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A method for T-antigen demonstration by a polyclonal antibody and peanut lectin; elimination of cross-reaction with naturally occurring antibodies*

Torben F. Örntoft
Institute of Cancer Research, Radiumstationen, DK-8000 Aarhus C, Denmark

Summary. Antisera and a lectin were used to demonstrate T-antigens in colorectal carcinomas. Rabbits were immunized with red blood cells on which the T-antigen had been exposed. The obtained polyclonal antiserum and a crude lectin (PNA) prepared from peanuts were used in an immunoperoxidase technique. The crude lectin and a chromatographic pure lectin showed the same staining specificity. Different fixatives, length of fixation and buffer compositions were tested on paraplast and frozen sections. Four per cent formaldehyde preserved the antigens well in paraplast embedded tissues when fixation was shorter than 48 h. In the immunoperoxidase technique the chromatogen used was 3-amino-9-ethyl-carbazole whose staining intensity was sensitive to the H_2O_2 concentration. Agglutination of T-exposed red blood cells was used to assess the anti-T titre of various sera. Normal animal serum contained anti-T antibodies, and the possibility of false positive reactions and methods to avoid it in immunohistochemistry is discussed.

Key words: T-antigen – Peanut lectin – Immunohistochemistry

The T-antigen, or Thomsen-Friedenreich's antigen, is present on normal human cell membranes and in certain glycolipids. It is part of the MN-blood-group antigen, substituted by N-acetyl-neuraminic acid, and therefore not immunologically reactive (Anstee 1981). In certain tumors this substitution is defect, and the T-antigen is potentially accessible to the immunne system. The immunoreactive structure in T-antigens is β-D-galactosyl-(1–3)α-N-acetyl-D-galactosamine linked to a protein backbone anchored in the plasma membrane (Bray et al. 1981; Springer et al. 1979). The substituted neu-

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Offprint requests to: T.F. Örntoft, Institute of Cancer Research, Aarhus Kommune Hospital, DK-8000 Aarhus C, Denmark

raminic acid can be removed and the cryptic T-antigen exposed by neuraminidase.

Various methods have been used to demonstrate T-antigens in malignant tissue of the gastrointestinal tract, urinary bladder, and breast. Springer and colleagues used hemagglutination inhibition with homogenized tumor tissue which made human serum unable to agglutinate neuraminidase treated red blood cells (RBC) (Springer et al. 1975). Immunohistochemical methods that apply either a lectin from peanuts, PNA, or an antibody from human serum have been used by others (Boland et al. 1982; Coon et al. 1982; Howard and Taylor 1979). PNA has a high affinity for the disaccharide in the T-antigen. However, its specificity has been questioned (Kania et al. 1980; Springer et al. 1982).

The purpose of the present study was to establish an optimal immunohistochemical method for the demonstration of T-antigens in human adenocarcinomas of the colon and the rectum. We describe the preparation of a polyclonal rabbit antiserum against T-antigens and an easy method to prepare PNA. The problem of cross-reaction with naturally occurring anti-T antibodies is evaluated.

Materials and methods

Tissues. Colorectal tissue samples from 46 patients were taken less than 30 min after surgical removal. Samples for cryostat sectioning were frozen at −140° C in precooled isopentane. The tissue was then stored at −80° C, and finally cut at 4 μ thickness. Samples for paraplast embedding were fixed in phosphate buffered 4% formaldehyde pH 7.2 for different time periods, stored in 70% ethanol until dehydrated in ascending alcohol concentrations, cleared in xylene, and embedded in paraplast (Sherwood Med. Ind. St. Louis, Miss.).

Immune rabbit anti-T serum (RAAT). The antiserum was prepared at Statens Seruminstitut (Copenhagen, Denmark) by means of immunizing rabbits with ON (Nero) RBC, T-exposed by treatment for 1 h with Vibrio Cholerae neuraminidase (Behringwerke AG, Marburg, FRG). 1 U per ml. Prior to use the serum was absorbed with ON (Nero), A and B RBC, and finally tested against a panel of RBC of known blood type.

PNA. This lectin was prepared from 250 g raw peanuts that were cleaned, homogenized with a blender at 20,000 revolutions per min, filtered through paper, and centrifuged at $10{,}000 \times g$ for 20 min (all procedures at 4° C). The supernatant was diluted 1:100 and tested for anti-T activity. PNA purified by affinity chromatography was purchased from E.Y. Lab. (San Mateo, CA).

Sera. The anti-T titre of sera was determined as the macroscopic hemagglutination titre by means of a 5% solution of T-exposed RBC at 20° C. Apart

from the rabbit anti-PNA serum, which was purchased from E.Y. Lab., all sera were purchased from Dakopatts (Copenhagen, Denmark).

Staining procedure. A standard indirect immunoperoxidase technique was applied to deparaffinized sections. RAAT and PNA were used as first layer. Rabbit anti-PNA serum was applied to PNA treated sections, and all reactions were visualized by swine anti-rabbit serum conjugated with peroxidase and reacted with 3-amino-9-ethyl-carbazole (Sigma Chem. Comp., St. Louis, Mo). Buffers tested were phosphate buffered saline (PBS) and TRIS containing PBS.

Results

Staining procedure

Temperature, salt, and hydrogen ion concentration are of importance for the interaction between ligand and receptor in this system; accordingly we varied these parameters.

Incubation with RAAT at 37° C for 1 h gave a weaker staining reaction than at 20° C. Incubation at 4° C for 24 h did not increase staining, neither quantitatively nor qualitatively. The buffer containing TRIS gave a significantly weaker background staining but not a weaker specific staining compared to PBS alone. The intensity of the staining was sensitive to alterations in hydrogen ion concentrations: with pH 7.6 the staining was optimal, but increased and decreased pH weakened the intensity radically.

We found the hydrogen peroxide concentration in the 3-amino-9-ethyl-carbazole solution to be an important factor that regulates the intensity of the staining reaction. PNA gave the strongest reaction with 5 µl hydrogen peroxide in the solution described above. RAAT gave the strongest reaction with 10 µl hydrogen peroxide.

Tissue preparation

Fixation of the tissue is of great importance for preservation of the antigen, and different methods are recommended (De Lellis et al. 1979; Sternberger 1979; Taylor 1978). In order to select the optimal fixative all the frozen sections were fixed in each of the following fixatives: 1) 1% H_2O_2 in absolute methanol, 20 min at 20° C; 2) 100% ethanol, 10 s at 20° C; 3) 100% ethanol, 10 min at 4° C; 4) 100% acetone, 10 min at 4° C; 5) 4% formaldehyde in phosphate buffer pH 7.2, 20 min at 20° C. For comparison an unfixed set of tissue sections was also included.

Tissue binding of PNA was optimal (high specific staining intensity, low background staining, good structural preservation) when formaldehyde or acetone was used. RAAT binding was optimal with the formaldehyde or methanol solution. The unfixed set of tissue showed the same antigen distribution, but there was diffusion of antigens throughout the section. Formaldehyde was chosen as standard fixative for both ligands. In order

Table 1. Optimal length of fixation (h) in 4% buffered formaldehyde for the immunoperoxidase demonstration of T-antigens in colorectal carcinomas

	Ligands	
	PNA	RAAT
Patient		
1	12–96	6–24
2	24	24–96
3	24–96	24–48
4	24–96	24–48

Table 2. Hemagglutination titre[a] of unabsorbed[b] sera and PNA against T-exposed human RBC[c]

	Titre
Normal rabbit serum	64
Normal rabbit serum immunoglobulin fraction	16
Normal swine serum	32
Immune rabbit anti-T (RAAT)	256
Rabbit anti-PNA batch A)	64
Rabbit anti-PNA (batch B)	8
PNA[d] (peanut agglutinin) crude	4,096
PNA[e] chromatographic pure	512
NaCl 0.9%	0

[a] Serial twofold dilutions in 0.9% NaCl, read macroscopically after 20 min

[b] T-absorbed sera of PNA did not agglutinate T-exposed RBC, neither were they agglutinated by PNA absorbed with 0.3 M D + Galactose

[c] No sera, nor PNA, agglutinated not-exposed RBC from the same person

[d] Prepared as described in the text

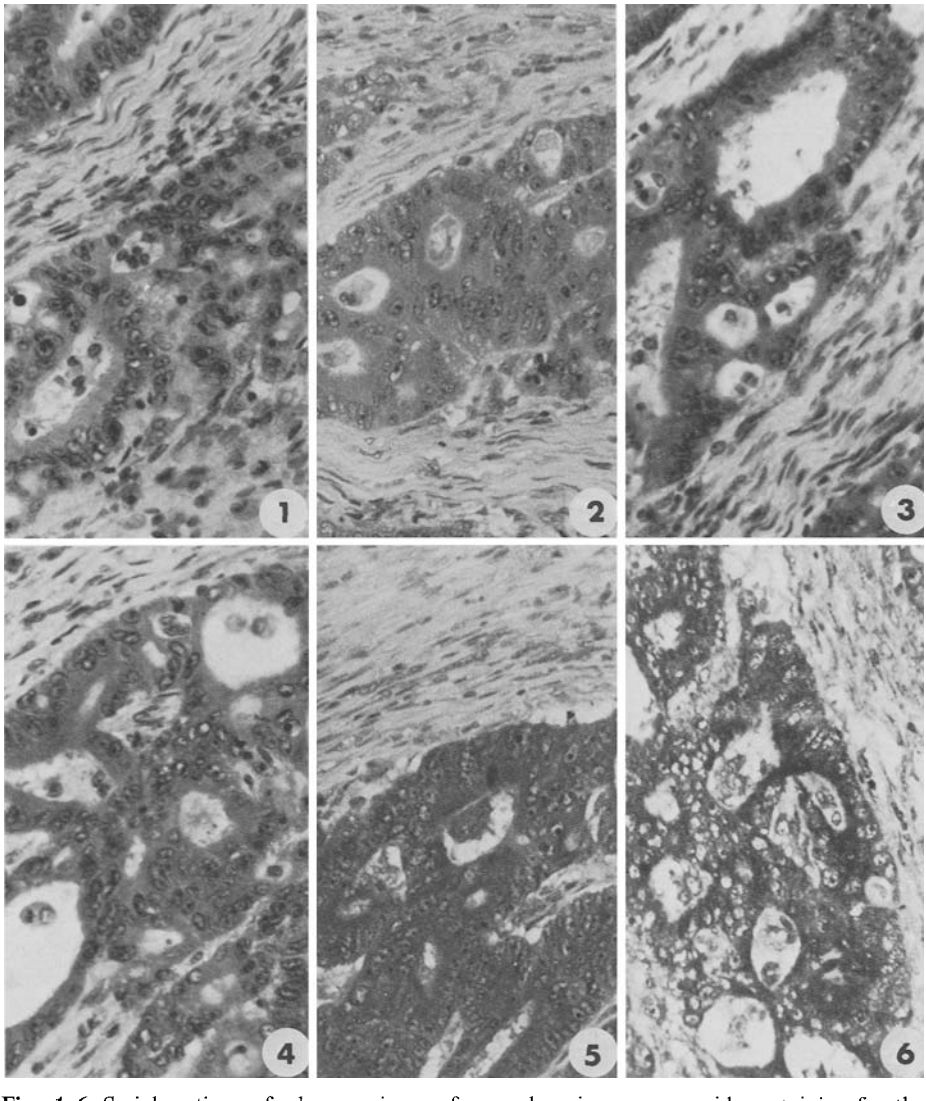
[e] Diluted to a protein concentration of 0.05 mg/ml

to establish the optimal fixation times for paraplast embedded tissues 4 big tumors were cut into 2–3 mm thick slices and fixed in the formaldehyde solution at room temperature for different time periods, from 6 h to 8 days (Table 1). Twenty-four hours proved ideal fixation time for both ligands, and an extension to more than 48 h led to gradual loss of staining intensity and reduction in the preserved number of binding sites. RAAT binding was more sensitive to the length of fixation than PNA.

When handling specimens of larger dimensions one has to compromise between loss of antigenecity at the periphery and insufficient fixation at the center. It is a well known fact that proteolytic enzymes increase sensitivity and reduce background staining on formalin fixed paraffin embedded material (Curran and Gregory 1977; Mepham et al. 1979). We did not find any benefit of incubating with trypsin.

Anti-T activity of sera and lectin

Two methods were used to assess the anti-T activity: 1) the hemagglutination titre with T-exposed RBC (Table 2); 2) immunoperoxidase staining of



Figs. 1–6. Serial sections of adenocarcinoma from colon; immunoperoxidase staining for the T-antigen with different ligands. Hematoxylin counterstain. **Fig. 1.** Swine anti-rabbit serum conjugated with peroxidase (diluted 1:15 in PBS) shows no staining of cytoplasm, membranes or secreted mucus. × 400

Fig. 2. Normal rabbit serum (diluted 1:10 in PBS); showing weak staining of cytoplasm and secreted mucus. × 400

Fig. 3. Normal rabbit serum, immunoglobulin fraction (diluted 1:10 in PBS) cytoplasm and mucus are stained more markedly than in Fig. 2. × 400

Fig. 4. Rabbit anti-PNA serum (diluted 1:30 in PBS). This is staining as the normal rabbit serum, immunoglobulin fraction. × 400

Fig. 5. Immune rabbit anti-T serum (diluted 1:50 in PBS). Marked staining of cytoplasm and secreted mucus. × 400

Fig. 6. Staining with PNA lectin. It shows marked staining of mucus and cytoplasm. × 400

serial tissue sections with the sera in increasing dilutions before and after T-absorption.

There was good correlation between the titre and the staining in the dilutional study. Before T-absorption identical structures were stained by all sera (Figs. 2–5). Normal swine serum was not tested by staining. Swine anti-rabbit serum conjugated with peroxidase as well as the T-absorbed sera did not show any staining (Fig. 1). PNA prepared as described in "Materials and methods" and the purchased PNA purified by affinity chromatography showed exactly the same staining in tissue sections from 14 different patients. In tissue sections from 4 patients the specific staining was the same, but the PNA prepared by our method showed a slightly increased background staining (Fig. 6).

Discussion

The increased use of immunoperoxidase techniques has made proper evaluation of methods essential. A comparison of results from different laboratories can lead to erroneous conclusions if the procedures that lead to positive or negative results are not thoroughly described.

Here we introduce the use of a polyclonal rabbit antiserum against T-antigens. Rabbit antibodies are often preferred in routine immunohistochemistry because they have broad precipitation curves and can be prepared in relative large numbers. A shortcoming of the method used is that the same T-antigen source is used for immunization and control of the immune serum. However, the T-antigen is defined as the structure exposed on human RBC treated with neuraminidase (Springer et al. 1979), and absorption with untreated RBC from the same person should ensure specificity. Furthermore, we could not detect any difference in agglutinating or staining properties between naturally occurring rabbit anti-T antibodies and the immune rabbit anti-T antibodies. There might be a difference in immunoglobulin composition, but this was not examined. In future studies absorption controls might be possible with the newly synthesized artificial T-hapten (Ratcliffe et al. 1981).

When performing the incubation at 37° C the reduction of staining intensity with RAAT might be caused by an instability of the immunoglobulin at this temperature. Others have found instability of human anti-T at temperatures above 32° C (Kania et al. 1980), or a heat sensitive fraction of human anti-T at 37° C (Kim 1980). Graham originally described the use of 3-amino-9-ethyl-carbazole in the demonstration of peroxidase activity (Graham et al. 1965). Our findings indicated that there was an optimal concentration of H_2O_2 for the oxidation of the carbazole compound by peroxidase. This optimum depended on which of the ligands we used to demonstrate the T-antigen. According to this we recommend titration of H_2O_2 in the carbazole solution in order to obtain the strongest staining intensity when applying immunoperoxidase technique to new antigen-antibody systems.

Anti-T antibodies are probably naturally occurring in all vertebrate sera

reflecting a continuous T-antigenic stimulation by the intestinal flora (Springer et al. 1979; Uhlenbruck et al. 1969; Uhlenbruck 1981). There was a great titer variation between the batches examined, which correlates with findings in humans of great interpersonal anti-T titer variation (Bray et al. 1982). We were able to demonstrate T-antigens on colorectal carcinomas by means of normal rabbit serum and an immune serum raised against T-exposed RBC. The immune serum with its higher titre could be diluted more in the staining procedure, which resulted in a minimal background staining. In the PNA procedure we used rabbit anti-PNA as second layer. If we omitted PNA and stained with rabbit anti-PNA as first layer we achieved a staining reaction identical with that obtained with normal rabbit serum and RAAT. This staining could be abolished by absorption with T-exposed RBC. To avoid this cross-reaction we had to T-absorb the rabbit anti-PNA serum before use. Our immunohistochemical procedure involved the use of normal swine serum to suppress nonspecific binding of immunoglobulins to the specimen. To avoid blocking of T-antigenic binding sites this serum was absorbed, too.

These findings should lead to great caution; not only when the distribution of T-antigens in tissues is studied but whenever a vertebrate serum is involved in immunohistochemistry. These techniques are sensitive, and careful controls are needed in order not to misinterpret T-anti-T reactions as positive when intending to study other antigen-antibody systems. Absorption with purified antigen is a recommended specificity control, but this absorption might be incomplete, or the antigen might contain blood group antigens or their precursors, as for example T-antigens. Carcino-embryonal-antigen has been shown to contain various blood group antigens (Mach et al. 1975).

We recommend a testing of sera applied in immunohistochemistry with T-exposed RBC. If they agglutinate these, staining of specimens before and after T-absorption is necessary so as to avoid false conclusions. Unfortunately the distribution of T-antigens in human tissues is not well known. So far they have been demonstrated in normal gastric mucosa (Kuhlmann et al. 1983), in structures where certain gangliosides occur, and in various cancers of epithelial and neuroectodermal origin (Springer et al. 1979; Springer et al. 1982).

Due to the association of T-antigens with various cancers the considerations of this paper gain particular importance for studies of malignant tissue.

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