Immunohistological Localization of Carcinoembryonic Antigen and Nonspecific Cross-reacting Antigen in Gastrointestinal Normal and Tumoral Tissues

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SUMMARY

The localization of the carcinoembryonic antigen (CEA) of gastrointestinal tumors and of the nonspecific cross-reacting antigen (NCA) was studied by immunocytological techniques in various cancerous and noncancerous digestive tissues.

NCA was found having the same centroglandular localization than CEA, in cancerous and noncancerous glands of colonic and gastric mucosae. The only difference was that NCA was detected much more often on adjacent cellular membranes.

Various patterns were observed in gastric adenocarcinomas, the most striking being the intracytoplasmic localization of CEA and NCA in signet ring cells of linitis plastica and the absence of labeling of anaplastic areas.

In noncancerous gastric mucosae, which were either peri-tumoral or excised for a gastric or duodenal ulcer, CEA and NCA were found only in glands having undergone an intestinal metaplasia.

INTRODUCTION

Several antigens present in gastrointestinal tumors have been identified. The 1st one was named CEA1 by Gold and Freedman (6). We demonstrated its typical centroglandular localization in tumoral and noncancerous colonic mucosae (12). Then a 2nd antigen was isolated from colonic tumors and normal lung, which was shown to cross-react with the CEA (7, 13). For that reason, it was called NCA (13). In this paper we describe the localization of NCA in tissues and compare it to that of CEA. We studied in particular some gastric tumors and noncancerous gastric mucosae, which yielded quite different results than those obtained in colonic tissues.

MATERIALS AND METHODS

Samples. The following samples, all obtained at surgery, were examined: 29 gastric adenocarcinomas, among them 6 cases of linitis plastica (9) and 3 totally anaplastic carcinomas; several other tumors contained anaplastic areas; 13 noncancerous gastric mucosae, excised for gastric or duodenal ulcers, and 7 samples of peritumoral gastric mucosae; 17 colonic adenocarcinomas; 8 noncancerous colonic mucosae.

Reference Antigens and Antisera. Purified NCA and CEA were prepared according to the immunopercloiric extraction method recently described (2), using a potent sheep anti-CEA antiserum, which contained a great amount of antibodies that cross-reacted with NCA. In a 1st step a precipitate was made by mixing this antiserum with a suitable amount of lung perchloric extract, used as a source of NCA. The precipitate, after careful washing with PBS, was suspended in 2 M glycine buffer, pH 2.2, and extracted by an equal volume of 1.2 M perchloric acid. The supernatant obtained by centrifugation at 38,000 x g for 20 min was neutralized by 5 N NaOH, dialyzed against several changes of deionized water, and lyophilized. It was found to contain only NCA. In a 2nd step, the sheep anti-CEA antiserum, deprived by absorption with lung perchloric extract of all its antibodies able to cross-react with NCA, as checked by Ouchterlony method, was mixed with the perchloric extract of the hepatic metastasis of a colonic cancer. The supernatant obtained by centrifugation at 38,000 x g for 20 min was neutralized by 5 N NaOH, dialyzed against several changes of deionized water, and lyophilized. It was found to contain only CEA. In a 2nd step, the sheep anti-CEA antiserum, deprived by absorption with lung perchloric extract of all its antibodies able to cross-react with NCA, as checked by Ouchterlony method, was mixed with the perchloric extract of the hepatic metastasis of a colonic cancer. The precipitate was extracted as above. The resulting supernatant contained only CEA.

Rabbits were immunized against CEA or NCA. They received each first an intracutaneous injection of about 100 µg of antigen emulsified in complete Freund's adjuvant, then 2 weeks later a series of 3 injections of alum-precipitated antigen, each of 50 µg of antigen, the 1st one being made s.c. and the 2 others i.v. They were bled 5 days after the last injection.

Antisera, because of the purity of the immunizing antigen, were free of antibodies against other tissue components. They sometimes contained some antibodies against plasma proteins; this was explainable by the presence of otherwise undetectable traces of these plasma proteins in the apparently pure antigens. When necessary, these antibodies were absorbed out by adding 30 mg of lyophilized plasma to 1 ml of antiserum.

Because of the cross-reaction between CEA and NCA, it was necessary to absorb anti-CEA antiserum with pure NCA and anti-NCA antiserum with pure CEA. The absorption was carefully controlled by the Ouchterlony tech-
P. Bunin, S. von Kleist, M. C. Sabine, and M. King

...nique, a small excess of antigen being desirable in order to be sure that absorption would be complete. Amounts of absorbing antigen were about 200 μg/ml of antiserum.

A portion was taken from all the samples for routine histological examination. The other portion was snap frozen and cut in a cryostat at 5 μm. The sections were fixed in ethanol for 20 min and then rehydrated and stained as follows.

Usually indirect immunofluorescence was used. The 1st layer was either antiserum or normal rabbit serum. After careful washing in PBS the sections were dipped for 1 min in a sodium nitrite solution (1% in 2% acetic acid), as previously described (4), in order to minimize the nonspecific fixation of fluorescent globulins, and washed in 2% acetic acid and then in PBS. As the 2nd layer, a fluorescein-labeled anti-rabbit globulin sheep antiserum (Institut Pasteur, Paris, France) was used, after a dilution to 1/10 with PBS.

In some cases, direct fluorescence was used in order to compare the localization of CEA and NCA on the same slides. We thus used fluorescein-labeled anti-NCA and rhodamin-labeled anti-CEA globulins. The labeling techniques were those reported by Nairn (10). The lissamine rhodamin sulfochloride was prepared by Dr. Dang (Faculté de Pharmacie, Toulouse, France). In this case, sections were first dipped in the sodium nitrite bath, washed, and stained with 1 globulin and then with the other (the 1st one being either anti-CEA or anti-NCA).

Some experiments were made with the immunoperoxidase technique according to Avrameas (1). The 2nd layer was then a purified anti-rabbit IgG sheep antibody labeled with horseradish peroxidase Type VI (Sigma Chemical Co., St. Louis, Mo.). The incubation time was 20 min. After washing, the slide was dipped in 0.5% diaminobenzidine in Tris buffer, 0.1 M, pH 7.4, for 10 min. A counterstaining was made with either toluidine blue or hematoxylin.

Control experiments were made with antiserum absorbed with their specific antigens. Absorption was made by successive increments of purified antigen in order to avoid the formation of soluble complexes and was checked by the Ouchterlony technique.

Observations were made with a Leitz Orthoplan microscope equipped with a vertical illuminator. The excitation filters were generally BG 12 plus BG 38 plus a 490-nm interference filter coupled to a TK 495 dichroic mirror. As barrier filters we used the K 495 of the illuminator and a K 490 before the eyepiece.

When we examined sections stained by both anti-CEA rhodamin-labeled globulins and anti-NCA fluorescein-labeled globulins, we used first the same combination of filters as previously described in order to have a general look of the preparation. To select the fluorescence of fluorescein, we replaced the barrier filter K 490 by an S 520 filter. To select fluorescence of rhodamin we used as excitation filters a BG 38 plus 548 nm interference filter plus a 2-mm BG 36 filter and TK 580 dichroic mirror and, as barrier filters, K 580 of the illuminator and a K 610 before the eyepiece.

Fluorescence photographs were taken on Fuji color films. In the case of the immunoperoxidase technique, we used Kodachrome II films.

RESULTS

**Colonial Adenocarcinomas.** As previously described, the CEA was found on the border of lumen of the glands and in intraglandular deposits (12).

The NCA was seen having the same localization, except that adjacent cellular membranes were often also stained (Figs. 1 and 2).

In direct immunofluorescence experiments, the use of fluorescein-labeled anti-NCA globulins and rhodamin-labeled anti-CEA globulin showed that CEA and NCA were found on the border of the lumen of the same glands. Some areas of this border were of a yellowish color (a color between red and green); in others either red (for CEA) or green fluorescence (for NCA) was predominant.

**Noncancerous Colonial Mucosa.** The CEA was often found bordering the lumen of the noncancerous glands as previously described. The NCA gave the same pattern and was often characterized on adjacent cellular membranes.

**Gastric adenocarcinomas.** Almost all the samples studied reacted with both anti-CEA and NCA antisera and generally in an identical manner. The images that were observed varied greatly from one case to another and can be summarized as follows.

Typical centroglanular localization, as in colonial tumors (Figs. 3 and 8), was seen in well-differentiated carcinomas.

Membrane staining was observed on invasive cancer cells having lost their glandular organization and either being isolated or forming small nodules (Fig. 4). Sometimes these cells or nodules were surrounded by a weakly stained mucus. This pattern was observed in colloid adenocarcinomas.

Cytoplasmic localization was found characteristic for linitis plastica. The signet ring cells (8) showed strongly stained intracytoplasmic deposits (Figs. 5 to 7) either small sized and only in a part of the cytoplasm or large and sometimes pushing the nucleus to 1 pole of the cell (Fig. 5). Most of these deposits looked granular at high magnification, especially after peroxidase staining. A staining of the cell membrane was often associated with cytoplasmic localization (Fig. 5).

In many cases, these various images were seen in different areas of the same tumor, which were in a whole much more polymorphic than colonic carcinomas.

As previously mentioned, anti-NCA antiserum gave images almost identical with those observed with anti-CEA antiserum, the only difference being the staining by anti-NCA antiserum alone of intercellular walls of cancerous glands. Both antisera reacted identically with signet ring cells of linitis plastica. By direct immunofluorescence, it was possible to show that CEA and NCA could be present in the same signet ring cell.
Three samples of gastric adenocarcinoma gave negative results. All 3 were made only of isolated cells having lost most of the morphological characteristics of epithelial cells. In 1 case, the diagnosis of lymphosarcoma was discussed and rejected only because the cancerous cells were stained by PAS reagent.

Several other carcinomas had areas of undifferentiated cells which did not react with anti-CEA or anti-NCA antisera, in contrast with more differentiated areas of the same tumors which were positive. We thus observed a parallelism between the differentiation of the carcinoma and the presence of such antigens as CEA or NCA. A similar correlation could be seen between the presence of these antigens and the positivity of the PAS staining. However, this correlation was far from being absolute, as cells stained at least slightly by PAS reagent were found in areas that did not contain any cell able to react with anti-CEA and anti-NCA antisera. This can be explained by the higher sensitivity of the PAS method or, more likely, by the fact that components different from CEA and NCA were able to react with PAS.

Noncancerous Gastric Mucosa. The 1st striking feature was that in many cases gastric mucosa was negative for CEA and NCA, even in the close vicinity of malignant adenocarcinomas. This was a great difference with the patterns that we observed in colonic peritumoral mucosa (12). Yet some areas were positive with each of the antisera. In some specimens (5 of 13 noncancerous mucosae, 3 of 7 peritumoral mucosae), a typical centroglandular pattern was obtained, the intensity of the staining being sometimes weaker than in the colonic mucosae. These positive glands could be always identified as containing many goblet cells, thus showing a typical intestinal metaplasia, as seen by hematoxylin counterstaining after immunofluorescence or immunoperoxidase labeling. In Fig. 9 a typical field can be seen where in close vicinity positive metaplastic glands and negative normal gastric glands are found after staining by anti-CEA antiserum and fluorescent antiglobulins. Fig. 10 shows a section of gastric mucosa, where some glands of metaplastic type are stained by anti-NCA antiserum and then peroxidase-labeled antitubulin, the other normal glands in the same field being negative.

DISCUSSION

Until the present time, the localization of NCA was never studied extensively, with the exception of a preliminary report on colonic tumors (11). As a whole, it was found to be very similar to that of CEA. Both antigens were found at the apical pole of the cancerous cells, either from colon or stomach, as far as they kept their glandular organization, and in intraglandular deposits. Both were present in the noncancerous colonic mucosae, especially in that of the inflammatory type, in metaplastic gastric glands, and in signet ring cells of linitis plastica. The only difference was that NCA was more often found than CEA on the adjacent cellular membranes of gastric and colonic glands, cancerous or not.

In a given tissue, images obtained by labeling with anti-CEA and anti-NCA antisera often had a comparable intensity. This could mean that the quantities of both antigens were in the same range. However, such an interpretation is rather hypothetical, as it is difficult to draw quantitative conclusions from qualitative immunofluorescence data.

One of the most striking features that we reported here is the intracytoplasmic localization of the CEA that we observed, H. Denk and G. Tappeiner (personal communication), in signet ring cells characterizing the special type of gastric adenocarcinomas, called linitis plastica. These findings are strong evidence favoring the idea of CEA being synthesized in the cytoplasm. Usually this site of synthesis cannot be demonstrated, because CEA very likely leaves the cytoplasm as soon as it is synthesized and becomes concentrated on the membrane of the apical pole of the cell before being excreted into the lumen of the gland. In the case of linitis plastica the excretion of CEA seems to be lowered, which results in an accumulation of the antigen in the cytoplasm. Granular deposits may be related to the storage of CEA in distended ergastoplasmic cisternae or Golgi apparatus. The same is logically true for the synthesis of NCA.

The relationship between the synthesis of the CEA and the cellular differentiation is especially important in the gastric mucosa.

As for gastric cancers, there is much less CEA in differentiated carcinomas than in the differentiated ones. Anaplastic areas, in our experiments, always showed negative results with anti-CEA and anti-NCA antisera. Our findings were thus in agreement with those observed by Denk et al. (5), who found no CEA in the anaplastic gastrointestinal carcinomas. Our observations were also similar to the results that we have obtained in the study of the colonic polyps. The more differentiated were the glands of the polyps, the more intense was their reactivity with anti-CEA antiserum (2).

In the nontumoral gastric mucosa, only the glands that had undergone an intestinal metaplasia were found able to synthetize CEA. A similar observation was made independently by H. Denk and G. Tappeiner (personal communication). Normal gastric mucosa does not contain CEA detectable either by immunocytological methods or radioimmunoassay (8); hence, it may be used, rather than colonic mucosa, to absorb out contaminant antibodies of anti-CEA antisera. These findings can be correlated with the results of our unpublished observations on fetal gastric mucosa. CEA was most often absent from this fetal tissue. When present, it was only found in a few glands.

Cellular differentiation also plays an important role in the expression of the gene(s) controlling the synthesis of NCA. It is clear from our results that the same pattern of differentiation is required for the synthesis of NCA and CEA.
P. Burtin, S. von Kleist, M. C. Sabine, and M. King

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REFERENCES


Figs. 1 and 2. Longitudinal and transversal sections of colonic cancerous glands stained by anti-NCA antiserum and then fluorescent anti-rabbit globulin. × 400.

Fig. 3. Immunoperoxidase staining of gastric tumoral glands and isolated signet ring cells, labeled by anti-CEA antiserum. × 400.

Fig. 4. Immunoperoxidase staining of gastric tumor signet ring cells (the same as those on Fig. 3), labeled by anti-CEA antiserum. × 900.

Fig. 5. Immunofluorescence staining of a signet ring cell, labeled by anti-CEA antiserum. × 900.

Fig. 6. Immunofluorescence staining of a gastric cancerous nodule, labeled by anti-CEA antiserum. × 900.

Fig. 7. Immunoperoxidase staining of gastric signet ring cells, labeled by anti-NCA antiserum. × 900.

Fig. 8. Immunoperoxidase staining of a gastric tumoral gland (longitudinal section), labeled by anti-NCA antiserum. × 900.
Fig. 9. Immunofluorescence staining of glands in gastric mucosa having undergone an intestinal metaplasia, labeled by anti-CEA antiserum. The normal glands on the right are negative. × 400.

Fig. 10. Immunoperoxidase staining of a gastric mucosa labeled by anti-NCA antiserum. Only some glands were positive, which had undergone an intestinal metaplasia. × 100.
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