Urol Res (1985) 13:47–50

Urological Research

Thomsen-Friedenreich Antigen in Bladder Tumors as Detected by Specific Antibody: A Possible Marker of Recurrence

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Accepted: July 11, 1984

Summary. TUR specimens of non-invasive transitional-cell carcinomas were examined for their expression of Thomsen-Friedenreich (T) antigen by indirect immunofluorescence staining using rabbit IgG antibody which was raised with desialated glycophorin. Nine (45%) out of 20 tumors of low grade (Grade I and II), and 5 (56%) of 9 tumors of high grade (III) were diffusely stained with anti-T (T-positive), whereas T-antigen in normal tissue was cryptic and stained only after neuraminidase treatment (cryptic T-positive). Of the T-negative tumors, 9 (45%) of the low grade but only one of the high grade tumors, were stained positively for the cryptic T-antigen. The rest of the tumors were devoid of the cryptic T-antigen. Eighty percent of both Tumors expressing T-antigen and those lacking the cryptic T-antigen recurred within two years. Recurrence was not influenced by initial histological grade.

Key words: T-antigen, Anti-T antibody, Urinary bladder cancer, Recurrence.

Introduction

The original observations by Springer and his collaborators [8, 9, 11] that Thomsen-Friedenreich antigen (T-antigen), non-sialated precursor disaccharide, D-galactose(β1-3)N-acetyl-D-galactosamine, is expressed by the majority of breast adenocarcinomas, whereas normal tissues generally carry fully sialated oligosaccharides in which the antigenicity of T-antigen is cryptic, has prompted subsequent investigations with carcinomas of other tissues. Evidence for expression of T-antigen by bladder carcinomas is being accumulated [2, 4, 13]. Whether such defect in sialyl transferation (positiveness in T-antigen expression) as well as in the precursor itself (negativeness in the cryptic T-antigen) is correlated with malignancy of bladder tumors is controversial [3]. Since only a lectin from *Arachis hypogaea* (peanut agglutinin, PNA) has been used for detection of T-antigen in these previous studies with bladder tumors, disparities among observations may have resulted from low avidity or degeneracy in the reactivity of PNA. In the present study antibody specific to the T-determinant was raised in rabbits and the IgG fraction was used for detecting T-antigen.

The results of examination with 29 cases of non-invasive, transitional-cell carcinomas of the urinary bladder were concordant with the previous observation with PNA by Coon et al. [2]. Tumors expressing T-antigen as well as those devoid of the cryptic T-antigen are likely to recur.

Materials and Method

Glycophorin (Gp) was isolated from pooled human erythrocyte ghosts of types O, NN, by the lithium diiodosalicylate-phenol method [5]. To obtain desialated Gp, which was used as T-antigen-bearing Gp (T-Gp) in this study, a solution of Gp at 1% in distilled water was adjusted at pH 2.0 with 1 N HCl and incubated for 12 h at 56 °C [10]. About 80% of sialic acid residues were removed by this treatment as judged by Warren's method [14]. The desialated material was applied to a column of PNA-conjugated gel (PNA-GEL, EY Laboratories) at 20 mg protein/5 ml column and, after being washed extensively to remove weakly bound material, the column was eluted with 0.2 M D-galactose in Tris-buffered saline (TBS) containing 0.2% Triton X-100 at pH 7.4.

Rabbits were given injections with 4 mg and then 1 mg of T-Gp Freund's complete adjuvant at an interval of one month, and antiserum was obtained 7–14 days after the second injection. The antiserum was absorbed with a mixture of fresh erythrocytes of blood groups A, B and 0, carrying also MM and NN. The completion of absorption was tested by the lack of hemagglutination of each blood group. The absorbed serum was then fractionated on a DEAE-cellulose column in 0.02 M sodium phosphate buffer, pH 7.2, to obtain the IgG fraction which gave a single precipitin line in gel with T-Gp but none with native Gp. This was used as anti-T.

Figure 1 illustrates the specificity of anti-T as tested by ELISA [1]. Antigen coated wells of microtitration plates and horseradish peroxidase-labelled guinea pig IgG antibody to Fab of rabbit IgG (HRPO-anti-Fab) were used for ELISA. The reaction with T-Gp predominated that with native Gp. When PNA (vector Laboratories) was added together with anti-T, the reaction of the latter with T-Gp

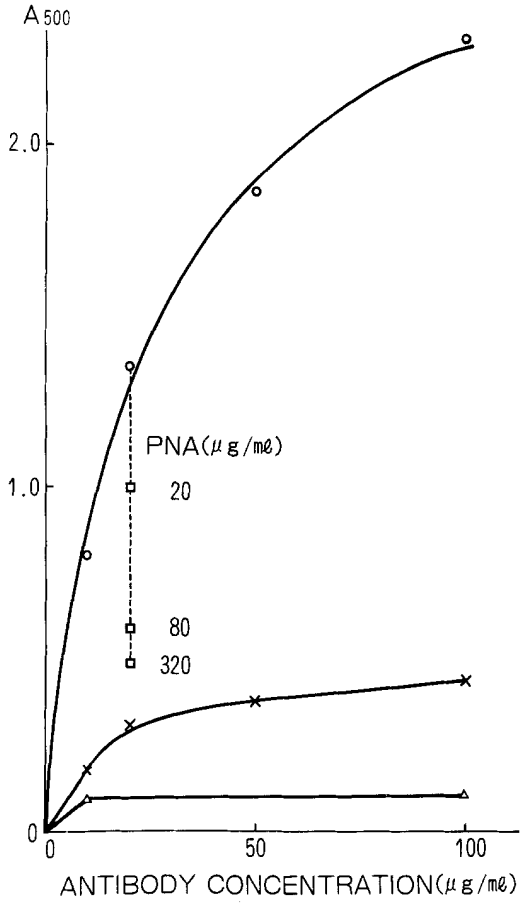

Fig. 1. Specific reactivity of anti-T in ELISA. Wells of a microtitration plate coated with either T-Gp or native Gp (10 μg/ml) were reacted with rabbit IgG anti-T at concentrations indicated, then with HRPO-labelled guinea pig IgG anti-Fab. ○—○: Reaction with T-Gp. x—x: Reaction with native Gp. □—□: Reaction with T-Gp in the presence of PNA at concentrations indicated (IgG anti-T, 20 μg/ml). △—△: Reaction with Gp in the presence of T-Gp at 500 μg/ml

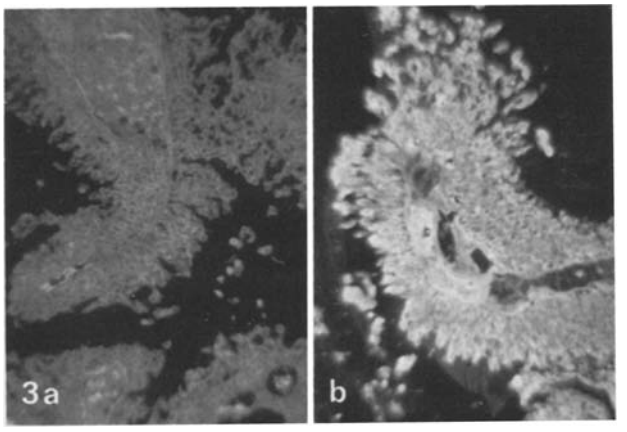

Fig. 2a, b. T-antigen positive staining of a grade II bladder tumor. a Background staining with normal rabbit serum and FITC-anti-Fab. b Staining with anti-T and FITC-anti-Fab without the neuraminidase pre-treatment. (x 200)

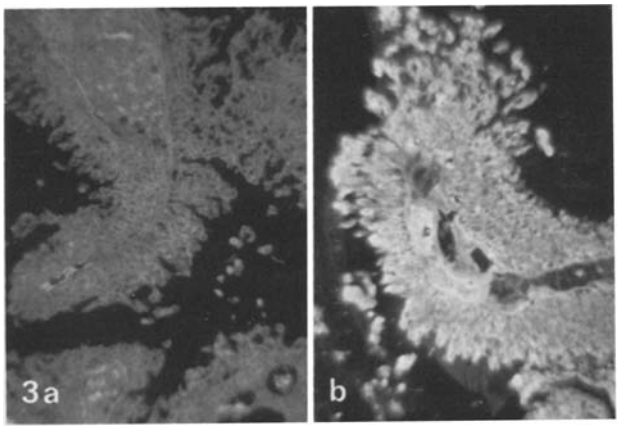

Fig. 3a, b. Cryptic T-antigen positive staining of a grade II bladder tumor. a Negative staining with anti-T and FITC-anti-Fab before neuraminidase treatment. b Staining with anti-T and FITC-anti-Fab after neuraminidase treatment. (x 200)

was inhibited in a PNA dose-responsive manner (Fig. 1). From the inhibition curve, a PNA concentration of 60 μg/ml was estimated to give a 50% inhibition against the reaction of anti-T at 20 μg/ml with respect to IgG.

TUR specimens were obtained from patients with noninvasive, transitional-cell carcinoma at stage 0 or A, including 8, 12 and 9 cases of grade I, II and III, respectively. These were fixed with neutral formalin and embedded in paraffin. After being deparaffinized, hydrated and washed thoroughly in a phosphate-buffered saline, pH 7.4 (PBS), sections were covered with 5 mg/ml of anti-T and incubated for 2 h at 37 °C then overnight at 4 °C. After being washed, the sections were reacted with fluorescein isothiocyanate (FITC)-labelled guinea pig IgG antibody to Fab of rabbit IgG (FITC-anti-Fab) for 3 h and then washed. These were suspended in 50% glycerol in PBS and examined with a fluorescence microscope. When specimens gave negative staining with anti-T, fresh sections were treated with neuraminidase (Type VIII, Sigma) at 0.2 units/ml in PBS, pH 6.5, for 1 h at 37 °C and subjected to the indirect immunofluorescence staining as above. Normal rabbit IgG which was absorbed with human erythrocytes was used for the background staining of each specimen.

The patients' clinical follow-up data during periods of two years or longer after the initial diagnosis were available.

Results

None of the normal tissue of urothelium, erythrocytes, lymphocytes and vascular endothelium so far examined were stained directly with anti-T, but all gave positive staining for the cryptic T-antigen after the neuraminidase treatment. Smooth muscle and connective tissue were not stained with anti-T even after the neruaminidase treatment.

Staining patterns of urothelial tumors with anti-T could be classified into the same three categories as those defined with PNA by Coon et al. [2], i.e. tumors which were direct-

Table 1. Expression of T-antigen or cryptic T-antigen and clinical fate of bladder tumors

Grade of tumor	T-antigen positive	Cryptic T-antigen	
		negative	positive
	(Recurrence; %)		
I	4 (3)	0 (0)	4 (1)
II	5 (4)	2 (2)	5 (1)
I, II	9; 45% (7)	2; 10% (2)	9; 45% (2)
III	5; 56% (4)	3; 33% (2)	1; 11% (0)
I, II, III	14 (11; 79%)	5 (4; 80%)	10 (2; 20%)

ly stained with anti-T without neuraminidase pretreatment (Fig. 2a, b), those stained only after neuraminidase treatment (Fig. 3a, b), and tumors which were not stained at all even after neuraminidase treatment (not shown). With most of the specimens expressing T-antigen, tumor masses were stained diffuesely and uniformly (Fig. 2b). In a few cases, the staining was focal, yet over 50% of the entire area were unambiguously stained. The positiveness or negativeness of staining after neuraminidase treatment was also uniform over whole areas of a given tumor specimen, provided that specimens were digested thoroughly with neuraminidase.

As summarized in Table 1, 40 to 50% of tumors of grade I and II, and 56% of high grade tumors (III), respectively, were T-antigen positive. In contrast to this similarity in frequency of T-antigen expression among tumors of various grades, T-antigen negative, cryptic T-antigen positive cases were more frequent in tumors of low grade (50%) than in those of high grade. Conversely, tumors which lacked the cryptic T-antigen appeared to increase as the histological grade increases.

On the average, 80% of patients whose tumors exhibited either positive T-antigen or negative cryptic T-antigen suffered from recurrence within two years, irrespective of the initial histological grading, whereas only 20% of patients with T-antigen negative, cryptic T-antigen positive tumors had recurrence (Table 1). The difference between these two groups was statistically significant ($p < 0.01$).

Discussion

By the use of specific antibody, a strong correlation was demonstrated between the expression of T-antigen or its complete deletion and the clinical prognosis of bladder carcinoma. The results are largely in agreement with the previous observations made with PNA [2, 4, 13]. However, the frequency of expression of T-antigen by low grade tumors (45%) was nearly comparable to that of high grade tumors (56%) and was much higher than that observed previously with PNA (28%) [2], although the status of cryptic T-antigen negativeness was more frequently associated with tumors of higher histological grades. Both T-antigen positive and cryptic T-antigen negative tumors subsequently proved to be highly recurrent (80%), irrespective of the histological grading at the time of TUR. Conversely, normal expression of the cryptic T-antigen was more frequently encountered in tumors of lower grades recurred at a much lower rate (20%) than tumors of the former two categories.

Most of the specimens examined in the present study were stained primarily along cell membranes and diffusely in an all-or-nothing fashion, although intracellular fine structures were not presently examined. This contrasted to the previous studies with PNA, by using either immunoperoxidase [2] or immunofluorescence [3] methods, where staining was often focal within individual tumors and a variability in the localization of PNA binding sites was noted among tumors, including tumors whose intracellular vesicles, rather than the cell membrane, were predominantly stained. Since PNA and IgG antibody, having molecular weights respectively of 110,000 and 150,000, are thought to be equally penetrable into cytoplasm and tissue matrices, this disparity may well be ascribed to the difference in avidity of these reagents.

In fact, as much as 60 μg of PNA were required to give a 50% inhibition of the reaction of T-Gp with 20 μg of the IgG fraction containing anti-T (Fig. 1). Considering that anti-T antibody constituted only 5–10% of the IgG fraction, as judged by the quantitative precipitin reaction with T-Gp (data not shown), the avidity of PNA for T-Gp is presumed to be at least one order of magnitude lower than that of the antibody used. This may make dissociation of the cell membrane bound PNA more rapid, depending on the density of T-antigen, whereas PNA inside the intracellular compartments or highly cross-linked matrices would still remain bound because of the restrained diffusion. Furthermore, it has been shown that the binding of PNA to the subterminal galactose residue or to the disaccharide core is less hindered by the presence of the terminal sialic acid than the binding of antibody is [6, 7, 12]. A comparative study with both PNA and anti-T is obviously needed to increase diagnostic value of T-antigen in bladder carcinomas.

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