Purification and Characterization of Carcinoembryonic Antigen-related Antigens in Normal Adult Feces

Masahide Kuroki, Yoshiko Koga, and Yuji Matsuoka

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ABSTRACT

We tried to purify carcinoembryonic antigen (CEA)-related antigens in normal human feces and found that, besides nonspecific cross-reacting antigen (fecal NCA), there are three other molecular species of CEA-related antigens which have been inclusively called normal fecal antigen (NFA). These were designated normal fecal antigen 1 (NFA-1), normal fecal antigen 2 (NFA-2), and normal fecal cross-reacting antigen (NFCA), respectively. Among these antigens, NFA-1, NFA-2, and fecal NCA were isolated in pure form. NFCA has not yet been obtained in pure form but was identified as an antigen different from three other antigens.

All antigens were glycoprotein in nature and migrated electrophoretically in the β region. Their molecular weights were estimated to be 20,000 to 30,000 for NFA-1, 160,000 to 170,000 for NFA-2, and 80,000 to 90,000 for NCA, respectively. NFA-2 had amino acid and carbohydrate compositions similar to those of CEA.

The results obtained by immunodiffusion analyses of antigens indicate that CEA and NFA-2 can be divided into four antigenic moieties: the first one is distinctive for CEA (cancer determinant) or NFA-2 (NFA-2-distinctive determinant); the second one corresponds to NFA-1 (NFA-1 determinant); and the remaining two moieties are present on NFCA, one of which is characteristic for NFCA (NFCA determinant) and the other of which is shared with NCA (NCA-common determinant).

INTRODUCTION

CEA was first described by Gold and Freedman (9, 10) as a cancer-specific fetal antigen in adenocarcinoma of the human digestive tract. Although the cancer specificity is, at present, rather controversial, CEA is considered to be one of cancer-related antigens, and its clinical significance is being widely accepted. In this context, several CEA-related antigens have been reported and can be categorized as follows: (a) NCA, which is partially cross-reactive with CEA (27) and antigens reported to be immunologically identical to CEA (1, 4, 11, 13, 19, 20, 26); (b) BGP I in human bile (23), which was shown to be cross-reactive with both CEA and NCA; (c) NFA in feces (16), NCA-2 in meconium and feces (2), CEA-like substances in gastric juice (28) and in NCW (5, 21), and BGP III in bile (23). These were found in the alimentary canal and were reported to be very similar to CEA; (d) CEA-No in normal colon mucosa (7), which was reported to be identical to the CEA present in tumor tissue.

Since the molecular relationship of these antigens has not yet been clarified, purification and characterization of these CEA-related antigens are of obvious importance not only for the elucidation of the structure and the biological role of CEA but also for the improvement of plasma CEA assay which is now widely applied in various clinical fields.

In the present study, we tried to purify the CEA-related antigens in normal feces and found that, besides fecal NCA, there were 3 other molecular species of CEA-related antigens which have been inclusively called NFA. These antigens were designated NFA-1, NFA-2, and NFCA, respectively. The immunological and physicochemical natures of these antigens as well as those of fecal NCA were characterized.

MATERIALS AND METHODS

Reference CEA and NCA Preparations. Preparations of CEA were isolated from metastatic liver tumors of colon adenocarcinomas obtained at autopsy. Extraction of CEA was done primarily according to the method of Kruepen et al. (12). Purification of CEA was performed by successive gel filtrations as reported previously (17).

NCA was prepared from the crude perchorlic acid extract of pooled normal lungs obtained at autopsy, by specific adsorption to and elution from antibody adsorbent, and by gel filtration.

The CEA and NCA preparations contained nearly pure antigens as demonstrated by immunoelectrophoresis (Fig. 1) and by SDS-PAGE electrophoresis (Fig. 2).

Antisera. Three different types of anti-CEA preparations were used in this study. R-α and Go-α were prepared by immunization with a purified CEA preparation (H.O.) as previously described (17). These 2 antisera were specifically purified by adsorption to CEA-Sepharose 4B (see below) and by elution with 0.175 M glycine·HCl buffer (pH 2.6). The purified antibody preparations were used without further absorption or after complete absorption with NCA. The third antiserum (AAP-1) was a guinea pig anti-CEA antiserum prepared by a special procedure reported elsewhere (18). In order to minimize the production of antibody reactive with fecal antigens, a guinea pig immunized with CEA-anti-CEA complex in complete Freund’s adjuvant (Difco Laboratories, Detroit, Mich.) was administered passively with a guinea pig anti-CEA antiserum containing antibodies reactive only with fecal antigens. The antiserum (AAP-1) thus prepared retained enough reactivity with CEA after complete absorption with fecal antigens.
A-NCA was prepared in the goat and rendered reactive only with NCA by complete absorption with CEA.

A-NFA-2 was raised in a rabbit and was reactive only with NFA-2 but not with any other antigens after absorption with CEA. Identification of these antisera is summarized in Table 1.

Antisera directed against crude extracts of tumors, lungs, or feces were raised in rabbits and used for immunoelectrophoresis.

Antigen and Antibody Adsorbents. Purified CEA or NCA was coupled with CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals AB, Uppsala, Sweden) (10 mg/g dry gel) according to the manufacturer’s instructions.

Goat anti-CEA antibody or goat anti-NCA antibody specifically purified by proper antigen adsorbent was conjugated to CNBr-activated Sepharose 4B (10 mg/g dry gel) and used for specific purification of fecal antigens and NCA.

Gel Filtration and Ion-Exchange Chromatography. Gel filtrations were performed with 0.05 M sodium phosphate-0.15 M NaCl (pH 5.2). DEAE-cellulose chromatography was done with PB.

Proteolytic Enzyme Treatment. Proteolytic digestion of purified antigens by Pronase E (Kaken Kagaku Co., Tokyo, Japan) was performed as described previously (17).

Immunoelectrophoresis and Immunodiffusion Tests. Immunoelectrophoresis and microscale immunodiffusion tests were carried out in a 1.2% agarose layer as described previously (17).

SDS-PAGE. Analytical electrophoresis was performed on 7.5% or 10% polyacrylamide gels containing 1% SDS following the method of Fairbanks et al. (6) with slight modifications. Samples were incubated in 1% 2-mercaptoethanol and 1% SDS for 2 hr at 37°C before application to the gels. Electrophoresis was carried out for 2 to 3 hr, and gels were stained with either Coomassie Brilliant Blue or periodic acid-Schiff reagent (8). Control gels were run with various reference proteins.

Analytical Ultracentrifugation. The sedimentation velocities of CEA and NFA-2 were estimated by measuring sedimentation of the band in a Hitachi Model LICA-1A ultracentrifuge (Hitachi Co., Ltd., Tokyo, Japan), revolving at 60,000 rpm at 20°C.

Amino Acid and Carbohydrate Analyses. Amino acid compositions of the purified CEA and NFA-2 preparations were analyzed by using a Hitachi Model 835-50 automatic amino acid analyzer by the method of Spackman et al. (22).

For identification and quantitation of all monosaccharides except sialic acid, gas-liquid chromatography was carried out after reduction of monosaccharides to the respective alditols and hexosaminols followed by trifluoroacetylation, according to the methods of Matsui et al. (14) and Tamura et al. (25). Sialic acid was determined according to the method of Warren (29) with N-acetylneuraminic acid as standard after samples were hydrolyzed in 0.1 N H2SO4 at 80°C for 1 hr.

RESULTS

Purification of CEA-related Antigens from Normal Adult Feces. In Chart 1, the purification procedures of CEA-related antigens are summarized. The feces from 4 healthy individuals of different blood types and ages 22 to 48 years were separately analyzed. Representative results obtained from an individual with Blood Type O will be described below. The antigens in feces were extracted with 0.6 M perchloric acid as described previously (16) and then adsorbed to and eluted from goat anti-CEA-antibody adsorbent. The eluate (NFA-rich eluate) was applied to a Sepharose 6B column. The elution profile is shown in Chart 2. In fractions II and IV were detected antigens reactive with specific anti-CEA. Fraction IV contained an additional antigen reactive with specific anti-NCA.

The concentrated Fraction II was chromatographed on another Sepharose 6B column and then on a Sephadex G-200 column. A CEA-related antigen was eluted from the column as a single peak. The antigen preparation thus obtained gave a single precipitate line in immunoelectrophoresis (Fig. 1) and revealed a single symmetrical peak in ultracentrifugation analysis and a single band in SDS-PAGE (Fig. 2). This high-molec-

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### Table 1

<table>
<thead>
<tr>
<th>Abbreviated name</th>
<th>Animal species</th>
<th>Immunizing antigen</th>
<th>Treatments</th>
</tr>
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<tr>
<td>R-8</td>
<td>Rabbit</td>
<td>CEA</td>
<td>Specificity purified with CEA-adsorbent, then used unabsorbed or after absorption with NCA</td>
</tr>
<tr>
<td>Go-8</td>
<td>Goat</td>
<td>CEA</td>
<td>Absorbed with fecal antigens and NCA</td>
</tr>
<tr>
<td>NFA-2</td>
<td>Rabbit</td>
<td>NFA-2</td>
<td>Unabsorbed or absorbed with CEA</td>
</tr>
<tr>
<td>NCA</td>
<td>Goat</td>
<td>NCA</td>
<td>Specifically purified with NCA adsorbent, then used after absorption with CEA</td>
</tr>
</tbody>
</table>

*a Prepared by a special procedure (see “Materials and Methods”) and reactive only with CEA.

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The concentrated Fraction II was chromatographed on another Sepharose 6B column and then on a Sephadex G-200 column. A CEA-related antigen was eluted from the column as a single peak. The antigen preparation thus obtained gave a single precipitate line in immunoelectrophoresis (Fig. 1) and revealed a single symmetrical peak in ultracentrifugation analysis and a single band in SDS-PAGE (Fig. 2). This high-molec-
CEA-related Antigens in Normal Feces

Upon ultracentrifugation analysis, CEA and NFA-2 showed sedimentation coefficients of 6.3 and 6.0S, respectively.

The amino acid composition of NFA-2 is shown in Table 2 in comparison with that of CEA. Essentially, a similar composition was observed. Table 3 shows the carbohydrate compositions of CEA and NFA-2. Both samples contained the same types of monosaccharides in similar weight percentage proportions. NFA-2 has 48.9% total carbohydrate, compared to 50.8% in CEA.

Immunological Properties. As can be seen in Fig. 3, the precipitate line of NFA-2 with R-8 coalesced completely with

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Amino acid composition of CEA and NFA-2</th>
<th>mol amino acid/10^5 g protein</th>
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<tr>
<td>Amino acid</td>
<td>CEA (H.O.) b</td>
<td>CEA (T.Y.) b</td>
</tr>
<tr>
<td>Lysine</td>
<td>23.8</td>
<td>25.6</td>
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<td>Histidine</td>
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<tr>
<td>Arginine</td>
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<td>Aspartic acid</td>
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<td>Threonine</td>
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<td>Serine</td>
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<td>Glutamic acid</td>
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<td>Proline</td>
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<td>Glycine</td>
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<td>Alanine</td>
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<td>Valine</td>
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<tr>
<td>Phenylalanine</td>
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<table>
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<tr>
<th>Table 3</th>
<th>Carbohydrate composition of CEA and NFA-2</th>
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<tr>
<td>Monosaccharide</td>
<td>CEA (T.Y.) b</td>
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<td>Fucose</td>
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<tr>
<td>Mannose</td>
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<td>Galactose</td>
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<tr>
<td>Sialic acid</td>
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<td>N-Acetylgalactosamine</td>
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<td>N-Acetylgalactosamine</td>
<td>0.6</td>
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a Initials in parentheses, initials of the patients.

Physicochemical Properties. Electrophoretically, all antigens revealed β-region mobility, but NFA-1 migrated slightly faster than did NFA-2 or CEA, and NCA migrated slower than did others, as shown in Fig. 1.

Apparent molecular weights of isolated antigens determined by SDS-PAGE were 20,000 to 30,000 for NFA-1, 160,000 to 170,000 for NFA-2, 170,000 to 180,000 for CEA, and 80,000 to 90,000 for fecal NCA as well as lung NCA, respectively. All purified antigens were stainable with both Coomassie Brilliant Blue and periodic acid-Schiff reagent, indicating that all antigens are glycoproteins.

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that of CEA. The precipitate lines of both NFA-2 and CEA spurred over the line of NFA-1 or fecal NCA, but the line of NFA-1 crossed completely over that of fecal NCA. The same results were obtained with Go-α. These results indicate that, for antigenic determinants reactive with R-α or Go-α, NFA-2 possessed all the antigenic determinants of CEA and that each NFA-1 and fecal NCA had a part of the antigenic determinants of NFA-2 or CEA, but NFA-1 and fecal NCA were antigenically unrelated to each other.

As can be seen in Fig. 4a, however, AAP-1 showed a reaction only with CEA but not with NFA-2, whereas α-NFA-2 showed a precipitate line only with NFA-2 but not with CEA. These AAP-1 and α-NFA-2 antisera did not react with NFA-1, NFCA, fecal NCA, and lung NCA (Fig. 4, b and c). The results indicate that each CEA and NFA-2 possessed the antigenic determinant which is unique to each CEA or NFA-2 and were referred to tentatively as cancer determinant and NFA-2-distinctive determinant, respectively.

As reported previously, the cancer determinant is sensitive to proteolytic digestion with Pronase E (17). The NFA-2-distinctive determinant was found to be resistant to the same digestion with Pronase E, indicating that the cancer determinant and the NFA-2-distinctive determinant have different chemical characteristics.

After absorption with CEA, α-NCA reacted only with NCA but not with CEA, NFA-1, NFA-2, or NFCA. This indicates that, in addition to the determinant common to CEA and NFA-2 (NCA-common determinant), NCA possessed a unique determinant (NCA-distinctive determinant) which was present neither on CEA nor on other CEA-related antigens in feces. No antigenic differences have been observed between fecal NCA and NCA prepared from lungs.

As demonstrated in Fig. 5, the NFCA-containing fraction developed 2 precipitate lines with R-α or Go-α. One of them was the NFA-1 line contaminating this fraction, and the other line was due to NFCA proper which fused with the NCA line. Both of them coalesced with the CEA line or the NFA-2 line (Fig. 5a), indicating that NFCA and NFA-1 possessed independently a part of antigenic determinants of the CEA or NFA-2 molecule. After absorption with NCA, R-α no longer developed the precipitate line of NFCA proper; however, Go-α still developed 2 precipitate lines of both NFA-1 and NFCA proper (Fig. 5b). Thus, it appears evident that antigenic determinants of NFCA can be divided into at least 2 moieties; one is common to NCA (NCA-common determinant), and the other is common to CEA or NFA-2 but unrelated to NFA-1, and detectable only with Go-α absorbed with NCA (NFCA determinant).

The conclusion of these observations is summarized in Chart 4 with particular reference to the antigenic determinants of these CEA-related molecules in normal feces.

**DISCUSSION**

Although CEA-related antigens in feces other than fecal NCA have been called inclusively NFA, it was found that there are 3 other molecular species of CEA-related antigens in feces, designated herein as NFA-1, NFA-2, and NFCA, respectively. These 4 CEA-related antigens were consistently found in feces of all individuals thus far analyzed, although some fluctuation in the contents of each antigen was observed.

NFA-1 which was antigenically unrelated to NCA had a molecular weight of 20,000 to 30,000 and seems to be the smallest CEA-related antigen reported thus far. In spite of its low molecular weight, NFA-1 showed a quite strong reactivity with R-α. The antigenic determinant corresponding to the NFA-1 (NFA-1 determinant) appears to be the most dominant immunogenic group among several antigenic moieties on the CEA molecule (see below) because the amount of antibody reactive with the NFA-1 was usually largest in conventional rabbit anti-CEA antiserum. It remains to be determined whether or not NFA-1 has a unique determinant.

NFA-2 was the largest antigen in feces, with a molecular weight (160,000 to 170,000) similar to that of the CEA molecule from tumor tissues. Amino acid and carbohydrate compositions of NFA-2 were also similar to those of CEA. Antigenically, as far as the reactivity with R-α or Go-α is concerned, NFA-2 was indistinguishable from CEA. As can be seen in Fig. 4, however, the CEA molecule had a unique determinant which was detectable by specially prepared guinea pig anti-CEA antiserum (18) in almost all CEA preparations from tumor tissues with a very few exceptions (15) and was designated tentatively as cancer determinant though the cancer specificity of the unique determinant is not conclusive yet. On the other hand, NFA-2 possessed a unique determinant (NFA-2-distinctive determinant) which was detectable by α-NFA-2 in all NFA-2 preparations obtained from individuals of various blood types. This determinant was present neither in CEA nor in other CEA-related antigens. The differences in sensitivity to proteolytic digestion with Pronase E suggest that these antigenic determinants unique to each CEA and NFA-2 reside in different parts of antigen molecule with different chemical characteristics.

The origin of NFA-2 in the alimentary canal is still unclear. Fritsche and Mach (7) reported that normal colon mucosa contained small amounts of CEA (CEA-No) which were identical to CEA in tumor tissues. The CEA-No might be a progenitor molecule of the NFA-2 in normal feces. The antigenic differences between NFA-2 and CEA from tumor tissues mentioned above would be an expression of molecular changes of CEA-No resulting from exposure to enzymes and/or bacteria in the digestive tract. Alternatively, CEA-No would be identical to NFA-2, and some antigenic differences between CEA-No and tumor CEA would be detectable provided that a special anti-CEA antiserum such as AAP-1 is usable to these antigens.
Most of anti-CEA antisera prepared by conventional immunization procedures could not distinguish the CEA in tumor tissues from the NFA-2 in feces (15). Thus, the clarification of the antigenic differences among CEA in tumor tissues, CEA-Nor in normal colon mucosa, and NFA-2 in normal feces must be very important especially for the elucidation of cancer specificity problems of CEA, but more detailed antigenic analyses must be necessary for this purpose. Another similar antigen, NCW, in normal colon washings reported by Egan et al. (5, 21) is worthwhile to note when the origin of NFA-2 is considered, but it is unclear at present whether NCW is identical to CEA- No or to NFA-2. The source material for NCW must contain both peeled normal epithelium cells and some fecal substances. CEA-like substances in gastric juice (28) and BGP III in bile (23) also must be taken into consideration; they seem to be very similar to NFA-2, but antigenic differences between these antigens and NFA-2 are unclear at present. It also remains to be clarified whether NFA-2 differs antigenically from NCA-2 purified from meconium (3).

NFCA was not separated in pure form yet, but it is evident from immunodiffusion analyses that the antigenic determinants of this antigen can be divided into at least 2 moieties, one common to NCA and the other common to CEA or NFA-2 but unrelated to NFA-1. Gel filtration profiles suggested that NFCA has a molecular size similar to that of NCA. Thus, NFCA seems to be an antigen quite similar to the BGP I purified from human bile by Svenberg et al. (24), as judged from their molecular size and cross-reactivity with CEA or NCA. Although BGP I was reported to have an antigenic determinant unique to BGP I itself, it is not clear at present whether NFCA possesses a similar unique determinant or not.

As concerns fecal NCA, no differences from NCA in lungs or spleens were observed. It had a molecular weight of about 80,000 to 90,000 and had at least 2 parts of antigenic determinants, one unique for NCA itself and the other common to CEA, NFA-2, and NFCA.

Taken together the results mentioned, it is clear that CEA and NFA-2 can be divided into 4 antigenic moieties: (a) distinctive for CEA (cancer determinant) or NFA-2 (NFA-2-distinctive determinant); (b) corresponding to NFA-1 (NFA-1 determinant); (c) moiety retained on NFCA molecule and characteristic for NFCA (NFCA determinant); (d) moiety retained on NFCA molecule and common to NCA (NCA-common determinant) (Chart 4).

In a previous paper (17), Matsuoka et al. have reported that, by proteolytic digestion with Pronase E, 2 antigenic fragments (NFA fragment and NCA fragment) were released from the CEA molecule. Since the antigenicities of proteolytic fragments were compared with the mixture of fecal antigens in the previous study, the antigenic relations of the proteolytic fragments of CEA to the fecal antigens were reexamined in this study with each isolated preparation of the fecal antigens. It was found that NFA-2 was the analog of the intact CEA, NFA-1 revealed the same antigenicity as did the NFA fragment although there existed some difference in molecular size, and NFCA was analogous to the NCA fragment. Furthermore, in a preliminary experiment, it was observed that, by the same digestion with Pronase E, NFA-2 was also cleaved into 2 antigenic fragments corresponding to NFA-1 and NFCA each. Thus, it seems possible that NFA-1 and NFCA may be due to bacterial and/or enzymatic degradation of NFA-2 in the alimentary canal. There is little probability, however, that the degradation of NFA-2 resulted from perchloric acid extraction, since extracts of feces with 0.9% NaCl solution contained the same family of CEA-related antigens and 0.6 m perchloric acid gave no effect on purified NFA-2 preparation.

As already reported (16), fecal extracts from patients with colorectal carcinomas contained antigens similar to those in feces of normal adults. No antigenic differences between them were found thus far in crude fecal extracts. It may be possible, however, that the CEA molecules possessing the cancer determinant can be detected in feces from colorectal cancer patients after separation of the antigens as a rather pure form. Another possibility is that the CEA molecules released into the alimentary canal from tumor tissue be altered to the NFA-2 molecules by exposure to enzymes and/or bacteria. In order to clarify these possibilities and to assess the clinical significance of CEA, purification and characterization of the CEA-related antigens in feces from patients with colorectal carcinomas must be important and are now under way.

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Fig. 1. Immunoelectrophoresis of the crude and purified antigen preparations. Antigens applied to the wells were: tumor crude extract (T. Crude Ext.); purified CEA (CEA); lung crude extract (L. Crude Ext.); purified lung NCA (L-NCA); fecal crude extract (F. Crude Ext.); purified NFA-2 (NFA-2); purified NFA-1 (NFA-1); purified fecal NCA (F-NCA). Antisera applied to the troughs were: rabbit anti-tumor crude extract antiserum (ä-T. Crude Ext.); rabbit anti-lung crude extract antiserum (ä-L. Crude Ext.); rabbit anti-fecal crude extract antiserum (ä-F. Crude Ext.).

Fig. 2. SDS-PAGE (7.5% polyacrylamide, 0.6 x 10.0 cm) electrophoresis of the purified antigen preparations: a, CEA; b, NFA-2; c, NFA-1; d, lung NCA; e, fecal NCA. C1 and C2, control gels. C1 contained membrane polypeptides prepared from human RBC marker proteins: Band 1 (Spectrin I), 240,000; Band 2 (Spectrin II), 220,000; Band 3, 90,000. C2 contained bovine serum albumin (BSA), 68,000; human IgG (H-chain), 50,000; human IgG (L-chain), 23,500; lysozyme (hen egg white), 14,300. All gels were stained with Coomassie Brilliant Blue. The same results were obtained by using 10% polyacrylamide gels.

Fig. 3. Immunodiffusion analysis of CEA and fecal antigens. Antibody (center well) was a conventional rabbit anti-CEA antibody (R-ä). For descriptions of CEA, NFA-1, NFA-2, and fecal NCA, see Fig. 1.
Fig. 4. a, presence of a unique determinant on each CEA and NFA-2 molecule; b and c, absence of these determinants on other fecal antigens. AAP-1, specially prepared anti-CEA (see "Materials and Methods"); δ-NFA-2, rabbit anti-NFA-2 absorbed with CEA; δ-NFA-2 nonabs., nonabsorbed rabbit anti-NFA-2 serum; R-ά, conventional rabbit anti-CEA. Antigens applied were purified CEA, purified NFA-2, purified NFA-1, NFCA-containing fraction (NFCA Fr.), fecal NCA (F-NCA), and lung NCA (L-NCA). The precipitate line between AAP-1 and δ-NFA-2 nonabs. or between δ-NFA-2 and R-ά was due to the excess of absorbing antigen, indicating clearly that the remaining reactivities were not due to insufficient absorption.

Fig. 5. Reactions of the NFCA-containing fraction (NFCA Fr.) with R-ά (a) and with Go-ά absorbed with NCA (b). For descriptions of L-NCA, NFA-1, NFA-2, and CEA, see Fig. 4.
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