Urol Res (1985) 13:117–121

Urological Research
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Preliminary Results on the Use of the Humoral Immune Response as a Serum Marker in Patients with Bladder Tumors*

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Accepted: February 27, 1985

Summary. An immunoassay using a human bladder tumor tissue culture cell line (J-82) as antigen and a modified avidin-biotin-complex (ABC) method were used to examine human serum samples. Optical density (OD) of 26 serum samples obtained from 19 patients with bladder tumors had a mean OD of 458 +/– 71 SD (328–570). This is significantly higher ($p < 0.0005$) when compared to the mean OD of 172 +/– 49 SD (107–301) found in serum samples from 28 patients with tumors other than bladder cancer and to the mean OD of 207 +/– 48 SD (136–310) of serum samples from 25 healthy donors. Thirty-one serum samples from 21 patients who had a bladder tumor surgically removed 1 to 36 months before were, with 2 exceptions, also higher than 320 (mean 412 [248–550]). All serum samples of patients with presence of a bladder tumor above the cut-off point of 320 and those from the 2 control groups below it shows, thus far, a high sensitivity as well as specificity of the method used and encourages further assessment of its potential usefulness as a serum marker assay in patients with bladder cancer.

Key words: Bladder cancer, Serum marker, Humoral immune response, Avidin-Biotin-Complex.

Circulating antibodies against various human malignant tumors, e.g., renal cell carcinoma [1], uterine cervix carcinoma [18], melanoma [11] or colon carcinoma [12], have been reported, as well as other serum factors which can modify cell-mediated immune reactions, such as tumor-associated antigens (TAA) shed by the tumor [3, 7], or antigen-antibody complexes [14].

In transitional cell carcinoma (TCC) of the human urinary bladder, membrane-associated antigens on tumor cells have been described [15]. Hakala and colleagues [8], and Troye and coworkers [17] found various amounts of circulating antibodies in some but not all sera of patients with TCC of the bladder when using the antibody-dependent lymphocyte-mediated cytotoxicity (ADCC) test on established TCC cell lines. The concept that ADCC can be induced by the donor's own antibodies is supported by findings from Akira and Takasugi [2].

Furthermore, Hansson and colleagues [9] reported that the ADCC was related to the tumor burden as well as to the tumor invasiveness in some but not all TCC patients.

There is an evident attempt to use the disease-related humoral immune response as a serum marker. However, the sensitivity or specificity of these as well as other procedures such as immunoadsorption, radio-immunoassays (RIA) and the peroxidase-antiperoxidase technique (PAP) were either unsatisfactory or technically too complicated to be used routinely for the detection or follow-up of most of the human cancers.

Hsu and coworkers [10] developed the avidin-biotin-complex (ABC) method. Unlike other indirect immunohistochemical methods, sensitivity is not only enhanced by an antigen-antibody reaction, but also by the particular affinity of the glycoprotein avidin (molecular weight 68,000) and biotin. Unlike antibody-antigen reactions, the binding of avidin to biotin is essentially irreversible. Amplification occurs because one antibody can be conjugated with several molecules of biotin and because avidin, used as a bridge between the biotinylated (second) antibody and a biotinylated enzyme (e.g., peroxidase), has 4 binding sites for biotin. By using this technique in our laboratory, together with a human tissue culture cell line serving as target cells and a water-soluble dye changing its color according to the peroxidase activity, a significantly higher optical density (OD) could be measured in sera from patients with renal cell carcinomas compared to sera from patients having cancer other than renal cell or the sera from healthy donors [5]. Experiments performed in an animal model for bladder

* Supported by the Blalock Foundation, USPHS Grant No. CA-16042, the Swiss Foundation for Medico-Biological Research and the Swiss Cancer League

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Table 1. Tumor stage of patient population studied

Stage	Active TCC	History of TCC
CIS	7	11
O/A	3	0
B_2	5	0
C–D	4	10
Total	19	21

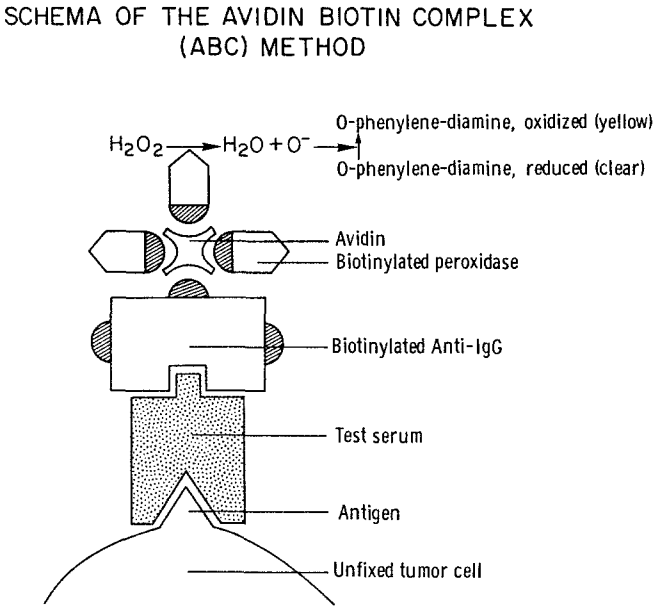

Fig. 1

cancer showed that the modified ABC method could be used for the diagnosis and follow-up of a specific cancer, with satisfactory sensitivity, specificity and reliability [16]. Based on these encouraging preliminary results, we investigated the basically same technique in patients with bladder tumors.

A serum marker in bladder cancer could be useful; eventually tumours could be classified according to their biological behaviour, which would reduce the unpredictability of superficial bladder tumors, or by making the staging more accurate. A serum marker might also be useful as a monitor of systemic treatments such as immuno- or chemotherapy.

Patients and Methods

According to a prospective study and after informed consent was obtained, 1 to 2 blood samples were collected (on different days) from 19 patients who had endoscopically diagnosed bladder tumors prior to treatment by surgery or chemotherapy (Table 1). Later, diagnosis was confirmed by histology; all but 3 had grade 3 lesions. Moreover, 31 serum samples (from 21 patients, 8 also in the former group) were collected 1 week, 1 month and/or thereafter in 3–6 months intervals following onset of treatment for histologically proven bladder tumor. Sera from 28 patients of comparable age and sex with carcinomas other than bladder (breast, lung, esophagus, melanoma, prostate, penis, testis or kidney) and sera from 25 healthy donors served as controls. Until analysis of the sera they were kept frozen at −70 °C.

Analysis of the sera was a follows: Monolayers of the human transitional cell carcinoma cell line J-82 [13] grown in tissue culture flasks with RPMI 1640 medium (Flow Laboratories) supplemented by 10% heat-inactivated fetal calf serum, were trypsinized (0.25%) for approximately 3 min, scrapped and mechanically dispersed (by pipette); 5×10^6 unfixed cells were placed in polyethylene microcentrifuge tubes and washed twice with 0.05 M TRIS buffer (pH 7.6). The latter procedure was repeated between each of the following steps:

0.1 ml of normal diluted goat serum (blocking serum, 45 µl/2.5 ml TRIS-buffer),

0.1 ml of 1:16 diluted test serum,

0.1 ml of biotinylated goat anti-human IgG (45 µl/10 ml TRIS buffer),

0.1 ml of diluted "Vectastain" (90 µl of avidin and 90 µl of biotinylated horseradish peroxidase in 10 ml TRIS buffer).

The cells are incubated with each of the reagents (Vector Laboratories, Burlingame, Ca 94010, USA) for 30 min at 37 °C. At each of the repeated intermediate cell-washings, the cells were resuspended (Vortex) in approximately 2 ml of TRIS-buffer, followed by centrifugation at 1,200 RPM for 5 min. The supernatant was aspirated.

After the last washing and centrifugation, 0.2 ml of the developer (60 mg of O-phenylene-diamine [Kodak] and 50 µl of H_2O_2 30% per 100 ml of 0.1 M phosphate buffer) were added to the pellet. After 10 min in the dark, the reaction was topped by adding 0.2 ml of 4 N H_2SO_4 and 2.5 ml of distilled water to each test tube. A schema of the method used is shown in Fig. 1. OD was measured by a spectrophotometer at a wavelength of 460 nm and the result multiplied by 1,000, in order to avoid using decimals. At each assay, 1 test was performed with 0.1 ml TRIS-buffer rather than test serum to adjust the spectrophotometer to zero (buffer control). OD values of the same serum differed little (< 5%) in the same assay and the proportion of the differences between any 2 given sera varied little from assay to assay. Greater differences (< 14%) between the absolute OD values of the same serum analyzed in different assays were found. Therefore, at each assay a control serum from a single healthy donor was used as a reference value and the other results were adjusted accordingly as follows. The test sera values were multiplied by the quotient:

$$\frac{\text{mean of the control serum values measured in all assays}}{\text{value of the control serum of the corresponding assay}}.$$

This enables the comparison of serum values analyzed in different assays.

For statistical comparison of the patients who had a bladder tumor with the other 2 control groups (Fig. 2), the Welch and Brown-Forsythe analysis of variance tests were used. For the estimation of sensitivity and specificity, 3 cut-points were chosen, two being the upper limits (95% confidence) of tolerance intervals [6] covering 90% and 95% of the normal population OD values. The third cut-point of 320 was chosen from visual comparison of the sample OD distributions for healthy donors and bladder cancer patients (Fig. 2).

Sensitivity is calculated as the proportion of bladder cancer patients in a (95% confidence) tolerance interval with the OD cut-point as a lower bounds.

Specificity is the proportion of healthy donors (95% confidence) with OD values below the OD cut-point. For these calculations, only the value of the first serum was used in patients having more than one serum sample. To evaluate the reliability of the assay, the average coefficient of variation (ratio of SD to mean) was calculated.

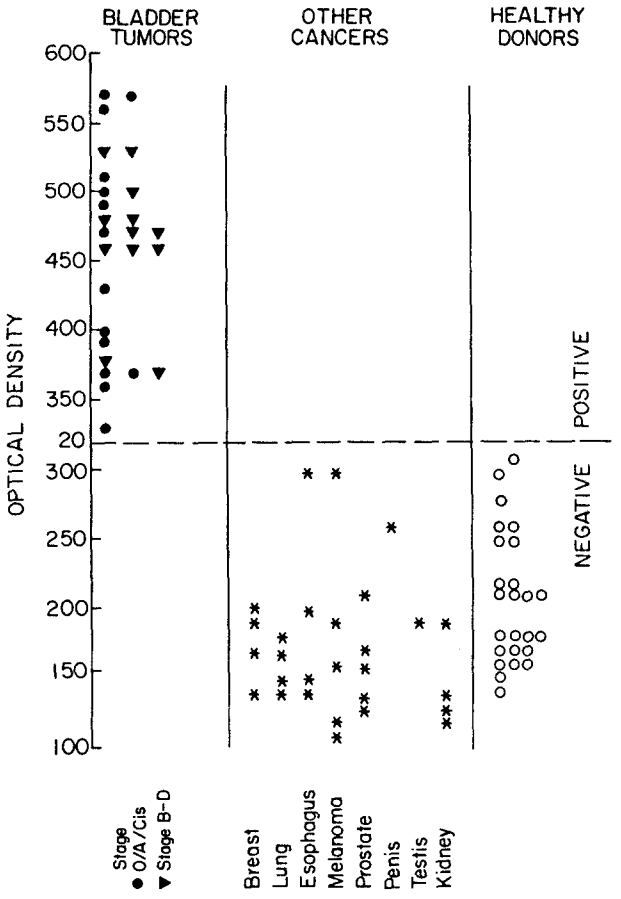

Fig. 2

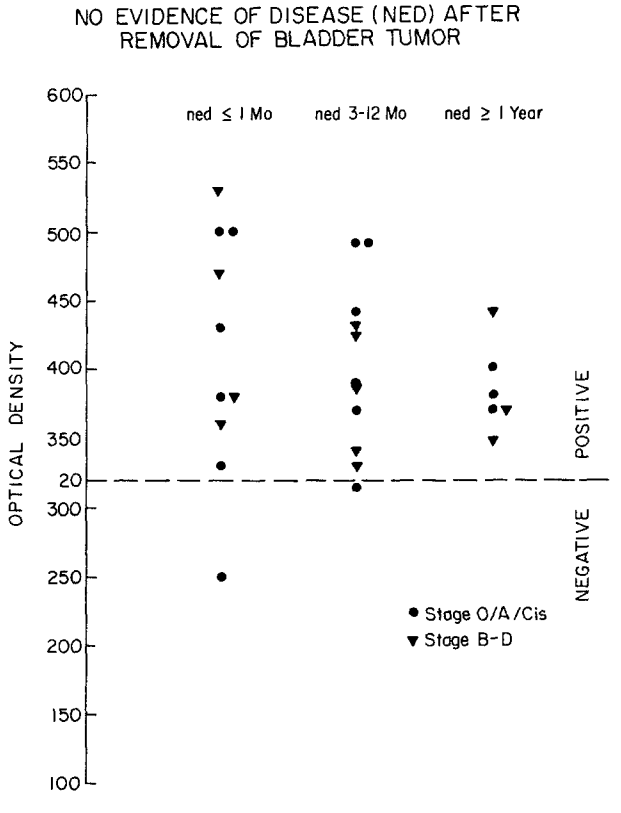

Fig. 3

Table 2. Calculated tolerance interval limits (95% confidence) for serum samples obtained from healthy donors and patients with bladder tumors

Population	Proportion covered	Tolerance interval limits (OD)	
		lower	upper
Patients with bladder tumors	90%	290	621
	95%	258	653
Healthy donors	90%	101	313[a]
	95%	81	333[a]

[a] Used as cutpoints for determination of sensitivity and specificity (Table 3)

Table 3. Sensitivity and specificity for 3 cutpoints, calculated on the basis of the tolerance interval limits shown in Table 2

Percent of healthy donors covered with 95% confidence	OD cutpoint	Estimated sensitivity	Estimated specificity
90%	313	91%	95%
92%	320	90%	96%
95%	333	89%	97.5%

Results

The mean value of ODs measured in sera of 19 patients who had a bladder tumor was 455 +/– 71 SD (range 328–570). The 7 repeated measurements (sera taken on different days) deriving from 5/19 patients were also within this range, i.e., all 25 sera samples had an OD higher than 320, with a mean OD of 458 (Fig. 2). Sera from 28 patients with tumors other than bladder cancer showed a mean OD of 172 +/– 49 SD (range 107–301), ie., no result above 320. The same applies for the sera deriving from 25 healthy donors which had a mean OD of 207 +/– 48 SD (range 136–310).

Thirty-one sera samples from 21 patients who had a bladder tumor macroscopically totally removed 1 to 36 months before serum sampling had a mean OD of 412 +/– 78 SD (248–550) (Fig. 3). An additional patient (not included in Fig. 3), who had undergone a radical cystectomy 14 years earlier and who had no signs of metastases and a normal urogram had a serum level of 302 (normal range).

The differences between the mean values of patients with bladder tumors compared to those with other carcinomas as well as to the healthy donors are highly significant ($p < 0.005$). The tolerance interval limits (95% confidence) for patients with bladder tumors and healthy donors are shown in Table 2. Sensitivity and specificity calculated for the 3 cut-off points are shown in Table 3. Seven serum samples (from different donors) were tested 2 to 5 times in

different assays; the calculated average coefficient of variation was 13%.

Six sera samples from bladder tumor patients who showed values higher than 320 when exposed to the bladder tumor cell line J-82 were indistinguishable from sera of patients with other carcinomas or from healthy donors when tested on a kidney cancer cell line (RC-Pa).

Discussion

Using an OD of 320 as a cut-off line, with all patients having bladder tumors above it, a sensitivity of 100% for the test could be calculated; combined with a specificity of 100%, since none of the serum samples deriving from 28 patients with tumors other than bladder cancer and from 25 healthy donors showed an OD higher than 320. However, since the number of examined patients is relatively small, sensitivity and specificity were calculated on the basis of the upper tolerance interval limits for the healthy donors (Table 2). This table also shows that some of the future serum samples from healthy donors are expected to be higher than the cut-off line of 320 shown in Fig. 2 and some patients with bladder tumors are expected to have values below that line. However, the rather high values for the upper limits of tolerance interval would be lower (and sensitivity as well as specificity shown in Table 3 consequently higher) if not only the results from the 25 healthy donors, but also those from the 28 patients with cancer other than bladder tumor had beed included in the calculations for the tolerance interval limits. Nevertheless, this was avoided since these 2 groups of patients were not identical statistically.

Because of the relatively low number of patients, it is too early to draw conclusions concerning correlations between the OD level and the tumor stage. While results in Figs. 2 and 3 actually suggest no difference between superficial and invasive tumors, it is interesting to see that all 7 patients with carcinoma in situ of the bladder had a positive result. No indication can be given concerning a relation between the OD level and the tumor grade since most of the patients examined had G3 lesions, the tumor form (papillary/sessile) or tumor burden. Furthermore, in future it will be interesting to see if the OD values of sera deriving from patients with transitional cell carcinoma elsewhere than in the bladder are likewise positive.

In patients who had previous bladder tumors, all but 2 serum values are above 320. Figure 3 suggests a fall with time; nevertheless, additional conclusions will be possible only when sufficient follow-up results of individual patients are available; moreover, it will be important to know if patients with persistently high serum values following treatment are at high risk for recurrence or if, after a radical cystectomy, thus justifying an adjuvant systemic treatment, they have an increased probability of developing metastases.

Basically the same method of serum analysis was used in animal experiments [16]. Sera samples from mice injected with living cells of a mouse bladder tumor cell line (MBT-2) have shown a correlation to the tumor burden. Also significantly higher levels were seen in animals with tumor recurrences than in those without. No significant increase was observed in the presence of purulent or granulomatous inflammatory reactions, or when carcinomas other than bladder tumors were present. The use of immune response modifiers (*Corynebacterium parvum* and/or irradiated tumor cells) which, when given alone caused increased serum values, did not falsify the results in the presence of a growing tumor. These findings support our preliminary results found in patients with bladder tumors as regards specificity of the immunoassay used. However, the influence of immunotherapy on test results in humans has not yet been studied.

Our results suggest that by using the modified ABC method and the bladder tumor cell line J-82, it might be possible to quantify the humoral immune response in patients with bladder carcinoma as described by Bubenik [4] 15 years ago. Further work is needed to simplify the assay and to determine its potential value as a serum marker.

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