Identification and Characterization of New Antigenic Fragments Related to Carcinoembryonic Antigen in Adult Feces

Jerry G. Henslel,1 Edward J. Vachula, Raymond J. Paxton,2 Matthew S. Matias, John E. Shively, Harry G. Rittenhouse,3 and Joseph T. Tomita

ABSTRACT

Antigens in human adult feces related to carcinoembryonic antigen (CEA) were analyzed with respect to their molecular masses, CEA domain compositions, and N-terminal amino acid sequences. By avoiding perchloric acid treatment, new fecal antigens related to CEA were identified. The fecal antigens revealed by Western blot were Mr 78,000, 70,000, 60,000, 50,000, 44,000, 36,000, 33,000, and 25,000 and a species Mr <14,000. Unlike native CEA, all of the fecal antigens were very poorly soluble in perchloric acid and did not bind to concanavalin A, suggesting that they had undergone significant deglycosylation in the digestive tract. The major fecal antigens were purified by immunoadfinity chromatography and their N-terminal amino acid sequences determined. FA78, FA60, FA33, and the Mr <14,000 antigen had the N-terminal amino acid sequence of the CEA N-domain, and FA44 and FA25, the sequence of the CEA A2 domain. The CEA domain compositions of the fecal antigens were investigated by probing them with anti-CEA monoclonal antibodies of known domain specificities. The N-terminal amino acid sequences, immunoreactivities with anti-CEA monoclonal antibodies, and apparent molecular masses of the fecal antigens allowed the following domain assignments (based on CEA as N-A1B1-A2B2-A3B3): FA78, N-A1B1-A2B2-A3B3; FA60, N-A1B1-A2B2; FA44, A2B2-A3B3; FA33, N-A1B1; and FA25, A2B2. The Mr <14,000 antigen was assigned to the N-domain of CEA or nonspecific cross-reacting antigen. FA36 was assigned the N-AB domain structure of nonspecific cross-reacting antigen. The results suggested that FA78, FA60, FA44, FA33, and FA25 were degradation products (including deglycosylation and proteolysis) of CEA and that FA36 was a degradation product of nonspecific cross-reacting antigen.

INTRODUCTION

CEA,4 first described by Gold and Freedman (1) in 1965, has gained an important role as a circulating tumor marker (2, 3). It was initially discovered in human colon adenocarcinomas (1) and soon thereafter found in other epithelial-derived tumors of the gastrointestinal tract (4). CEA in normal colon was more difficult to demonstrate because of its low level and the presence of CEA cross-reacting antigens in this tissue, but the combination of biochemical and immunological analyses eventually confirmed the expression of CEA in the normal colon (5, 6). Recently, CEA and CEA mRNA were shown to be actively expressed by normal colon mucosa maintained in organ culture (7).

The glyocalyx of colonic cancer cells was found to contain CEA, which suggested that CEA could be released into the intestinal lumen and appear in the stool (8). Accordingly, CEA immunoreactivity was detected in perchloric acid extracts of fecal material from colon cancer patients and normal individuals (9, 10). Several studies using immunoassays to measure fecal CEA have shown that levels from colon cancer patients are sometimes elevated over the levels from normal individuals (11–13).

The quantitative and specific measurement of CEA in fecal specimens is complicated by: (a) the presence in feces of CEA cross-reacting antigens (9); and (b) possible degradation of colonic CEA during transit through the digestive tract (14). These fecal components may act as interfering substances during the measurement of CEA in fecal specimens. It is important, therefore, to characterize CEA-related antigens in feces in order to develop tests with improved specificity and sensitivity for fecal CEA. Several distinct CEA-related antigens have been identified in feces. The most extensively characterized species were designated NFA. NFA included 3 molecular species, NFA-1, NFA-2, and NFCA, approximately Mr 20,000, 170,000, and 90,000, respectively (14–17). Matsuoka et al. (18) recently postulated that NFA-2 may result from cleavage of CEA from the plasma membrane of cells on the luminal surface of the intestine. NFA-1 and NFCA were proposed to be breakdown products of NFA-2 (14, 18). NCA has also been found in feces (9, 14, 15). The presence of NCA in feces (Mr 80,000–90,000) was supported by the ability of this antigen to bind antibody specific for NCA of normal human lung (14).

The present report describes new CEA-related antigens from human feces that have not been described previously. Our investigation avoids perchloric acid extraction of feces, which has been a standard step in previous studies (10, 14). Western blotting and immunoadfinity purification have been used in characterizing CEA-related antigens from various sources (19, 20) and were utilized in the present study for analyzing CEA-related antigens in feces.

MATERIALS AND METHODS

Antibodies. Mouse monoclonal antibodies to CEA were generated by standard procedures. MAb HGC8, HBC2, and H19C91 (21) were provided by Dr. Ilse Tribby of Abbott Laboratories and MAb T84.1E3 (22, 23) by Dr. John Shively of the City of Hope. All of the MAbs used were IgG. Purified CEA and affinity-purified goat anti-CEA were provided by Dr. Dennis Delfert of Abbott Laboratories.

Aqueous Extraction of CEA-related Antigens from Human Fecal Specimens. Fecal specimens from individuals were immediately frozen after collection. Cold PBS containing 1 mm phenylmethylsulfonyl fluoride, 1 mm benzamidine, 7 × 10−3 trypsin inhibitor unit/ml aprotinin, 5 mm EDTA, and 0.1% sodium azide (PBS-I) was added to fecal material in a disposable 50-ml screw-cap tube to yield an approximate 30% fecal wet weight suspension. (Biochemicals used in this study were obtained from Sigma Chemical Co., St. Louis, MO.) The fecal suspension was vigorously agitated for 2 min and then centrifuged at 5000 × g for 30 min at 4°C. The 5000 × g supernatant was carefully decanted and filtered through cheesecloth to yield the PBS fecal extract.
Gel Filtration Chromatography of Fecal CEA-related Antigens. PBS extraction of feces as described above was scaled-up for purification of the CEA-related antigens. The initial fecal suspension was homogenized in a blender for 5 min. The homogenate was centrifuged and filtered as described above, yielding the PBS fecal extract. Mixed alkyltrimethylammonium bromide (24) was used to decrease the mucin content of the PBS fecal extract. One hundred μl of a 10% w/v solution of this cationic detergent per g wet weight of starting fecal material were added to the PBS fecal extract, which was stirred for 10 min. After centrifugation, the supernatant was recovered as the mucin-depleted PBS fecal extract. The extract was made 70% ammonium sulfate and the resulting precipitate was dissolved in a volume of PBS-I, which was about 5% of the volume of the initial fecal homogenate.

The dissolved pellet was chromatographed on a Sephacryl S-400 column, 5 × 90 cm (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) equilibrated in PBS-I. Fractions were assayed for CEA immunoreactivity as described below. Fractions containing CEA immunoreactivity were selected, pooled, and concentrated over a YM10 membrane (Amicon Corp., Danvers, MA) to yield the S-400 CEA active pool. For further purification of the CEA-related antigens, the S-400 CEA active pool was similarly chromatographed on a Sephacryl S-100 column.

Perchloric Acid Fractionation of Fecal CEA-related Antigens. The S-400 CEA active pool was extracted with 0.6 M perchloric acid. The soluble fraction was exhaustively dialyzed against PBS and concentrated over a YM10 membrane to yield the perchorlic acid-soluble fraction. The insoluble fraction was dissolved in PBS and dialyzed and concentrated as above to yield the perchorlic acid-insoluble fraction. The perchorlic acid-soluble and -insoluble fractions were adjusted to the initial volume of the S-400 CEA active pool used in this experiment. A control soluble fraction was obtained by substituting PBS for perchorlic acid followed by the same dialysis and concentration steps described above.

Con A Affinity Fractionation of Fecal CEA-related Antigens. To 250 μl of S-400 CEA active pool were added 250 μl of pure CEA at 20 μg/ml, and 100 μl of this mixture was immediately frozen. The remaining 400 μl of the mixture were added to 400 μl of 0.2 M sodium acetate, pH 6.0, 2.0 M NaCl, 2 mM in each of CaCl2, MgCl2, and MnCl2 (2 X Con A buffer) (25). The diluted mixture was chromatographed through a Con A-agarose column. A Con A-unbound fraction was obtained by eluting the column with PBS, it was eluted with 10 to 15 bed volumes of 0.1 M glycine, 0.5 M NaCl, pH 2.8. The glycine eluate was neutralized with NaOH, concentrated over a YM10 membrane, and dialyzed against PBS.

RESULTS

CEA-related Antigens in PBS Fecal Extracts. PBS fecal extracts were prepared from fecal specimens of 8 individuals and analyzed by Western blotting (Fig. 1). All of the fecal extracts had components that stained intensely with goat anti-CEA. The Western blot profiles did not change if the PBS fecal extracts were dialyzed against PBS at 4°C before SDS gel electrophoresis (data not shown).

At least 7 discrete bands of different sizes can be observed in this blot. Several bands appeared to be common between some samples.
of the extracts. The extracts in Fig. 1, Lanes 1, 3, 4, and 5, stained broadly near the top of the blot where larger components would be located. These profiles revealed the high degree of size heterogeneity of fecal antigens that are cross-reactive with CEA.

Separation of Fecal CEA-related Antigens by Gel Filtration. Proteolytic activity in a PBS fecal extract was measured by following the degradation of denatured, dye-derivatized collagen (28) added to the extract and was negligible when the extract was at 4°C (data not shown). If the collagen-extract mixture was incubated at 37°C, however, proteolytic activity was detected. Care, therefore, was taken to perform all fractionation steps at 4°C.

CEA immunoreactive components in fractions 42 through 87 were separated from viscous material eluting near the void volume by S-400 chromatography (Fig. 2). The sandwich assay detected high levels of CEA immunoreactivity in fractions 42 through 70. These fractions, designated “A” in Fig. 2, were also strongly immunoreactive by the competition assay, which indicated that these fractions contained significant levels of the epitope defined by MAb H8C2. In contrast, fractions 71 through 87, designated “B,” contained very little or no CEA immunoreactivity by the sandwich assay while showing strong immunoreactivity in the competition assay. These results suggest that the fecal CEA-related antigen(s) in the “B” fractions did not contain both epitopes defined by the capture and probe anti-CEA MAbs of the sandwich assay (29), but did contain the epitope defined by MAb H8C2 as revealed by the competition assay.

Western blots were performed on the “A” and “B” fractions (data not shown). The “A” fractions contained CEA immunoreactive components of various sizes, while the “B” fractions were characterized by a single immunoreactive component of about Mr 25,000, which will be described below. The competition assay was, therefore, more sensitive in detecting this fecal CEA-related antigen in the S-400 fractions than the sandwich assay. Fractions 42 through 87 were pooled and concentrated to yield the S-400 CEA active pool. The low level of CEA immunoreactivity detected near the void volume was not investigated in this study.

The S-400 CEA active pool was subjected to S-100 chromatography, and a CEA immunoreactivity elution profile was obtained, which was very similar to that of the S-400 profile. S-100 provided better separation of the CEA-related antigens from contaminating material that eluted in larger elution volumes (data not shown).

Western Blot Analyses of S-400 CEA Active Pool. A S-400 CEA active pool was subjected to Western blotting and probed with 3 anti-CEA MAbs, H8C2, H6C8, and H19C91 (Fig. 3A), which define 3 different CEA epitopes (21). The 8 fecal components observed in the blot were approximately Mr 78,000, 60,000, 44,000, 36,000, 33,000, and 25,000 (Fig. 3B). Two minor staining components were Mr 70,000 and 50,000.

Different CEA immunoreactivity profiles resulted from the different antibody probes (Fig. 3A). FA78 (designation for fecal antigen of Mr 78,000) was the only antigen stained by all 3 MAbs. The smaller fecal antigens were stained by one or 2 of the MAbs, e.g., the smallest band, FA25, was only stained by H8C2. The binding of anti-CEA MAbs to these fecal components further supported their characterization as CEA-related antigens and demonstrated their diversity with respect to CEA epitope structure.

The same S-400 CEA active pool was used throughout this study. The starting material was derived from specimens pooled from 2 individuals. Specimens from some other individuals also produced the blotting profile of this pool, suggesting that it was a representative one (data not shown).

The possibility of nonspecific binding by the anti-CEA MAbs in the Western blots was shown to be unlikely by demonstrating the ability of soluble, pure CEA to inhibit binding of MAbs H8C2 and H19C91 to blotted S-400 CEA active pool (Fig. 4). This inhibition was specific to CEA since human IgG did not inhibit MAb binding.

Perchloric Acid Treatment. Perchloric acid treatment of a S-400 CEA active pool (Fig. 5) resulted in very poor recoveries of FA78, FA70, FA60, FA50, FA44, and FA36 in the acid-soluble fraction (Fig. 5C) compared to their levels in the starting material (Fig. 5A) and in the control soluble fraction (Fig. 5D). These 6 components were primarily found in the acid-insoluble fraction (Fig. 5B). FA33 and FA25 levels were
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Fig. 3. Molecular mass determination of fecal CEA-related antigens from Western blots. 4. 500 ng of CEA immunoreactivity of a S-400 CEA active pool were loaded per well of an 8–16% acrylamide SDS gel (Lanes 1–3). Lanes 1–5, blotted fecal antigens (FA) were probed with MAbs H8C2 (Lane 1), H6C8 (Lane 2), and H19C91 (Lane 3). Molecular weight standards were run and stained with Coomassie blue (Lanes 4 and 5). B, molecular masses of the standards were plotted against their relative mobilities by linear regression (O). Relative mobilities of the stained FA are shown, from which their molecular masses were determined.

Fig. 4. Competition between blotted fecal CEA-related antigens (FA) and soluble CEA for anti-CEA MAbs. A S-400 CEA active pool was electrophoresed on a 10% acrylamide SDS gel and blotted. The blots were probed with MAbs H8C2 (A) and H19C91 (B) at 1 μg/ml MAb in the presence of CEA at 0, 5, 10, and 20 μg/ml (Lanes 1–4, respectively) or in the presence of human IgG at 5, 10, and 20 μg/ml (Lanes 5–7, respectively).

also significantly reduced in the acid-soluble fraction and appeared to distribute similarly between the acid-insoluble and -soluble fractions (Fig. 5, B and C).

Con A Analysis. A mixture of exogenous native CEA and S-400 CEA active pool was eluted through a Con A affinity column. Most of the exogenously added CEA apparently was bound by the Con A affinity column, and very little appeared in the Con A unbound fraction (Fig. 6, Lane 3). In contrast, the fecal antigens did not bind to the Con A column as seen by the near quantitative recovery in the Con A unbound fraction (Fig. 6, Lane 3). Binding of exogenous CEA to Con A was confirmed by its recovery in the Con A bound fraction (Fig. 6, Lane 4). Very low levels of FA78 and FA60 and none of the smaller antigens were detected in the Con A bound fraction (Fig. 6, Lane 4).

Immunoaffinity Fractionation of Fecal CEA-related Antigens. The S-400 CEA active pool was chromatographed on a S-100 column as described in “Materials and Methods.” The S-100 column fractions containing CEA immunoreactivity could also be grouped into “A” and “B” fractions as already described for the S-400 column fractions. The A fractions and the B fractions were separately pooled. The A pool contained significant levels of both H8C2 and H19C91 immunoreactive components. It was applied to a MAb H19C91 immunoaffinity column, and the bound proteins were recovered. The type B pool contained H8C2 immunoreactive components at significantly higher levels than those that were H19C91 immunoreactive (data not shown). This pool was applied to an H8C2 immunoaffinity column, and bound proteins were recovered. Because of very low protein concentrations of these fractions, protein was measured by A280, which were 0.74 and 1.59 for the
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Fig. 6. Interaction of fecal CEA-related antigens (FA) with Con A. A Western blot was performed and probed as in Fig. 5. Lanes 1–4, S-400 CEA active pool (Lane 1); S-400 CEA active pool plus exogenous CEA (Lane 2); Con A unbound fraction (Lane 3); and Con A bound fraction (Lane 4).

H8C2 immunoabsorbent purified and H19C91 immunoabsorbent purified fractions, respectively.

In a Western blot, the crude S-400 pool was a complex mixture of proteins staining the length of the blot by Coomassie blue, but each affinity-purified fraction contained only 3 discrete bands (Fig. 7, top). FA78, FA60, and FA33 were significantly enriched in the H19C91 affinity-purified fraction (Fig. 7, Lane 3 in all blots). FA44 and FA25 were enriched in the H8C2 affinity-purified fraction, which also contained some FA33 (Fig. 7, Lane 2 in all blots).

FA36 was not recovered in either affinity-purified fraction. The elution of FA36 through the S-100 column was predominantly in the fractions that were pooled and loaded on the H19C91 affinity column. Since the Western blots showed that FA36 does not contain the H19C91 defined epitope (Fig. 7B, Lane 1), FA36 would not be enriched by the H19C91 affinity column. In the blots of the S-400 CEA pool, MAb T84.1E3 bound FA36 (Fig. 7C, Lane 1), while MAb H19C91 did not (Fig. 7B, Lane 1).

N-Terminal Amino Acid Sequence Analysis. FA78, FA60, FA33, and a component at the dye front had an N-terminal amino acid sequence identical to the first 10 amino acid residues of the CEA N-terminal domain, KLTIESTPFN (30). The initial yields were 23, 52, 41, and 7 pmol, respectively. The component at the dye front was Mr < 14,000. It was never detected in Western blots probed with anti-CEA antibodies, but was discovered in a Coomassie blue-stained blot.

Two other components, FA44 and FA25, had the N-terminal amino acid sequence of the CEA A2 domain, [Y]AEPPKPFITS (31, 32). The initial yields were 9 and 44 pmol, respectively. In each case, the predominant sequence began with alanine (A), but there were also sequences beginning with tyrosine (Y), which precedes the alanine, and with glutamic acid (E), which follows the alanine. These 2 components, therefore, had “ragged” amino termini, with the sequence beginning with alanine being predominant.

DISCUSSION

Since CEA was discovered, other naturally occurring antigens have been described that demonstrate immunological cross-reactivity with CEA. In a recent review, 36 antigens were listed as members of this CEA family (33). The similarities and differences between these antigens are becoming better understood, especially as their amino acid sequences and carbohydrate compositions are being delineated. For example, the 2 antigens NCA (Mr 45,000) and TEX (Mr 75,000) were shown to be significantly different in size because of variations in glycosylation while having the same primary amino acid sequence (34). Included in the CEA family were adult fecal antigens that were cross-reactive with CEA.

NFA-2, NFA-1, NFCA, and NCA from feces were prepared by first treating feces with perchloric acid and using the acid-soluble fraction for further purification (10, 13, 15). In the present study, however, very poor recoveries of fecal CEA cross-reacting antigens were obtained in the acid-soluble fraction (Fig. 5). Since only highly glycosylated glycoproteins are soluble in perchloric acid, it is likely that the CEA fecal antigens are less glycosylated than CEA. We speculate that the action of glycosidases present in the intestinal lumen has rendered them poorly soluble in perchloric acid. By avoiding perchloric acid extraction and using the sensitive Western blot technique for...
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detection, we observed at least twice the number of CEA-related antigens in human feces than the 4 antigens reported previously.

These fecal antigens were compared to CEA in their ability to bind to Con A. Seventy to 90% of total CEA from tumor tissue has been shown to bind to Con A (35-37). In contrast, the fecal CEA-related antigens showed little to no binding to this lectin (Fig. 6). If the carbohydrate compositions of the antigens have been affected by deglycosylation as postulated above, these antigens may have lost the ability to bind to Con A. Kuroki et al. (38) found NFA-1 to contain 13% carbohydrate compared to 52% for CEA. The lower carbohydrate content of NFA-1 may be due, at least in part, to deglycosylation. Carbohydrate analysis of the fecal CEA-related antigens described in this study will be required to determine their state of deglycosylation.

The fecal antigens were further characterized by determining their: (a) apparent molecular masses by SDS-PAGE; (b) CEA domain compositions by Western blotting and probing with anti-CEA MAbs of defined specificities; and (c) N-terminal amino acid sequences. These results are summarized in Table 1, which shows their relation to the domain structure of the CEA polypeptide, N-A1B1-A2B2-A3B3 (31). The amino acid sequence of the intact CEA polypeptide and, therefore, of each domain are known (39). The polypeptide making up the N-domain is approximately M, 12,000 and the A and B domain polypeptides are similar, ranging between about M, 9,000 and 10,500. Different combinations of intact or nearly intact polypeptides should, therefore, have predictable sizes if they have been subjected to significant deglycosylation.

The deglycosylated intact polypeptide of CEA based on its amino acid sequence is about M, 73,000 (39), which is very close to the M, 78,000 of FA78 determined by SDS-PAGE. The size of FA78 suggests that this antigen may be a largely intact CEA polypeptide stripped of most of its carbohydrate. The Western blots of FA78 support this conclusion. FA78 was strongly stained by 3 anti-CEA MAbs of different domain specificities, which together approximate the full length CEA polypeptide. MAb H19C91 has been shown to be specific for an epitope in the N-domain; H8C2 binds preferentially to the A1B1 and A2B2 domain regions, and H6C8 is highly specific for the A3B3 domain region (21). Furthermore, addition of exogenous CEA to a fecal extract results in a component of Mr 78,000, indicating that FA78 was produced directly from CEA (data not shown). M, 78,000 was also reported for the unglycosylated polypeptide moiety of CEA synthesized by several human tumor cell lines in the presence of tunicamycin (40).

The N-terminal amino acid sequence of FA78 is identical to the CEA N-domain for the first 10 amino acid residues. This observation confirmed the presence of the N-domain in FA78, proving the relationship between CEA. The relationship between FA78 and NFCA cannot be established from this study. Both FA78 and NFCA (41) share MAb-defined reactivities with CEA that are absent on NCA.

CEA domain compositions were also proposed for the fecal antigens smaller than FA78 (Table 1). For each one, the molecular mass observed by SDS-PAGE, the CEA immunoreactivities determined by Western blotting, and the N-terminal amino acid sequences closely agreed with the proposed domain structures. FA25 is most likely NFA-1, which was also shown to start with the A2 sequence and was between M, 20,000 and 30,000 (38). One difference between NFA-1 and FA25 found in this study was their relationship with NCA. NFA-1 was not enriched during the immunoaffinity steps used in this study and so could not be subjected to N-terminal amino acid sequencing. It was stained by MAb T84.1E3 but not by H19C91 in a Western blot (Fig. 7). Both of these MAbs recognize epitopes of the N-domain, but they differ in their specificities toward CEA and NCA. H19C91 binds CEA only (21), while T84.1E3 binds both CEA and NCA (22). FA36 may be a degradation product of NCA since it binds T84.1E3 but not H19C91. FA36 was very similar in size to the polypeptide moiety of NCA, M, 35,000 (34, 44). FA36 may be the intact NCA polypeptide stripped of its carbohydrate in the gut.

The carbohydrate content of CEA confers resistance to cleavage by proteolytic enzymes (45, 46). CEA becomes susceptible to proteolysis after desialylation or deglycosylation (47, 48). CEA, when first released into the intestinal lumen, may also be resistant to proteolytic degradation and initially undergo significant deglycosylation by intestinal glycosidases. Deglucosylated regions of the CEA polypeptide may then become susceptible to cleavage by proteolytic enzymes in the intestine.

### Table 1 Summary and proposed domain compositions of fecal CEA-related antigens

<table>
<thead>
<tr>
<th>Antigen</th>
<th>MAb profile</th>
<th>N-terminal sequence</th>
<th>Proposed structure</th>
<th>Calculated M,</th>
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<td>FA78</td>
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<td>H19−, H8−, H6−</td>
<td>N</td>
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<td>12,000</td>
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</table>

* Antigen staining (+, positive; −, negative) on Western blots by MAbs H19C91 (H19), H8C2 (H8), H6C8 (H6), and T84.1E3.

* CEA domain defined by N-terminal amino acid sequence of antigen.

* Proposed domain compositions of antigens.

* Calculated mass of proposed domain composition.

* ND, not determined.

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generating discrete fragments of CEA and possibly of NCA. Western blots of the individual crude fecal extracts may be reflecting this degradation process (Fig. 1). The high degree of heterogeneity near the top of the blot may be CEA in a spectrum of deglycosylation states. As a result of being deglycosylated, the CEA polypeptide is proteolytically cleaved at specific sites, resulting in smaller discrete fragments.

The complete amino acid sequences of the isolated fecal CEA-related antigens in this study will completely define their polypeptide and CEA domain compositions. It will also identify the peptide bonds of the CEA polypeptide, which are especially sensitive to proteolytic cleavage in the digestive tract. Analyses of the carbohydrate compositions of the fecal antigens will further characterize them. Other degradation products of CEA may exist in adult human feces that were not identified in this study. The use of immunoabsorbsents with specificities for different CEA epitopes may enrich and identify other fecal CEA antigens.

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