Comparative Immunoperoxidase Demonstration of T-Antigens in Human Colorectal Carcinomas and Morphologically Abnormal Mucosa

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ABSTRACT

The T-antigen (Thomsen-Friedenreich antigen) is a well-characterized tumor-associated glycoprotein that is immunologically reactive in humans. In order to demonstrate the presence of T-antigens in colorectal tissue, benign and malignant tissue from 46 patients with colorectal cancer were examined by means of an immunoperoxidase method. Peanut agglutinin and a polyclonal immune rabbit antiserum were used to demonstrate T-antigens on 72% of formalin-fixed malignant specimens and on more than 92% of frozen malignant specimens. Both ligands bound to the cell membrane and secreted mucus, but only the rabbit serum showed routine staining of the cytoplasm. The T-antigen distribution was heterogeneous without relation to degree of differentiation.

Transitional mucosa adjacent to malignant tissue showed a strong anti-T binding to secreted mucus. Slightly morphologically altered crypts remote from the carcinoma expressed T-antigens. Unexpectedly, both ligands bound to nerve cells of the enteric ganglia. These contain gangliosides with immunodominant oligosaccharides identical with those on the T-antigen. Therefore, cross-reactions might have occurred between the gangliosides and the used ligands. The T-antigens now seem to be present in various widespread cancers, and they probably occur early in malignant transformation.

INTRODUCTION

The T-antigen is presumed to be present in most human tissues, either substituted by other carbohydrates or by sialic acid residues only, as in the MN blood group antigens (21). The expression of the antigen in unsubstituted form has been demonstrated in cancers of epithelial and possibly neuroectodermal origin (22).

The T-antigen is immunologically reactive in contrast to onco-fetal antigens which are tolerated by the host. The T-antigenic determinant β→6-galactosyl-(1→3)-α→N-acetyl-d-galactosamine mounts a cellular immune response, which can be assessed by delayed hypersensitivity reactions to skin testing with an antigen prepared from RBC (22).

All humans possess naturally occurring circulating anti-T which probably reflect a continuous T-antigenic stimulation from the intestinal flora. These antibodies seem to interact with carcinoma cell surfaces and their shed components which results in a depression of anti-T hemagglutinin titer among patients with colorectal carcinomas (20); 42% of patients with gastrointestinal carcino-

In order to demonstrate T-antigens in tissues, either human anti-T or a lectin from peanuts (PNA) has been used as ligand (5, 10-14, 16). However, it seems that PNA does not bind to immunoreactive T-antigen exclusively, which has led to some controversy (21, 22). Until now, the emphasis has mainly been on breast and bladder cancer. Springer et al. (23) were not able to apply their method to colorectal carcinomas because contamination with T-antigenic bacteria and their products was difficult to rule out. Boland et al. (2) demonstrated alterations in mucin secreted from cancer cells or transitional mucosa compared to normal colonic cells. This altered mucin was able to bind PNA. In the present study, we describe the presence of T-antigens in colorectal carcinomas, in transitional mucosa, and in atypical mucosa remote from the carcinoma. Apart from being present in the transformed cells, T-antigens are demonstrated in the nerve plexuses of Auerbach and Meissner. The study was undertaken with both PNA and a rabbit polyclonal anti-T serum for comparison of their binding specificities.

MATERIALS AND METHODS

Patient Material. Colorectal tissue samples were obtained from 46 patients undergoing surgical resections for colorectal carcinomas. From each of 24 patients, one surgical specimen was taken from the proximal and one from the distal part of the primary tumor. These specimens were divided into halves; one half was frozen for cryostat sectioning, and the other was fixed in formaldehyde. In addition to this, a full-thickness specimen of normal colonic wall was taken as far from the tumor as possible. All specimens were obtained fresh from surgery. Five of the tumors were from cecum or ascending colon, and 19 from descending colon, sigmoid colon, or rectum. The distribution according to Dukes' system was: Stage A, 2; Stage B, 12; Stage C, 10. They were graded by 2 of the authors according to the method of Grinell (7), and in cases of disagreement, the higher grade was used: Grade I, 1; Grade II, 14; Grade III, 9. Five were colloid carcinomas. The distance from the mucosal samples to the tumor was from 4 to 26 cm with a mean of 15 cm.

Paraffin tissue blocks from 22 patients were selected from the pathology files of the Institute of Pathology, Aarhus Municipal Hospital, Aarhus, Denmark. These had been fixed in phosphate-buffered 4% formaldehyde, pH 7.2, and stored at room temperature in accordance with routine laboratory procedure. They were less than 3 years old. From each patient tissue from the primary carcinoma and the proximal resection edge was studied. Seven of these tumors were from the cecum or ascending colon, one from the transverse colon, and 18 from the sigmoid colon or rectum. (One patient had 3 tumors). According to Dukes, their distribution was: Stage A, 4; Stage B, 12; Stage C, 8. They were graded as mentioned above: Grade I, 4; Grade II, 10; Grade III, 10. The one tumor from the transverse colon was anaplastic and 4 were colloid carcinomas. The distance from the tumor to the proximal resection edge was from 6 to 40 cm with a mean of 17 cm.

Tissue Preparation. Unfixed samples for cryostat sectioning were frozen at ~140° C in precooled isopentane. The tissues were stored at...
--80° and cut at 4-μm thicknesses. Frozen sections were subjected to various fixatives at different temperatures and time conditions, including ethanol, acetone, formalin, and hydrogen peroxide containing methanol; 4% formaldehyde in phosphate buffer, pH 7.2, for 20 min at 20° proved to be optimal. The specimens used for embedding (1 x 1.5 cm) were subjected to this latter fixative for different time periods, varying from 6 hr to 8 days. Ideal fixation time was 24 hr, which was used routinely. After fixation, specimens were dehydrated in increasing concentrations of alcohol, cleared in xylene, and embedded in Paraplast (Sherwood Medical Industries, St. Louis, MO).

Immune Rabbit Anti-T Serum. The antiserum was prepared at Statens Seruminstitut (Copenhagen, Denmark) by means of immunizing rabbits with ON (Nero) RBC, T-exposed by treatment for 1 hr with Vibrio cholerae neuraminidase, 1 unit/ml (Behringwerke AG, Marburg, West Germany). Each rabbit received 2 ml T-exposed RBC i.p. at Day 1 and i.v. at Days 3, 5, and 7. Fourteen days after the last immunization, the animals were bled. The serum was then absorbed with ON (Nero) A and B RBC for 2 hr at 20° and for 20 hr at 4° and finally tested against a panel of RBC of known blood type.

PNA. This was prepared as described by Howard (9), or purchased as purified by affinity chromatography from E. Y. Laboratories (San Mateo, CA). The PNA preparations showed identical reactions to 9 frozen and 9 embedded specimens, whereupon PNA prepared at our laboratory was used routinely.

T-Absorption. Sera were absorbed with packed T-exposed RBC, v/v, rendering them free from anti-T activity after 2 absorptions. For further details, see Ref. 9.

Naturally Occurring Anti-T Antibodies. Anti-T antibodies probably occur naturally in all vertebrate sera, reflecting a continuous T-antigenic stimulation by the intestinal flora (21). The ability of normal rabbit serum and an immunoglobulin fraction of this serum (both from Dakopatts, Copenhagen, Denmark) to identify T-antigens in tissue sections was compared to that of RAAT. The normal rabbit serum and immunoglobulin fraction showed a more pronounced background staining but an identical specific staining. The anti-T titer of all sera used was determined by hemagglutination assay (24) and varied greatly in the batches examined. To avoid an uncontrolled decrease in staining sensitivity, T-absorbed normal swine serum was used for blocking nonspecific staining. At the stated dilutions (see below), rabbit anti-PNA serum (E. Y. Laboratories), in contrast to the peroxidase-conjugated swine anti-swine serum (Dakopatts), contained significant amounts of anti-T so that it exhibited positive staining reactions. This was abolished by T-absorption.

Immunohistological Methods. All specimens were stained with both RAAT and PNA by means of an indirect, peroxidase-labeled antibody procedure. The embedded specimens were cut at 6 μm and deparaffinized immediately before staining. All sections had endogenous peroxidase blocked by 3.5% hydrogen peroxide in methanol for 10 min. After washing, they were incubated for 15 min with normal swine serum diluted 1:5, which had been absorbed with T-exposed RBC. Optimal dilutions of reagents were assessed by checkerboard titrations. RAAT-treated sections were incubated with RAAT at a dilution of 1:40 for 1 hr. PNA-treated sections were incubated for 30 min with PNA at a protein concentration of 0.05 mg/ml, followed by washing and incubation for 30 min with rabbit anti-PNA (E. Y. Laboratories) diluted 1:30, which had been absorbed with T-exposed RBC. All sections were washed again and incubated for 30 min with swine anti-rabbit serum conjugated with peroxidase (Dakopatts) diluted 1:15. Sections were washed again and then incubated in 0.04% 3-amin-9-ethylcarbazole (Sigma Chemical Co., St. Louis, MO) with 0.01% hydrogen peroxide, washed in tap water, counterstained with Meyer's hematoxylin, washed again in tap water, and mounted in gelatin.

Washings and dilutions were performed with 0.5 M Tris-HCl in PBS (NaCl, 8.0 g; K2HPO4, 0.34 g; KH2PO4·3H2O, 1.58 g in 1000 ml H2O) 1:10, pH 7.6, unless otherwise indicated. The staining reaction was scored by 2 of the authors: 0, no staining; 1+, weak; 2+, moderate; 3+, marked staining.

Controls. Absorption controls of sera were performed with T-exposed RBC, PNA was absorbed with 0.3 M α-d-galactose (Sigma). The different steps in the immunoperoxidase procedure were omitted in turn in order to exclude the possibility of cross-reactions. Each staining procedure involved positive as well as negative controls. A few breast cancers were stained in order to compare our staining reactions with those found by others (10-12, 16).

RESULTS

Malignant Colorectal Tissue. The staining pattern was the same for frozen and Paraplast-embedded tissues with both ligands. Binding of RAAT showed a granular staining throughout the cytoplasm, with highest intensity along the glandular lumen. Also, the cell membrane was stained intensely (Figs. 1 to 3). In 40% of the positive specimens, there was a strong staining of the mucin secreted into the glandular lumens (Fig. 1). Binding of PNA showed a marked staining of the luminal cell membrane and in a few cells staining of the cytoplasm. In 90% of the positive specimens, the mucin secreted into the glandular lumens was stained (Figs. 4 and 5).

Tumors were counted as positive if more than 5% of the malignant cells were stained moderately or markedly, even if the rest of the cells were negative. In cases where 2 biopsies from the same tumor were examined, they were counted as positive if one or both were positive. The extent of staining within different categories of positive cells can be seen in Table 1. Of 19 negative tumors, 4 had doubtful, weak staining which could reflect a restricted number of antigens.

Staining for the T-antigen with PNA was positive in 72% of the examined 47 embedded specimens. RAAT also bound to 72% of these. We could not detect any correlation between T-antigen expression and grade of malignancy of these specimens (Table 2); neither was there any correlation with the tumor localization in the colon or rectum, the size of the tumor, or the patient’s sex.

To determine the influence of fixation and embedding on the T-antigen preservation, we examined fresh frozen tissue from 24 of the tumors; 92% of these bound PNA and 96% bound RAAT. This increased binding to frozen tissue might indicate a reduction of preserved T-antigenic binding sites. The detection of T-antigens by PNA or RAAT seemed to correlate well on both frozen and Paraplast-embedded tissues; 88% of the frozen tissues and 63% of the Paraplast-embedded tissues bound both ligands. All frozen malignant tissues and 88% of the Paraplast-
embedded tissues bound either PNA or RAAT (Table 3). The embedded specimens from the pathology files and the specimens fixed and embedded under standard conditions showed identical reactions.

There was a considerable intratumoral heterogeneity. In some tumors, all malignant cells in the specimen were stained, whereas in others areas with stained cells alternated with unstained. In some specimens we observed marked staining of a single cell in the border of cells surrounding a glandular lumen (Fig. 6). In order to estimate antigenic heterogeneity between different parts of the tumor, one biopsy from the proximal and one from the distal part of the tumor were taken from 22 patients. In one of four of the tumors, one biopsy was negative whereas the other was positive (Table 4).

Transitional Mucosa. In some sections, this epithelium immediately adjacent to the cancer was included. It was histologically normal or showed morphological abnormalities but did not meet the criteria for cancer (2). It showed intense staining with both PNA and RAAT of the secreted mucin (Fig. 7). Furthermore, RAAT stained the pericellular cytoplasm of some of the goblet cells. The staining was localized to both the upper and the lower crypt. PNA stained only the transitional mucosa when the carcinoma or its mucin was stained. In contrast to this, RAAT stained transitional mucosa in 7 cases, even if the carcinoma cells or their secreted mucin was unstained.

Mucosa Remote from the Carcinoma. Normal colonic epithelium did not bind any of the ligands. However, a few of the crypts were morphologically abnormal and showed some of the characteristics that have been described by Shamsuddin et al. (19): dilatation and distortion, overcrowning; lining with surface cells; and focal stratification. We found these alterations to be associated with the expression of T-antigens (Table 5; Fig. 8).

### Table 3

<table>
<thead>
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<th>Ligand</th>
<th>Paraplast-embedded tissue binding RAAT</th>
<th>Frozen tissue binding RAAT</th>
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<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
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<tr>
<td>PNA</td>
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### Table 4

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<th>No. of tumors examined</th>
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<td></td>
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<tr>
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<td>RAAT</td>
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<tr>
<td>Paraplast embedded</td>
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* Tumors where one biopsy was positive while the other was negative.

### Table 5

<table>
<thead>
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<th>Ligand</th>
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<tr>
<td>PNA</td>
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<tr>
<td>RAAT</td>
<td></td>
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<tr>
<td>Superficial crypt</td>
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<tr>
<td>Lower crypt</td>
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DISCUSSION

Our findings indicate that T-antigens are commonly expressed by carcinoma cells of the colon and rectum and not by the normal mucosa. Their distribution is heterogeneous and reflects a biological variation in the malignant cell population. This is concordant with the findings by others, as they show heterogeneous distribution of carcinoembryonal antigens among malignant cells (25), variation in glycoprotein content between clonal isolates from a single malignant transformed cell line (15), and tumor cell subpopulations with different DNA content (17). The heterogeneity between different tumor parts in one-fourth of the examined tumors makes it imperative to examine 2 or even more biopsies from these tumors before considering the tumor as not expressing T-antigens.

In breast cancer, there seems to be a reduction in T-antigenic expression among the most poorly differentiated carcinomas (10). We could not detect any such relation among colorectal carcinomas.

There is a great difference between the 2 ligands. PNA is a compactly folded tetrameric (M, 98,000) (6), and RAAT is composed of several antibodies directed towards the T-antigen and recognize different parts of this antigen. According to Springer et al. (22), vertebrate antibodies require a much lower sialylation in the vicinity of the immunodominant structure than does PNA when they are tested with agglutination inhibition. There was nevertheless a good correlation between the 2 ligands in the identification of T-antigens in malignant tissue (Table 3) with our method. A staining difference between them, apart from the ability to identify lesions far from the cancer, was the staining of the cell membrane and cytoplasm by RAAT compared to PNA that preferentially stained the membrane and secreted mucin but rarely stained the cytoplasm. This made staining with RAAT more evident and thus easier to grade.

It is well known that transitional mucosa adjacent to the carcinoma is abnormal compared to normal mucosa (2, 19). Our finding of T-antigen expression of secreted mucin and in the cytoplasm of some of the cells in this area confirms these findings. T-antigen expression in similarly altered solitary crypts located several cm proximal to the carcinoma in normal mucosa makes it unlikely that these alterations are local phenomena due to the presence of carcinoma. Both findings probably reflect the same biological phenomenon, as they are identical morphologically and histochemically. It has been proposed that these changes are premalignant (2, 19). If this is so, T-antigens occur early in malignant transformation and could be of potential value in the identification of patients at risk for developing colorectal cancer. RAAT was superior to PNA in terms of identifying these alterations.

Part of the colonic microbial flora is known to possess T-specificity (21) and should for this reason bind RAAT and PNA.
However, the fact that normal colonic epithelial cells and their mucus were consistently negative makes it unlikely that T-antigens of bacterial origin contribute to staining. Besides, malignant glandular formations located deep within the colonic wall are considered sterile as they do not communicate with the intestinal lumen.

We have not examined mucosa from patients without cancer. However, T-antigen expression was absent from histologically normal mucosa, as it was only present in mucosa containing morphologically abnormal crypts, and according to Shamssudin et al. (19) these abnormal crypts are absent in humans without colonic cancer.

The intense staining of the cell membrane with both ligands could be explained by the natural localization of the immunoreactive disaccharide in membrane glycoproteins expressing T-antigens (1). The cytoplasmic staining by RAAT could reflect an ability to identify T-antigens at an earlier step of their synthesis. Sialic acid, which substitutes the immunoreactive group of the T-antigen, could be present to a lesser extent because of reduced sialyltransferase activity or increased sialidase activity. However, reports on sialic acid metabolism are conflicting (27).

Alterations of membrane constituents have been shown to be due to neosynthesis of unique glycolipids and glycoproteins or a shift in synthesis from one series of glycolipids to another (8). Our finding of the binding of anti-T ligands to nerve cells of the enteric ganglia favors the hypothesis that gangliosides are complementary to our ligands. The immunoreactive disaccharide is not restricted to T-antigens; it is also expressed by some of the major gangliosides (GM1, GD1a). These are present in large amounts in nervous tissue, but they are not limited to this location as they are present in probably all cell types located in the cell membrane and other membrane systems (18). Thus, the staining of colon cancer cells could be explained by a ganglioside pattern that differs from that of normal cells. The microscopic heterogeneity of our staining would then be in agreement with findings by Murray et al. (15) that the ganglioside content and pattern varies between clonal isolates from one malignant transformed cell line.

With this report, T-antigens have been verified now in breast, bladder, and colorectal carcinomas. Their presence in some of the most widespread human cancers could have great clinical implications. At a histological level, T-antigens might be useful in the identification of premalignant changes. Serological assays of anti-T titer have shown correlation with disease burden in colorectal carcinomas (4). The use of T-antigens in immunolocalization is under current investigation (28).

REFERENCES

Figs. 1 and 2. Replicate sections from colon carcinoma. Immunoperoxidase staining for the T-antigen with RAAT before and after absorption with T-exposed RBC. Fig. 1 (before absorption) shows strong uniform staining of secreted mucin and tumor cell cytoplasm. Fig. 2 (after absorption) is negative. Hematoxylin counterstain, × 200.

Fig. 3. Increased magnification of Fig. 1 showing strong granular staining of cytoplasm, most intense along the lumen. Membranes are strongly positive. Hematoxylin counterstain, × 400.

Figs. 4 and 5. Immunoperoxidase demonstration of T-antigens in colon carcinoma using PNA. Fig. 4 shows strong staining of luminal cell membranes and secreted mucin. × 200. Fig. 5 shows additional staining of the cytoplasm of a single cell. × 400. Arrows, stained cell membranes and mucin. Hematoxylin counterstain.

Fig. 6. T-antigens demonstrated with RAAT immunoperoxidase staining. Marked heterogeneity with only a few positive cells (arrow). Hematoxylin counterstain, × 100.
Fig. 7. T-antigens demonstrated with PNA immunoperoxidase staining. Normal mucosa negative. Transitional mucosa shows staining of secreted mucin and part of the luminal cell membranes (upper arrow). Malignant tissue shows uniform staining of mucus and luminal membranes (lower arrow). Hematoxylin counterstain, x 100.

Fig. 8. Mucosa 12 cm from Grade II, Dukes’ Stage C rectal adenocarcinoma. Immunoperoxidase staining for the T-antigens with RAAT. The morphologically abnormal crypt shows strong staining of luminal cell membranes (arrow). Normal crypts are negative. Hematoxylin counterstain, x 200.

Fig. 9. Nerve cells from the plexus of Auerbach. T-antigens demonstrated with RAAT immunoperoxidase staining. There is marked staining of nerve cell cytoplasm. Insert shows lack of staining after absorption with T-exposed RBC. Hematoxylin counterstain, x 400.
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