N6, see Table I) purified from normal plasma were further concentrated about 7 to 10 times for Western blotting. About 200 ng of purified antigen was added to each lane. After electrophoresis, the proteins were transferred to a hydrophobic Durapore filter, and immunostained with a Group C MAb (F4-82) or a Group F MAb (F33-104) or a control MAb (C: MOPC 21). Vertical scales, mol. wt. markers ($\times 10^{-3}$).

Table II. Reactivity of Spontaneously Secreted CEA and PI-PLC-solubilized CEA from Tumor Cell Lines with MAbs of Groups C, E and F

Tumor cell line[a]	Sample	CEA value (ng/ml)[b]	Reactivity (cpm)[c] of 100 ng antigen with		
			Group C MAb[d] (F4-82)	Group E MAb[d] (F3-30)	Group F MAb[d] (F33-104)
M7609	s-CEA[e]	1,800	27,300	24,400	40,800
	p-CEA[e]	2,600	26,900	25,000	42,000
KNS-62	s-CEA	54	27,100	27,200	51,000
	p-CEA	105	28,000	27,500	57,800
MKN-45	s-CEA	2,300	25,900	28,000	40,500
	p-CEA	7,800	27,800	32,200	53,000
QGP-1	s-CEA	7,800	27,200	24,600	39,000
	p-CEA	9,400	28,400	28,200	37,800

a) M7609, a colon carcinoma line; MKN-45, a gastric carcinoma line; KNS-62, a lung carcinoma line; and QGP-1, a pancreatic carcinoma line.
b) CEA values determined by a sandwich-type solid-phase RIA using polyclonal goat anti-CEA antibodies, which do not cross-react with NCAs.
c) Reactivity was normalized for 100 ng of antigens estimated by the sandwich-type solid-phase RIA using polyclonal goat anti-CEA antibodies as described in *b*).
d) Each MAb was used as the solid-phase antibody and one and the same polyclonal goat anti-CEA antibody absorbed with NCA was used as the tracer.
e) s-CEA and p-CEA mean soluble CEA spontaneously secreted into culture medium and CEA solubilized by PI-PLC treatment, respectively.

cancer, gastric cancer, lung cancer, and pancreatic cancer, which were visualized by immunostaining with a Group C MAb F4-82 or a Group F MAb F33-104. The antigens with high molecular masses of about 180 to 200 kDa reacted with both MAbs. When stained with F33-104, two bands were more clearly visualized in the high-molecular-weight region. An antigen with a molecular mass of about 100 kDa was detected with F4-82 in two samples M1 and M9, but not with F33-104. These results indicate that the antigens with high molecular masses of about 180 to 200 kDa in malignant plasma have the epitopes for the Group F MAbs.

On the other hand, as can be seen in Fig. 4, the antigens from normal plasma showed several broad bands with molecular masses ranging from 100 to 200 kDa stained with F4-82. Similar staining profiles were also obtained with F3-30 (data not shown). The antigens with high molecular masses of 180 to 200 kDa, which seem to account for only a small part of the antigens in the normal plasma, were stained with F33-104. This result indicates that a large part of the antigens in normal plasma with molecular masses of 100 to 180 kDa do not possess the epitopes for the Group F MAbs.

Reactivity of the MAbs with CEA released from cultured tumor cells Then, we tested whether CEA released spontaneously from tumor cells has reactivity with the Group F MAbs, in comparison with CEA solubilized from tumor cells with PI-PLC by using several cultured human tumor cell lines. As reported previously,[30] a variable but considerable amount of CEA was released into culture medium from cultured cells of all the CEA-producing tumor cell lines so far tested. The reactivity of the antigens with MAbs of Groups C, E and F obtained by solid-phase RIAs is shown in Table II. The soluble CEA (s-CEA) spontaneously released from tumor cells, as well as the PI-PLC-solubilized CEA (p-CEA), showed strong reactivity with all three MAbs, a Group C MAb F4-82, a Group E MAb F3-30 and a Group F MAb F33-104. Similar results were obtained with another Group F MAb F36-96 (data not shown). These results indicate the presence of the epitopes for the Group F MAbs on these antigen molecules.

DISCUSSION

Since the membrane-bound, PI-PLC-solubilized antigen in normal adult feces reacted with the Group F MAbs,[18] the "CEA-distinctive" antigenicity recognized by them seems to be by no means cancer-specific. The recognition of this antigenicity in patients' plasma is, however, critical for cancer diagnosis with CEA because a new monoclonal RIA using a Group F MAb[10] showed improved cancer specificity, sensitivity and accuracy in clinical trials as compared with a polyclonal RIA.[19] Thus, to clarify the epitopes recognized by the Group F MAbs is of obvious importance for the improvement of the specificity of CEA detection and measurement, which are now widely applied in various clinical fields.

It has recently been shown that the Group F MAbs recognize the protein epitopes on domains A3-B3 of the CEA molecule by using recombinant CEA proteins expressed in CHO cells.[14] In the present study, the reactivity with three new recombinant CEA proteins with restricted domain structures revealed that the epitopes for the Group F MAbs are present on domain B3 close to the anchoring device of the CEA molecule. But the Group F MAbs did not react with CEA-N-M, which contains a 14-amino acid sequence adjacent to the anchoring device (Fig. 1 and 2). This result indicates that the C-terminal 14-amino acid sequence of domain B3 does not constitute the epitopes for the Group F MAbs, although it is questionable whether or not the 14 amino acids were adequately able to take up the conformation corresponding to that part of the native CEA molecule.

We then wondered whether CEA released into the circulation from cancer tissues has the epitopes for the Group F MAbs. When the immunoreactivity was tested by solid-phase RIAs, the antigens in the plasma of all 10 patients with various cancers were found to react well with a Group F MAb, F33-104. The Western blotting profile of the antigens from the plasma of 4 patients with colon cancer, gastric cancer, lung cancer, and pancreatic cancer showed that the antigens with high molecular masses of about 180 to 200 kDa, which seem to account for a large part of the antigens in the malignant plasma, have the epitopes for the Group F MAbs. The antigen with a molecular mass of about 100 kDa, which was detected only with F4-82 in two samples M1 and M9, seems to be a tumor-associated antigen, TEX, which possesses antigenic determinants found in CEA but not in NCA and has often been found in plasma of patients with carcinomas.[33, 34] TEX, however, does not always increase in the sera of patients with malignant tumors.[34] This may be the reason why two samples M5 and M7 lack the 100 kDa band. Although the number of cases tested is too small to draw a conclusion, this result implies that, in cancer patients, the CEA molecules possessing the epitopes for the Group F MAbs may be steadily released into the circulation from tumor tissues by the action of some enzyme presently unknown, but probably a kind of phospholipase, or by cell breakdown. This possibility was supported by the fact that the CEA spontaneously released from cultured tumor cell lines into the culture medium possessed the epitopes for the Group F MAbs, as the PI-PLC solubilized CEA did, although the mechanism by which CEA is released from tumor cells remains obscure. The fact that two bands

were visualized with F33-104 in the high-molecular-weight region of the Western blotting profile of the antigens from malignant plasma samples may suggest the presence of two different releasing mechanisms of CEA *in vivo*. Another possible explanation is that the smaller band may be a degraded product of the larger one, formed after its release.

On the other hand, when the immunoreactivity was tested by solid-phase RIAs, the antigens in the plasma of normal individuals showed a very weak reactivity with a Group F MAb F33-104. The Western blotting profile of the antigens from the plasma of normal individuals showed that only the antigens with high molecular masses of 180 to 200 kDa, which seem to account for only a small part of the antigens in the normal plasma, were weakly stained with F33-104, indicating that a large part of the antigens in normal plasma with molecular masses of 100 to 180 kDa do not retain the epitopes for the Group F MAbs. Recently, Neumaier *et al.* isolated antigens related to CEA from normal human plasma by perchloric acid extraction, gel filtration, and immunoaffinity chromatography and delineated four CEA-related antigens in normal plasma with molecular masses of 85 to >200 kDa.[35] They have also found that only the antigens with high molecular masses of >200 and 177 kDa showed reactivity with their two MAbs with a high degree of CEA specificity. One of them has since been classified into the same epitope group Gold 1 in the International Workshop on anti-CEA MAbs as has been our MAb F33-104.[13] Thus, it seems likely that a large proportion of the CEA molecules in plasma of normal individuals may have lost its GPI anchor moiety and probably an additional C-terminal peptide of some length, which may constitute the epitopes for the Group F MAbs. It is not known at present, however, whether the epitopes are cleaved while CEA is being released from normal CEA-producing epithelial cells or in the circulation after it has been released, or both.

In conclusion, the results obtained in this study indicate that the Group F MAbs are of potential clinical utility and provide a basis for the improved cancer diagnosis by using our CEA assay system utilizing a Group F MAb.[10] For further understanding of the tumor specificity problem of CEA as a tumor marker, it would be of interest to elucidate the releasing mechanism of CEA from cancerous tissues and/or normal epithelial tissues into the circulation in relation to the epitopes for the Group F MAbs. Such a study is under way by establishing organ cultures which represent malignant or normal epithelial conditions as far as possible.

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REFERENCES

1) National Institutes of Health Consensus Development Conference Statement. Carcinoembryonic antigen: its role as a marker in the management of cancer. *Cancer Res.*, **41**, 2017–2018 (1981).
2) Hedin, A., Carlsson, L., Berglung, A. and Hammarstrom, S. A monoclonal antibody-enzyme immunoassay for serum carcinoembryonic antigen with increased specificity for carcinomas. *Proc. Natl. Acad. Sci. USA*, **80**, 3470–3474 (1983).
3) Liu, Y.-S. V., Tobias, R. J. and Zurawski, V. R., Jr. A more specific, simpler radioimmunoassay for carcinoembryonic antigen, with use of monoclonal antibodies. *Clin. Chem.*, **31**, 191–195 (1985).
4) Yachi, A., Imai, K., Fujita, H., Moriya, Y., Tanda, M., Endo, T., Tsujisaki, M. and Kawaharada, M. Immunohistochemical distribution of the antigenic determinants detected by monoclonal antibodies to carcinoembryonic antigen. *J. Immunol.*, **132**, 2998–3004 (1984).
5) Sheahan, K., O'Brien, M. J., Burke, B., Dervan, P. A., O'Keane, J. C., Gottli, eb, L. S. and Zamcheck, N. Differential reactivities of carcinoembryonic antigen (CEA) and CEA-related monoclonal and polyclonal antibodies in common epithelial malignancies. *Am. J. Clin. Pathol.*, **94**, 157–164 (1990).
6) Sharkey, R. M., Goldenberg, D. M., Goldenberg, H., Lee, R. E., Ballance, C., Pawlyk, D., Varga, D. and Hansen, H. J. Murine monoclonal antibodies against carcinoembryonic antigen: immunological, pharmacokinetic, and targeting properties in humans. *Cancer Res.*, **50**, 2823–2831 (1990).
7) Lind, P., Lechner, P., Arian-Schad, K., Klimpfinger, M., Cesnik, H., Kammerhuber, F. and Eber, O. Anti-carcinoembryonic antigen immunoscintigraphy (technetium-99m-monoclonal antibody BW 431/26) and serum CEA levels in patients with suspected primary and recurrent colorectal carcinoma. *J. Nucl. Med.*, **32**, 1319–1325 (1991).
8) Sharkey, R. M., Motta-Hennessy, C., Pawlyk, D., Siegel, J. A. and Goldenberg, D. M. Biodistribution and radiation dose estimates for yttrium- and iodine-labeled monoclonal antibody IgG and fragments in nude mice bearing human colonic tumor xenografts. *Cancer Res.*, **50**, 2330–

2336 (1990).
9) Ledermann, J. A., Begent, R. H. J., Massof, C., Kelly, A. M. B., Adam, T. and Bagshawe, K. D. A phase-I study of repeated therapy with radiolabelled antibody to carcinoembryonic antigen using intermittent or continuous administration of cyclosporin A to suppress the immune response. *Int. J. Cancer,* **47,** 659–664 (1991).
10) Matsuoka, Y., Kuroki, Ma., Okamoto, N., Ikeda, S., Hara, Y., Minamizawa, T., Tachibana, S., Ogawa, H. and Tabata, N. Evaluation of a new monoclonal radioimmunoassay system for carcinoembryonic antigen. *Jpn. J. Clin. Oncol.,* **18,** 97–103 (1988).
11) Kuroki, Ma., Arakawa, F., Higuchi, H., Matsunaga, A., Okamoto, N., Takakura, K. and Matsuoka, Y. Epitope mapping of the carcinoembryonic antigen by monoclonal antibodies and establishment of a new improved radioimmunoassay system. *Jpn. J. Cancer Res.,* **78,** 386–396 (1987).
12) Kuroki, Ma., Koga, Y. and Matsuoka, Y. Purification and characterization of carcinoembryonic antigen-related antigens in normal adult feces. *Cancer Res.,* **41,** 713–720 (1981).
13) Hammarstrom, S., Shively, J., Paxton, R. J., Beatty, B. G., Larsson, A., Ghosh, R., Borner, O., Buchegger, F., Mach, J.-P., Burtin, P., Seguin, P., Darbouret, B., Degorce, F., Sertour, J., Jolu, J. P., Fuks, A., Kalthoff, H., Schmiegel, W., Arnat, R., Kloppel, G., von Kleist, S., Grunert, F., Schwarz, K., Matsuoka, Y., Kuroki, Ma., Wagener, C., Weber, T., Yachi, A., Imai, K., Nishikawa, N. and Tsujisaki, M. Antigenic sites in carcinoembryonic antigen. *Cancer Res.,* **49,** 4852–4858 (1989).
14) Ikeda, S., Kuroki, Ma., Haruno, M., Oikawa, S., Nakazato, H., Kosaki, G. and Matsuoka, Y. Epitope mapping of the carcinoembryonic antigen with various related recombinant proteins expressed in Chinese hamster ovary cells and 25 distinct monoclonal antibodies. *Mol. Immunol.,* **29,** 229–240 (1992).
15) Kuroki, Mo., Arakawa, F., Yamamoto, H., Shimura, H., Ikehara, Y. and Matsuoka, Y. Active production and membrane anchoring of carcinoembryonic antigen observed in normal colon mucosa. *Cancer Lett.,* **43,** 151–157 (1988).
16) Counoyer, D., Beauchemin, N., Boucher, D., Benchimol, S., Fuks, A. and Stanners, C. P. Transcription of genes of the carcinoembryonic antigen family in malignant and nonmalignant human tissues. *Cancer Res.,* **48,** 3153–3157 (1988).
17) Sato, C., Miyaki, M., Oikawa, S., Nakazato, H. and Kosaki, G. Differential expression of carcinoembryonic antigen and nonspecific crossreacting antigen genes in human colon adenocarcinomas and normal colon mucosa. *Jpn. J. Cancer Res.,* **79,** 433–437 (1988).
18) Matsuoka, Y., Matsuo, Y., Sugano, K., Ohkura, H., Kuroki, Mo. and Kuroki, Ma. Characterization of carcinoembryonic antigen-related antigens in normal adult feces. *Jpn. J. Cancer Res.,* **81,** 514–519 (1990).
19) Matsuoka, Y., Kuroki, Ma., Koga, Y., Ogawa, H., Nakazawa, N., Tachibana, S. and Minamizawa, T. A new direct solid-phase radioimmunoassay for carcinoembryonic antigen without pretreatment of serum samples. *J. Immunol. Methods,* **58,** 31–47 (1983).
20) Kuroki, Ma., Haruno, M., Arakawa, F., Wakisaka, M. and Matsuoka, Y. Reaction profiles of seven enzyme immunoassay kits for carcinoembryonic antigen (CEA) analyzed with purified preparations of CEA and related normal antigens. *Clin. Biochem.,* **25,** 29–35 (1992).
21) Kuroki, Ma., Ichiki, S., Kuroki, Mo. and Matsuoka, Y. Coproduction of carcinoembryonic antigen and nonspecific cross-reacting antigen by a continuous cell line from a human pancreatic tumor. *J. Natl. Cancer Inst.,* **69,** 401–408 (1982).
22) Kuroki, Ma., Kuroki, Mo., Koga, Y. and Matsuoka, Y. Monoclonal antibodies to carcinoembryonic antigen: a systematic analysis of antibody specificities by using related normal antigens and evidence for allotypic determinants on carcinoembryonic antigen. *J. Immunol.,* **133,** 2090–2097 (1984).
23) Kuroki, Ma., Greiner, J. W., Simpson, J. F., Primus, F. J., Guadagni, F. and Schlom, J. Serologic mapping and biochemical characterization of the carcinoembryonic antigen epitopes using fourteen distinct monoclonal antibodies. *Int. J. Cancer,* **44,** 208–218 (1989).
24) Matsuoka, Y., Koga, Y., Maruta, H., Yoshino, M. and Tsuru, E. Proteolytic release of antigenic fragments corresponding to normal fecal antigen and non-specific cross-reacting antigen from carcinoembryonic antigen. *Int. J. Cancer,* **21,** 604–610 (1978).
25) Oikawa, S., Inuzuka, C., Kuroki, Mo., Matsuoka, Y., Kosaki, G. and Nakazato, S. Cell adhesion activity of non-specific cross-reacting antigen (NCA) and carcinoembryonic antigen (CEA) expressed on CHO cell surface: homophilic and heterophilic adhesion. *Biochem. Biophys. Res. Commun.,* **164,** 39–45 (1989).
26) Oikawa, S., Inuzuka, C., Kuroki, Mo., Arakawa, F., Matsuoka, Y., Kosaki, G. and Nakazato, H. A specific heterotypic cell adhesion activity between members of carcinoembryonic antigen family, W272 and NCA, is mediated by N-domains. *J. Biol. Chem.,* **266,** 7995–8001 (1991).
27) Sanger, F., Nicklen, S. and Coulson, A. R. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA,* **74,** 5463–5467 (1977).
28) Oikawa, S. Nakazato, H. and Kosaki, G. Primary structure of human carcinoembryonic antigen (CEA) deduced from cDNA sequence. *Biochem. Biophys. Res. Commun.,* **142,** 511–518 (1987).
29) Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. Measurement of protein using bicinchoninic acid. *Anal. Biochem.,* **150,** 76–85 (1985).
30) Ichiki, S., Kuroki, Ma., Kuroki, Mo., Koga, Y. and

Matsuoka, Y. Production kinetics and immunochemical properties of carcinoembryonic antigen and nonspecific cross-reacting antigen synthesized by various human tumor cell lines. *Jpn. J. Cancer Res.*, **77**, 462–472 (1986).

31) Bayer, E. A. and Wilchek, M. Protein biotinylation. *Methods Enzymol.*, **184**, 138–160 (1991).

32) Kuroki, Ma., Fernsten, P. D., Wunderlich, D., Colcher, D., Simpson, J. F., Poole, D. J. and Schlom, J. Serological mapping of the TAG-72 tumor-associated antigen using 19 distinct monoclonal antibodies. *Cancer Res.*, **50**, 4872–4879 (1990).

33) Kessler, M. J., Shively, J. E., Pritchard, D. G. and Todd, C. W. Isolation, immunological characterization, and structural studies of a tumor antigen related to carcinoembryonic antigen. *Cancer Res.*, **38**, 1041–1048 (1978).

34) Shively, J. E., Spayth, V., Chang, F.-F., Metter, G. E., Klein, L., Presant, C. A. and Todd, C. W. Serum levels of carcinoembryonic antigen and a tumor-extracted carcinoembryonic antigen-related antigen in cancer patients. *Cancer Res.*, **42**, 2506–2513 (1982).

35) Neumaier, M., Fenger, U. and Wagener, C. Delineation of four carcinoembryonic antigen (CEA) related antigens in normal plasma by transblot studies using monoclonal anti-CEA antibodies with different epitope specificities. *Mol. Immunol.*, **22**, 1273–1277 (1985).