Establishment and Evaluation of a New Chemiluminescent Enzyme Immunoassay for Carcinoembryonic Antigen Adapted to the Fully Automated ACCESS® System

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Summary: We have established a new chemiluminescent enzyme immunoassay for carcinoembryonic antigen (CEA), designated ACCESS CEA, which is adapted to the fully automated ACCESS® immunoassay analyzer. The assay is based on a one step sandwich-type method using two monoclonal antibodies, one of which is immobilized on micrometer-size paramagnetic particles and the other is conjugated to alkaline phosphatase. Ten microliters of calibrators or sera are incubated for 5 minutes at 37 °C with the particles and with the alkaline phosphatase conjugate. The particles are then magnetically separated and washed to remove unbound components. Time needed to obtain the first result is less than 15 minutes.

The assay range was 0.04−1000 µg/l of CEA, and the possible high-dose hook effect was prevented at CEA concentrations up to 100 000 µg/l in this working range. The coefficient of variation (CV) for intra-assay precision was 3.0 to 4.7%, and inter-assay CV was 3.4 to 5.6%. The sample carryover was less than 0.001%. The analytical recovery ranged from 98 to 104% and a dilution linearity was demonstrated. No interference was detected in any sample with levels up to 300 mg/l for bilirubin, 12 000 mg/l for haemoglobin, 50 000 mg/l for human serum albumin, 8 500 mg/l for triacylglycerol, and 500 000 IU/l for rheumatoid factor. The ACCESS CEA assay also showed very homogeneous reactivity with purified CEA preparations from different tumours and could discriminate CEA from four CEA-related normal antigens tested.

Serum samples (n = 362) from patients with malignant or non-malignant disease, as well as from healthy individuals, were analyzed by the ACCESS CEA assay and by the established IMx CEA assay. The CEA values determined by the ACCESS CEA assay were in good agreement with those determined by the IMx CEA assay, and the ACCESS CEA assay significantly increased the sensitivity and specificity of tumour diagnosis as compared with the IMx CEA assay.

Introduction

Carcinoembryonic antigen (CEA) is a heavily glycosylated protein of the immunoglobulin gene superfamily with a relative molecular mass of 180 000 (1). The CEA peptide is composed of an N-terminal domain (N) and six very homologous immunoglobulin constant region-like domains (A1, B1, A2, A3 and B3) (1). The measurement of CEA levels in blood is considered a useful laboratory aid for the management of patients with various cancers (2).

Although various immunoassay systems for CEA are currently available, the CEA values obtained are highly variable depending on the assay kit used (3−5). This is thought to be due to the diverse structures of the carbohydrate chains of the CEA molecule (6, 7), which lead to variation in specificity of anti-CEA antibody preparations used in the assay kits (8). On the other hand, about

20 CEA-related antigens, such as non-specific cross-reacting antigens and normal faecal antigens, have been detected in many tissues unrelated to malignant diseases (1). Thus, it seems important to improve the accuracy of the CEA assays by selecting monoclonal antibodies which are independent of the influence of variations in the carbohydrate chains and are not cross-reactive with the CEA-related antigens (5). In previous studies, *Kuroki* et al. have generated 146 anti-CEA monoclonal antibodies and clarified in detail their reactivities with CEA and main CEA-related antigens (9, 10). Furthermore, these monoclonal antibodies have been classified into 7 groups (groups A−G) in light of the domain structure of the CEA molecule (11, 12). Among them, group F monoclonal antibodies have been found to discriminate CEA in tumour tissues from the CEA-related antigens (13). The immunoassay systems for CEA utilizing at least one

group F monoclonal antibody have shown improved cancer diagnosis (14, 15).

Recent attempts, however, have concentrated on the development of automated immunoassay analyzers with sensitive and precise assay reagents, which also reduce turn-around time, stuff usage and volume of disposables and assay costs, and several automated non-isotopic immunoassay systems have been introduced for estimation of various analytes including CEA (16). Of these, the ACCESS® immunoassay analyzer (Sanofi Diagnostics Pasteur, Inc., Chaska, MN, USA) is a fully automated laboratory assay system for enzyme immunoassays (EIAs) with the term *random* access relating to the concept of flexible assay processing and with *continuous* access i. e., the ability to interrupt an analyzer run, add stat or additional routine specimens, and continue the run with no loss of results (16, 17).

In the present study, by using two defined monoclonal antibodies, including a .Group F monoclonal antibody, we tried to establish a new immunoassay for CEA, designated ACCESS CEA, which is adapted to the fully automated ACCESS® immunoassay analyzer. We further evaluated the ACCESS CEA assay in the CEA determinations of patients' serum samples, and compared it with the established IMx CEA assay.

Materials and Methods

Carcinoembryonic antigen and related normal antigens

Four different CEA preparations, designated CEA-DB, CEA-MY, CEA-TT, and CEA-TY, were purified from liver metastases from colorectal carcinomas by treatment with phosphatidylinositol-specific phospholipase C followed by perchloric acid extraction and gel filtration as previously described (18). Non-specific cross-reacting antigen and non-specific cross-reacting antigen-2 were purified from pooled normal human lung (19) and meconium (20), respectively. Normal faecal antigen-1 and normal faecal antigen-2 were obtained from normal adult faeces (21). The non-specific cross-reacting antigen and normal faecal antigen-1 are partially cross-reactive with CEA but antigenically unrelated to each other (22). The purity of each antigen preparation was ensured by the demonstration of a single component on immunoelectrophoresis and by sodium dodecyl sulphate polyacrylamide gel electrophoresis (20, 21), and was also defined by a single NH_2-terminal amino acid (22, 23). The concentrations in weight of all antigens were estimated from the absorption coefficient of each antigen (5).

Monoclonal antibodies

Two mouse anti-CEA monoclonal antibodies (F82-61 and F11-39) were prepared as described by *Kuroki* et al. (10, 12). Both monoclonal antibodies were of IgG1 (12). The monoclonal antibodies were purified from ascitic fluids by ammonium sulphate precipitation and protein A chromatography. Purified monoclonal antibody F82-61 was immobilized on micrometre-size paramagnetic particles (Rhone-Poulenc Chimie, Lyon Cedex, France) as capture antibody as described previously (24). The antibody fragment Fab' of monoclonal antibody F11-39 was prepared with pepsin digestion followed by gel filtration on a TSK G3000SW column and reduction with 2-mercaptoethylamine HCl as described by *Hashida* et al. with slight modifications (25), and conjugated to alkaline phosphatase (Boehringer Mannheim GmbH, Mannheim, Germany) as tracer antibody.

Instruments

The ACCESS® immunoassay analyzer has a unique solid-phase washing procedure and a chemiluminescent detection system (16, 17). These provide for immunoassays characterized by high sensitivity and/or broad dynamic working ranges. During the wash cycles, magnets are used to pull the paramagnetic particles to the side of the reaction vessel to allow complete aspiration of the wash fluid. The particles are resuspended by forcefully adding buffer and then gently agitating the vessel. The ACCESS system assays use Lumi-Phos 530 (Lumigen, Inc., Detroit, MI, USA), a dioxetane-based chemiluminescent substrate, which is dephosphorylated upon the addition of alkaline phosphatase, resulting in the release of light. The light emitted is measured by the luminometer and the system converts the signals (relative luminescence units: RLUs) into a sample test result.

Commercial enzyme immunoassay for carcinoembryonic antigen

A commercially available EIA kit, Abbott IMx® CEA (Dainabot Co., Tokyo, Japan), was used for comparative studies. The assay employed as sandwich-type method with two different monoclonal antibodies and was performed according to the instructions of the manufacturer.

Serum samples

Sera from patients with various malignant or benign diseases and from healthy individuals were collected and stored at −20 °C until use.

Interfering substances

Bilirubin, haemoglobin, rheumatoid factor and triacylglycerol were obtained from International Reagents Corp. (Kobe, Japan); and human serum albumin from Sigma Chemical Co. (St. Louis, MO, USA).

Results

Assay protocol obtained

Ten microlitres of calibrators or sera and 40 µl of the diluent are incubated for 5 minutes at 37 °C with 50 µl of monoclonal antibody F82-61-immobilized particles and with 50 µl of monoclonal antibody F11-39 Fab' conjugated with alkaline phosphatase. To eliminate interference from human anti-mouse immunoglobulin antibody, the diluent contains 250 mg/l of Heterophilic Blocking Reagent (Scantibodies Laboratories, Santee, CA, USA), which is a mixture of purified mouse IgG subclasses. After washing the unbound components, 200 µl of the Lumi-Phos® 530 solution are added and the particles are incubated for 5 minutes at 37 °C. Time needed to obtain the first result is less than 15 minutes and thereafter one result is obtained every thirty-six seconds. Results are interpolated automatically from a previously run stored standard curve (stable for up to four weeks).

Dose-response curve, detection limit, and hook effect

The CEA calibrators with the concentrations ranging from 0 to 100 000 µg/l were tested. Figure 1 represents a typical dose-response curve in which the luminescence obtained is plotted against the CEA calibrator concentra-
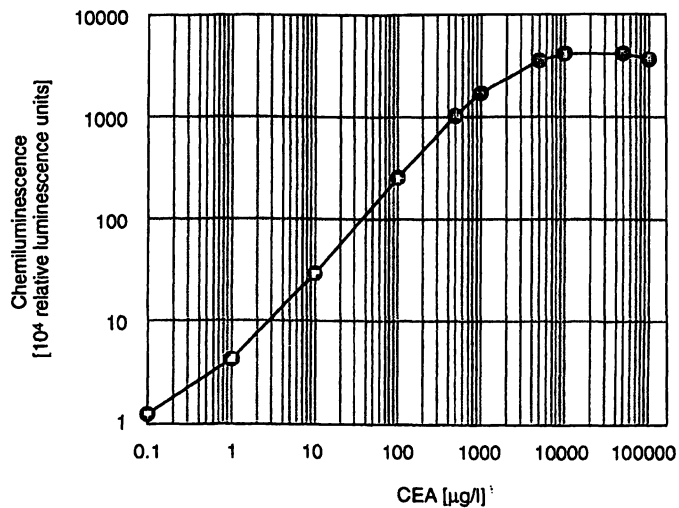
Fig. 1 Typical calibration curve obtained by the ACCESS CEA assay. Note that the first point against the zero calibrator is not included because of logarithmic scale.

tion. The detection limit of the assay, defined as the concentration of CEA equivalent to the mean luminescence plus 2 standard deviations of the zero calibrator, was calculated based on the respective 10 measurements on three different instruments and found to be 0.04 μg/l. Although the dose-response curve extended up to about 10 000 μg/l, moderate high-dose hook effect was observed with the concentrations of 50 000 and 100 000 μg/l (fig. 1). Therefore, the concentrations of calibrators ranging from 0 to 1000 μg/l were selected for determination of CEA in serum samples because in this working range it is virtually impossible to underestimate the concentration due to the hook effect except when the concentration is extremely high (> 100 000 μg/l).

Precision study

The intra-assay precision was determined by replication (n = 20) of three serum samples with different CEA levels. To assess inter-assay precision, three concentrations of control sera were assayed in duplicate in at least 20 runs over a period of four weeks. As shown in table 1, the intra-assay CV ranged from 3.0 to 4.7%, while inter-assay CV ranged from 3.4 to 5.6%.

Tab. 1 Intra- and inter-assay precision using control sera.

Intra-assay precision[a]			Inter-assay precision[b]		
Mean (μg/l)	SD (μg/l)	CV (%)	Mean (μg/l)	SD (μg/l)	CV (%)
4.5	0.2	4.7	5.1	0.2	3.4
46.4	1.4	3.0	49.0	1.8	3.7
483.4	21.6	4.5	497.3	27.7	5.6

[a] Three concentrations of control sera were measured (n = 20) in one assay.
[b] Three concentrations of control sera were measured in duplicate over four weeks (n = 20 runs).

Carryover

The carryover was checked by running a serum sample spiked with a high amount of CEA (10 000 μg/l). After analyzing 4 replicates of the zero calibrator, 4 replicates of the sample were then analyzed, as well as 4 replicates of the zero calibrator. The first replicate of the zero calibrator, immediately after the sample, could then be read. Three values obtained by 3 runs were all < 0.1 μg/l, indicating that the carryover was less than 0.001%.

Recovery

The recovery of the assay was also determined by spiking known quantities of CEA to 4 different serum samples, assaying the serum samples and determining the recovery of the amount added. As shown in table 2, analytical recovery ranged from 98 to 104%.

Dilution

Three different serum samples containing elevated concentrations of CEA within the assay range were diluted in the calibrator diluent. The samples and their dilutions were then assayed to determine linearity on dilution. Good linearity was observed in each case (fig. 2).

Interference

Assay interference was assessed by measuring the apparent response of the ACCESS CEA assay to various concentrations of potential interfering substances spiked into human sera. The basal sera and the spiked serum samples were then assayed. As shown in table 3, no interference was demonstrated in any sample with levels

Tab. 2 Analytical recovery of carcinoembryonic antigen (CEA).

Serum No.	Endogenous CEA (μg/l)	CEA spiked (μg/l)	CEA expected (μg/l)	CEA measured (μg/l)	Recovery[a] (%)
A	12.0	2.5	14.5	14.3	98
		5.0	17.0	16.7	98
		7.5	19.5	20.0	102
		10.0	22.0	21.8	99
B	45.7	2.5	48.2	48.8	101
		5.0	50.7	50.9	100
		7.5	53.2	52.2	98
		10.0	55.7	56.8	102
C	116.5	12.5	129.0	133.8	104
		25.0	141.5	146.9	104
		50.0	166.5	164.8	99
		100.0	216.5	215.8	100
D	594.3	25.0	619.3	618.0	100
		50.0	644.3	633.4	98
		75.0	669.3	659.2	99
		100.0	694.3	688.7	99

[a] The percentage of recovery was defined as the ratio of the CEA measured divided by the CEA expected, multiplied by 100!
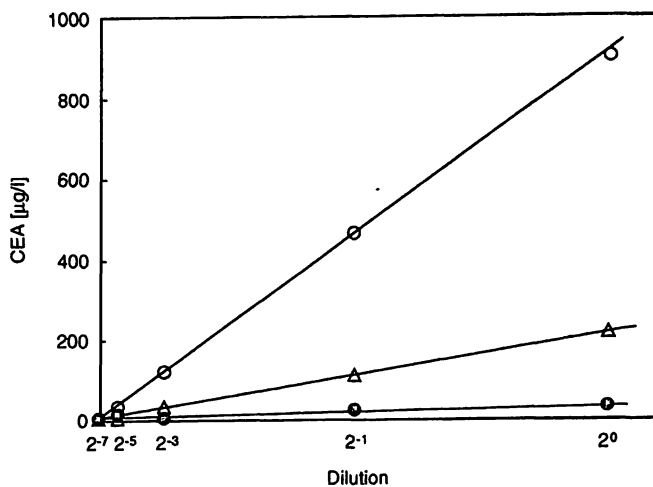
Fig. 2 Dilution test for the ACCESS CEA assay. Three patients' sera with CEA values of 28.9 (●), 221.0 (△) and 902.0 µg/l (o) were diluted with the zero calibrator buffer.

up to 300 mg/l for bilirubin, 12 000 mg/l for haemoglobin, 50 000 mg/l for human serum albumin, 8 500 mg/l for triacylglyerol, or 500 000 IU/l for rheumatoid factor.

Reactivity with carcinoembryonic antigens and related normal antigens

As shown in figure 3, the ACCESS CEA assay gave no reaction with non-specific cross-reacting antigen (NCA), NCA-2, normal faecal antigen (NFA)-1, and NFA-2.

Tab. 3 Assay interference

Interference substance	Interference spiked (mg/l)	Endogenous CEA (µg/l)	CEA measured (µg/l)	Recovery[a] (%)
Bilirubin	50	4.1	4.2	102
	150		4.0	98
	500		4.0	98
Haemoglobin	2 000	4.1	4.1	100
	4 000		4.0	98
	8 000		3.9	95
	12 000		4.1	100
Human serum albumin	10 000	6.2	6.2	100
	20 000		6.2	100
	30 000		6.2	100
	40 000		6.2	100
	50 000		6.1	98
Triacylglycerol	1 062	4.1	4.3	105
	2 125		4.3	105
	4 250		4.3	105
	8 500		4.2	102
Rheumatoid factor	100 000[b]	6.5	6.6	102
	200 000[b]		6.6	102
	300 000[b]		6.7	103
	400 000[b]		7.1	110
	500 000[b]		6.8	105

[a] The percentage of recovery was defined as the ratio of the CEA measured divided by the endogenous CEA, multiplied by 100!
[b] IU/l.

Furthermore, the ACCESS CEA assay showed quite homogeneous reactivity with all CEA preparations tested (fig. 3).

Normal cutoff level

The normal cutoff level was determined by analyzing sera from 477 healthy blood donors including smokers aged from 18 to 82 years, showing the values ranging from 0.2 to 9.9 µg/l and the average value of 2.03 µg/l. Since 5 µg/l defined the 95th percentile in the ACCESS CEA assay and the kit documentation of the IMx CEA assay demonstrates a similar distribution of CEA values in reference populations, we therefore used a tentative common reference limit of 5 µg/l for both the ACCESS CEA and the IMx CEA assays.

Clinical evaluation of the ACCESS CEA assay and comparison with a commercial kit

Serum samples of 133 patients with malignant disease and 72 patients with benign disease as well as of 157 normal subjects were tested with the ACCESS CEA and IMx CEA assays. The regression analyses of the ACCESS CEA assay with the IMx CEA assay showed satisfactory correlations, although the correlation coefficient (r) in sera from normal subjects is slightly lower than those in sera from benign or malignant disease (fig. 4). However, when frequency of elevated CEA levels for respective diseases was compared, some differences were seen between the ACCESS CEA assay and the IMx CEA assay (tab. 4). It is evident that the total positive rate for malignant disease showed an definite increase with the ACCESS CEA assay (56.4%) compared to that of the IMx CEA assay (48.9%). In gastric cancer, colorectal cancer, lung cancer, and hepatoma,
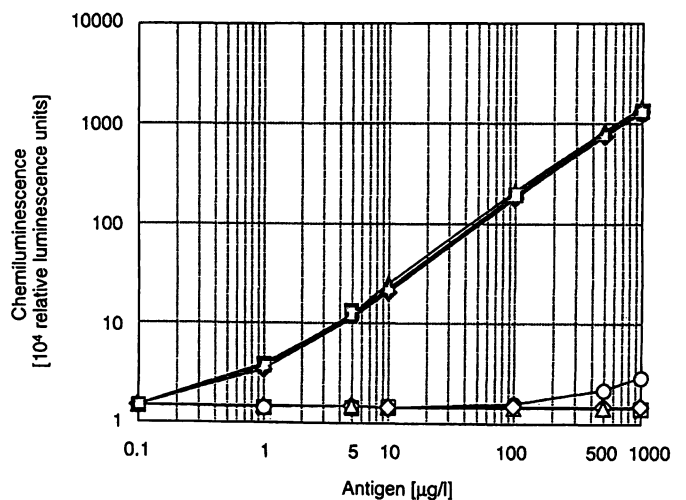

Fig. 3 Reactivity of carcinoembryonic antigens (CEAs) and related normal antigens in the ACCESS CEA assay. Antigens tested: CEA-DB (◆), CEA-MY (□), CEA-TT (▲), CEA-TY (●), non-specific cross-reacting antigen (NCA) (□), NCA-2 (◇), normal faecal antigen (NFA)-1 (△), and NFA-2 (o). The first point against the zero calibrator is not included.
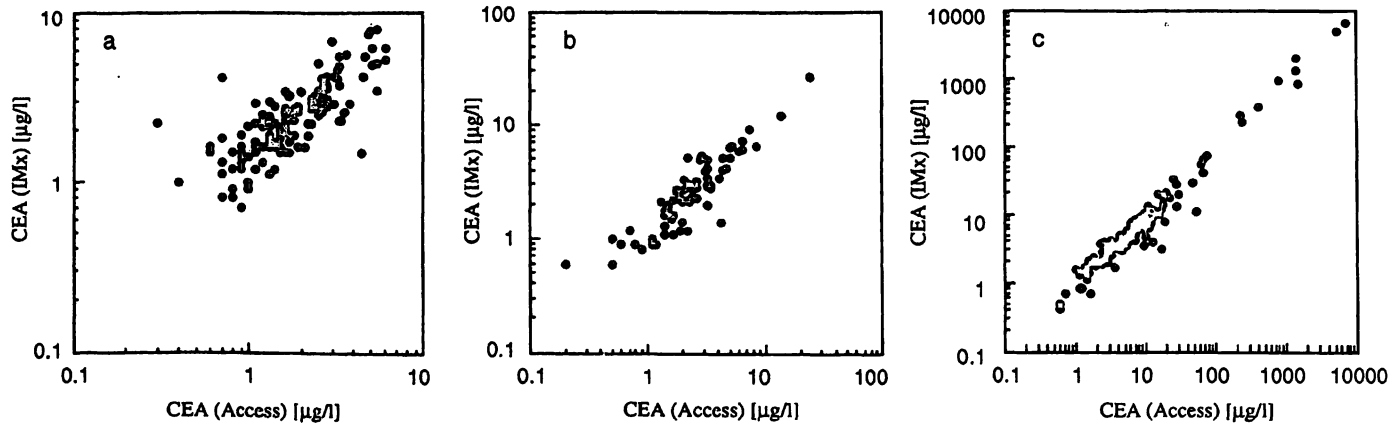
Fig. 4 Correlations of the ACCESS CEA (x) and the IMx CEA (y) assays. (a) 157 normal subjects, y = 0.952x + 0.682, r = 0.812, (b) 72 patients with benign disorder, y = 1.047x

+ 0.133, r = 0.962, and (c) 133 patients with malignant disease, y = 1.001x + 1.088, r = 0.995.

prominently increased positive rates were observed with the ACCESS CEA assay. The total false positive rate (11.1%) for benign disease with the ACCESS CEA assay was certainly lower than that with the IMx CEA assay (19.4%). Relatively high false positivities with the IMx CEA assay were observed in sera from patients with gastric disease, liver disease or diabetes mellitus.

Tab. 4 Positivity of serum CEA in 205 patients with malignant or benign disease and 157 normal subjects determined with AC-CESS CEA or IMx CEA.

Diagnosis	No. of cases	Access® CEA >5.0 µg/l n* (%)	IMx® CEA >5.0 µg/l n* (%)
Malignant disease	133	75 (56.4)	65 (48.9)
Oesophageal cancer	10	4 (40.0)	4 (40.0)
Gastric cancer	31	18 (58.1)	15 (48.4)
Colorectal cancer	26	17 (65.4)	15 (57.7)
Hepatoma	14	11 (78.6)	7 (50.0)
Pancreatic cancer	6	4 (66.7)	4 (66.7)
Lung cancer	11	9 (81.8)	8 (72.7)
Breast cancer	20	12 (60.0)	12 (60.0)
Cervical cancer	9	0 (0.0)	0 (0.0)
Ovarian cancer	6	0 (0.0)	0 (0.0)
Benign disease	72	8 (11.1)	14 (19.4)
Gastric disease	13	0 (0.0)	4 (30.8)
Chronic gastritis	8	0 (0.0)	2 (25.0)
Gastric ulcer	3	0 (0.0)	1 (33.3)
Gastric polyp	2	0 (0.0)	1 (50.0)
Colorectal disease	7	1 (14.3)	1 (14.3)
Acute colitis	2	1 (50.0)	1 (50.0)
Ulcerative colitis	2	0 (0.0)	0 (0.0)
Crohn's disease	3	0 (0.0)	0 (0.0)
Liver disease	34	3 (8.8)	4 (11.8)
Chronic hepatitis	25	2 (8.0)	3 (12.0)
Liver cirrhosis	9	1 (11.1)	1 (11.1)
Pancreatic disease	2	0 (0.0)	0 (0.0)
Pancreatitis	2	0 (0.0)	0 (0.0)
Uterine disease	4	0 (0.0)	0 (0.0)
Myoma uteri	4	0 (0.0)	0 (0.0)
Diabetes mellitus	12	4 (33.3)	5 (41.7)
Normal subjects	157	7 (4.5)	12 (7.6)

* Numbers of positives.

In sera from normal individuals, the ACCESS CEA assay gave a decreased false positive rate (4.5%) as compared with the IMx CEA assay (7.6%).

Discussion

Group F anti-CEA monoclonal antibodies have been found to recognize the protein epitopes present on domain B3 of the CEA molecule and to discriminate CEA in tumour tissues from the CEA-related normal antigens (13). By using a group F monoclonal antibody as tracer antibody, Kuroki et al. have developed a new radio-immunoassay and an EIA with increased tumour specificity and independence from the carbohydrate structure of the CEA molecule (10, 15). Although those assay systems revealed improved features in cancer sensitivity and efficiency in clinical trials (14, 15), it has been impossible to employ those assays on the fully automated immunoassay analyzers. In the present study, by using two defined monoclonal antibodies with higher specificity and affinity for CEA, we tried to establish a new immunoassay for CEA, designated ACCESS CEA, which is adapted to the fully automated ACCESS® immunoassay analyzer.

The ACCESS CEA assay employed a one step sandwich-type method using a combination in which monoclonal antibody F82-61 from group C was used as the capture and monoclonal antibody F11-39 from group F as the tracer antibody. Each monoclonal antibody recognizes an epitope present on the N-terminal domain (N) or C-terminal domain (B3) of the CEA molecule, respectively (11, 12), suggesting that the ACCESS CEA assay detects only the whole CEA molecules with both terminal domains.

The assay range of the ACCESS CEA assay was 0.04–1000 µg/l of CEA and the possible high-dose hook effect was prevented up to 100 000 µg/l in the working range of the assay. The evaluation of assay performance, such as reproducibility, recovery and dilution tests, and

interference studies demonstrated the excellent performance of the ACCESS CEA assay.

Since many CEA-related antigens have been found in various tissues unrelated to malignant diseases (1), the discrimination of CEA from the normal related antigens, especially from normal faecal antigen-2 in normal adult faeces, seems to be essential for a CEA-assay system (5). Although non-specific cross-reacting antigen-2 is a foetal type of CEA (20, 26) and has not yet been found in any normal tissues or tumour tissues, the reactivity with this antigen was also tested in this study. No reactivity of the ACCESS CEA assay with NCA, NFA-1, NFA-2, and NCA-2 indicates that this assay system is able to distinguish CEA from the related normal antigens.

The independence of a CEA-assay system from the influence of variations in the sugar chains of the CEA molecule also seems to be very important, because apparent variation in CEA determination appeared to be mainly due to variation in the structures of carbohydrate chains (8). Furthermore, the expression of epitopes on the sugar chains of the CEA molecule is changeable during synthesis and secretion of CEA by tumour cells, and thus is unstable during the course of malignancy (27). This may result in false apparent fluctuations of the CEA level in the course of diseases unless the assay system is independent of carbohydrate epitopes. The ACCESS CEA assay showed quite homogeneous reactivity with different CEA preparations tested, showing its independence from the effect of variations in the sugar chains of the CEA molecule.

Since the ACCESS CEA assay certainly showed improved features with respect to the reactivities with purified CEA and related antigen preparations and exhibited excellent assay performance, the clinical features of the ACCESS CEA assay, compared with those of the IMx CEA assay, were estimated in this study. For a fair comparison, the same 362 serum samples from patients with malignant or non-malignant disease, as well as from healthy individuals, were simultaneously assayed by the ACCESS CEA and the IMx CEA assays. The improved diagnostic sensitivity of the ACCESS CEA assay is demonstrated in table 4. In comparison with the IMx CEA assay, 7.5% increases in the diagnostic sensitivity were observed with the ACCESS CEA assay. This was not simply due to an unsuitable cutoff level in the ACCESS CEA assay since the false positive rate with this assay in benign disease or normal subjects was less than that of the IMx CEA assay; that is, the diagnostic specificity (93.4%, 214/229 cases) with the ACCESS CEA assay was also better than that (88.6%, 203/229 cases) with the IMx CEA assay. The ACCESS CEA assay, however, deserves further evaluation using longitudinal collected serum samples from cancer patients to determine how well CEA levels correlate with clinical course.

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