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Studies on Urinary Isozymes of Lactic Dehydrogenase and β-Glucuronidase in Patients with Bladder Tumors

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Summary. Isozymes of urinary lactic dehydrogenase (LDH) were studied in 55 subjects, including 17 patients with bladder tumors. Normal clear urine from healthy persons showed little activity of LDH_5, but in 11 out of 17 patients with bladder tumors LDH_5 was increased sufficiently to invert the ratio of LDH_5/LDH_1, although the urine was not contaminated appreciably with leucocytes. Studies on tissue LDH isozymes in 16 tumor specimens strongly suggested that the increased LDH_5 in the urine of patients with bladder tumors originated from the tumors themselves. β-Glucuronidase (β-G) isozymes were studied in urine specimens from 10 normal subjects, and 10 patients with bladder tumors and in 5 specimens of normal epithelium and 5 of tumor tissue. Two or three distinct bands of β-G were separated from specimens of urine and tumor tissue from patients with bladder tumors, but only a single band was found in specimens from normal subjects.

Key words: Urinary isozymes, lactic dehydrogenase, β-glucuronidase, bladder cancer.

Introduction

Recently isozymes have become widely used in diagnosis and the serum of patients with malignant conditions have sometimes been found to show unusual isozyme patterns (6, 22). However, physical examinations are still the most important in establishing the diagnosis of malignant conditions and of suitable treatment, while laboratory data are only of relatively minor value. With recent developments in physical procedures it is now possible to assess details of the growth of tumors, sometimes to the order of millimeters. However, using physical procedures it is not possible to assess the character of tumor cells or their degree of malignancy, which are clearly important factors in prognosis. Studies on the isozymes of tumor cells should provide a more precise assessment of the nature of these cells, since isozymes furnish a direct visualization of gene products. An accurate method is urgently required to determine the degree of malignancy of bladder tumors, since it is known that even relatively non malignant tumors sometimes show heterotopic recurrence in a more malignant grade (7).

At present, after the initial treatment of bladder tumors, the course of therapy is monitored by endoscopic examination and by urinary cytodiagnosis. However, these methods are not entirely satisfactory, since they do not give quantitative results and often provide only subjective impressions. These disadvantages should be avoided by monitoring therapy with examination of urinary isozymes. To test this possibility we examined two enzymes in the urine of patients with bladder tumors: lactic dehydrogenase (LDH), the isozyme pattern of which has been extensively studied, and β-glucuronidase (β-G), which has been found to increase in the urine of patients with bladder tumors. We compared the isozymes of these enzymes in the urine and tissues of normal subjects and of patients with bladder tumors.

Materials and Methods

Urine specimens were obtained from 1): 10 healthy subjects, 2): 7 cases of mild hematuria due to pyelosinous backflow, 3): 9 cases of mild pyuria, 4): 8 cases of severe pyuria and 5): 17 cases of bladder tumor with negligible microscopical findings in the urine. All these cases appeared normal on routine clinical laboratory examination. In all specimens from benign cases of hematuria more than 30 erythrocytes were seen in one high

power field and the Hb content was more than 30 mg/dl. In specimens from the 9 cases of mild pyuria less than 15 leucocytes were seen in one high power field and no bacterial growth was observed on culture. In the specimens from the 8 cases of severe pyuria more than 50 leucocytes were seen in one high power field and bacterial contamination was detected. In these specimens more than 98% of the leucocytes were neutrophils and scarcely any thrombocytes were detected. Urine specimens were not treated in any way before examination and were usually collected early in the morning without using a catheter.

Urine specimens which contained in the sediment more than 15 leucocytes or erythrocytes in a highpower field were excluded from the study on the patients with bladder cancer. Urine specimens were centrifuged at 1 000 r.p.m. for 10 min. and then the precipitate was examined microscopically and tested for bacterial contamination. A urinary content of leucocytes and erythrocytes were counted according to Addis method and presented as the number of leucocytes or ethrocytes per mm^3 urine. Then the supernatant of urine specimens was dialysed in a cellophane membrane against running tap water for 2 hours and concentrated 30-fold to a volume of 1 ml using a collodion described below. The specimens were then subjected to electrophoresis as described below. LDH and β-G activities were assayed on fresh, undialysed urine within 1 hour of collection.

The urinary LDH activity was measured by the spectrophotometric method using LDH-UV-Test from Boehringer-Mannheim Co. Ltd. and defined in terms of Wroblewski unit per milligram of urinary creatinine. The urinary β-glucuronidase activity was measured by a modification of the procedure of Talalay (22) using p-nitrophenyl- β-glucuronide instead of phenolphthalein- β-glucuronide as substrate. Units of β-glucuronidase were expressed as a numbers of 250 times of μg of phenolphthalein released per milligram of urinary creatinine.

All patients with bladder tumors were treated surgically and the tumors were confirmed not to be more advanced than stage T_2 of the TNM classification. In all, 23 tissue specimens were studied: 7 of normal bladder epithelium and 16 of bladder tumors. The normal epithelium used was the mucosa of the bladder neck, which was obtained surgically from patients with prostatic hypertrophy and confirmed histologically not to show neoplastic changes. Tissue homogenates were prepared as follows: fresh superficial portions of tumors were excised, rinsed throughly with physiological saline, blotted and weighed. Then the tissue was homogenised in 10 times its weight of physiologic saline in a potter-Ekvehjen teflon pestle at 500 r.p.m. for 10 min at 4°C. The homogenate was centrifuged at 10 000 r.p.m. for 15 min at 4°C and the supernatant was subjected to electrophoresis. In addition to these studies on urine and homogenate, the serum LDH isozyme patterns were investigated in 29 patients with the bladder cancer. All of them were divided by their clinical staging according to the TNM classification.

Electrophoresis was performed at a constant current at 4°C using three kinds of supporting medium: agar gel, cellulose acetate membranes and polyacrylamide gel (3). Agar gel electrophoresis was carried out at 10mA for 45 min in barbital buffer of μ-0.04 at pH 8.4. Electrophoresis on cellulose acetate was carried out at 1.5 mA for 45 min in barbital buffer of μ-0.06 at pH 8.6. Disc electrophoresis was carried out according to Davis' original technique at 2 mA for 120 min. in

Table 1. Urinary LDH and β-G activities and LDH isozyme patterns in various conditions

Condition	No. of Cases	β-G Activities (u./mg creatinine)	LDH Activities (u./mg creatinine)	% LDH isozyme distribution I	II	III	IV	V
Normal	10	10.38 ± 4.90	0.25 ± 0.19	45.40 ± 12.10	34.60 ± 6.10	12.50 ± 10.90	3.70 ± 5.80	1.30 ± 1.30
Hematuria[a]	7	10.82 ± 3.01	0.25 ± 0.09	40.40 ± 6.60	33.70 ± 4.70	16.90 ± 6.40	2.20 ± 1.40	3.30 ± 3.20
Pyuria Mild	9	12.20 ± 7.42	0.26 ± 0.17	40.80 ± 11.60	29.20 ± 5.70	14.20 ± 3.80	7.00 ± 7.60	8.80 ± 7.30
Pyuria Severe	8	31.69 ± 23.34	1.99 ± 0.94	17.40 ± 8.20	16.90 ± 4.50	22.20 ± 6.10	15.20 ± 5.70	27.80 ± 7.00
Bladder Tumor	17	20.39 ± 9.72	0.92 ± 0.71	18.80 ± 11.40	18.30 ± 8.40	20.00 ± 8.60	16.40 ± 7.30	26.50 ± 12.20

Values are means ± standard errors

[a] Hematuria due to essential renal bleeding
[b] Pyuria due to non-specific infection of the urinary tract

Table 2. Serum LDH isozyme patterns of patients with bladder tumors

Clinical Stage	No. of Cases	% LDH isozyme distribution I	II	III	IV	V
T_1	11	34.1 ± 7.3	35.1 ± 2.2	25.6 ± 7.5	1.2 ± 1.7	3.5 ± 4.1
T_2	5	30.7 ± 8.3	28.2 ± 6.9	25.2 ± 3.8	5.9 ± 6.8	9.6 ± 7.9
T_3	8	23.4 ± 9.9	33.0 ± 6.8	30.5 ± 5.5	5.4 ± 3.8	7.8 ± 7.4
T_4	5	30.7 ± 5.3	30.5 ± 5.6	20.0 ± 8.5	9.1 ± 3.2	13.2 ± 2.2

Values are means ± Standard errors

Table 3. Urinary LDH isozyme patterns of patients with bladder tumors

Case No.	Urinary contents (/mm^3) leucocytes	erythrocytes	% LDH isozyme distribution I	II	III	IV	V
1	<50	<25	21.4	32.2	28.5	8.1	9.8
2	< 2	>50	29.6	29.0	20.9	8.9	11.5
3	< 2	< 2	27.3	21.0	18.1	20.2	13.5
4	<50	< 2	9.1	15.7	19.2	22.9	33.0
5	<25	< 2	10.6	11.4	33.8	10.1	32.5
6	< 2	< 2	46.7	30.9	3.1	0.2	19.1
7	< 2	< 2	18.2	15.9	10.8	22.7	32.6
8	<50	<25	20.1	20.9	20.9	18.1	20.1
9	< 2	< 2	18.5	15.6	30.0	17.4	18.5
10	<50	<25	16.4	11.2	13.5	25.2	33.6
11	< 2	< 2	36.3	26.4	12.2	9.9	15.0
12	< 2	< 2	9.3	7.3	10.4	20.0	53.3
13	< 2	< 2	27.0	19.6	18.0	13.7	21.3
14	<50	< 2	11.1	19.7	31.7	14.7	22.9
15	<50	< 2	10.6	11.0	12.5	21.9	44.0
16	< 2	< 2	8.0	23.0	30.3	13.3	25.4
17	<50	<50	0	0	25.3	30.6	44.1

Table 4. The LDH isozyme patterns of bladder tumor tissues

Specimen No.	Histological Grade	Stage	% LDH isozyme distribution I	II	III	IV	V
1	II	T_2	10.2	14.4	18.6	31.3	24.7
2	III	T_3	1.6	14.4	23.6	26.7	33.7
3	III	T_3	19.5	18.2	19.2	20.7	21.9
4	III	T_2	16.3	19.9	18.3	23.1	22.2
5	II	T_2	4.1	15.4	23.2	28.1	28.8
6	II	T_2	4.2	17.6	19.5	26.4	32.1
7	I	T_1	9.6	12.7	20.5	24.8	32.3
8	II	T_1	23.0	17.6	17.1	20.1	21.9
9	II	T_1	7.1	15.3	22.9	24.5	30.3
10	II	T_2	23.8	23.4	24.0	18.9	9.5
11	Adenocarc.	T_4	1.6	11.6	17.4	27.5	41.9
12	III	T_3	6.7	18.0	19.1	24.4	31.8
13	I	T_1	8.2	20.0	31.3	29.7	10.8
14	I	T_1	6.4	16.3	34.4	36.1	2.1
15	I	T_1	0	21.4	29.3	23.2	26.1
16	II	T_2	11.4	2.8	29.7	50.0	6.1
Control[a]	Normal Epithelium		28.2 ± 16.2	32.3 ± 6.5	32.8 ± 15.7	4.7 ± 8.3	2.5 ± 3.3

a Average from normal bladder epithelium of 8 cases

Fig. 1. LDH isozyme patterns of urine from a normal subject on the left and clear urine from a patient with bladder cancer on the right obtained by agar gel electrophoresis.
(The fastest migrating anodal band is numbered LDH_1 and numbering is continued in a cathodal direction)

Fig. 2. Tissue LDH isozyme patterns of normal urinary bladder epithelium on the left and of a urinary bladder cancer on the right obtained by agar gel electrophoresis

Fig. 3. β-G isozyme patterns of urine from normal subjects on the left and clear urine from a patient with bladder cancer on the right obtained by cellulose acetate electrophoresis

Fig. 4. β-G isozyme patterns of urine from normal subjects on the left and clear urine from patients with bladder cancer on the right obtained by disc electrophoresis.

Fig. 5. Tissue β-G isozyme patterns of a normal urinary bladder epithelium on the left and of a urinary bladder cancer on the right obtained by agar gel electrophoresis

Fig. 6. Tissue β-G isozyme patterns of normal urinary bladder epithelium on the left and of a urinary bladder cancer on the right obtained by disc electrophoresis.

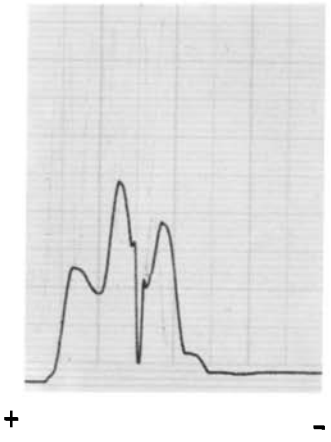

Fig. 1

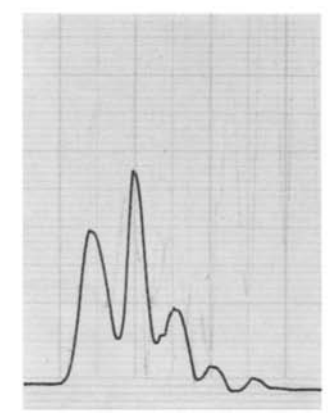

Fig. 2

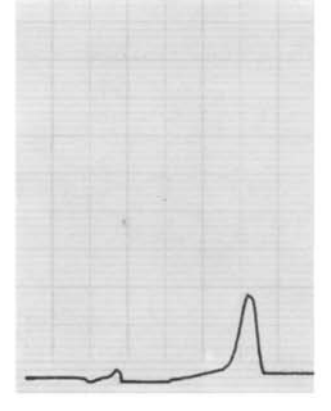

Fig. 3

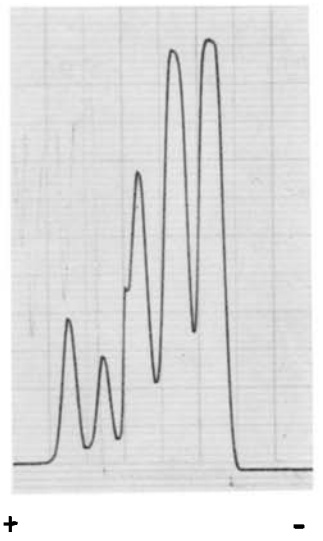

Fig. 4

Fig. 5

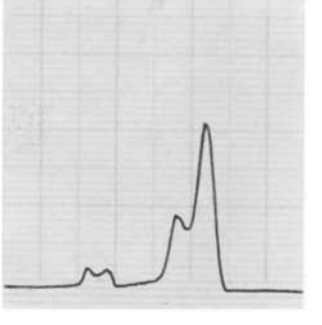

Fig. 6

glycine buffer, pH 9.0 or according to Leisfeld's modification of the method in β-alkaline buffer, pH 4.0 (11).

Zymograms of LDH were stained by immersion for 60 min. or 120 min. at 37°C in tris-HCL buffer, pH 7.4, containing 40 mg of NAD, 5 ml of sodium lactate, 1 mg of PMS and 30 mg of nitro BT in a total volume of 56 ml. Zymograms of β-G were stained by the methods of Hayashi et al. using naphthol AS-BI- β-D-glucuronide as substrate and fast red violet LB salt as coupler in 0.2 M acetate buffer, pH 5.0 (7, 8, 9). Zymograms of β-G were also visualised by the post-coupling technique of Fishman and Goldman (8).

Results

Studies of urinary LDH and β-G activities: Before studies on urinary isozymes, the activities of lactic dehydrogenase and β-G were measured in 51 urine specimens from normal subjects and patients with various diseases (Table 1).

The average levels of LDH and β-G in 13 specimens from normal subjects were 0.25 ± 0.19 / mg creatinine, and 10.38 ± 4.90 / mg creatinine respectively.

No significant increases in the levels were observed in either mild hematuria or mild pyuria. However, in specimens from cases of hematuria containing more than 30 erythrocytes in one high power field and in specimens from cases with severe pyuria containing more than 50 leucocytes in one high power field statistically significant increases in the levels of these enzymes were observed ($P < 0.01$, $P < 0.01$).

Except in 3 specimens the urines from the cases of bladder tumor, which were not appreciably contaminated with erythrocytes and leucocytes, all showed moderate, but significant, increase in LDH and β-G activities ($P < 0.005$, $P < 0.005$).

Studies on LDH isozymes: In normal urine LDH consisted almost entirely of LDH_1 and LDH_2 with only traces of LDH_4 and LDH_5. The isozyme pattern in specimens from cases of hematuria due to benign renal disease, was similar to that of normal urine. Pyuria was associated with an increase in LDH_5 (Table 1). This increase in LDH_5 was not detected in specimens from mild cases with less than 15 leucocytes in one high power field, but it was significant in specimens from severe cases with more than 50 leucocytes in one high power field. In specimens from 6 out of 8 cases of severe pyuria the LDH_5/LDH_1 ratio was reversed.

Table 2 shows that the serum LDH isozyme patterns of operable cases of bladder tumor have no diagnostic value. In patients with highly advanced tumors a slight increase in LDH_5 is seen with predominance of LDH_2 over LDH_1; but these changes are not significant. Table 3 shows the urinary lactic dehyrogenase isozyme patterns of 17 patients with bladder tumor. Predominance of LDH_5 and LDH_4 was observed in all cases except those where microscopical findings were negligible. Eleven of seventeen specimens showed an abnormal pattern of LDH isozymes, with a reversed LDH_5/LDH_1 ratio.

The isozyme patterns of normal epithelium and

Table 5. Comparison of the LDH isozyme patterns in urine and tumor tissues

Case	Urine % LDH isozyme distribution					Tumor % LDH isozyme distribution				
	I	II	III	IV	V	I	II	III	IV	V
S. M.	29.6	29.0	20.9	8.9	11.5	19.5	18.2	19.2	20.7	21.9
M. Y.	18.5	15.6	30.0	17.4	18.5	16.3	19.9	18.3	23.1	22.2
T. M. [a]	36.3	26.4	12.2	9.9	15.0	9.6	12.7	20.5	24.8	32.3
M. Y.	9.3	7.3	10.4	20.0	53.3	4.2	17.6	19.5	26.4	32.1
T. M.	0	0	25.3	30.6	44.1	1.6	14.4	23.6	26.7	33.7
M. O.	21.4	32.2	28.5	8.1	9.8	23.8	23.4	24.0	18.9	9.5
S. Y.	8.0	23.0	30.3	13.3	25.4	11.7	15.7	27.6	33.2	11.7
M. U.	11.1	19.7	31.7	14.7	22.9	11.3	16.7	25.9	29.6	16.2

a : Urethral carcinoma

of tumor tissue correlated well with those of urine specimens. Seven specimens of normal bladder epithelium contained only LDH_1 and LDH_2, while in tumor tissues LDH_4 and LDH_5 were predominant (Table 4, 5). In 12 of 16 specimens of tumor tissue the LDH_5/LDH_1 ratio was reversed. Figs. 1 and 2 show the clear difference between the isozyme patterns of LDH in urine and tissue from normal subjects and patients with bladder tumour.

Studies on the isozymes of β-G: Electrophoresis was carried out on 10 specimens of normal urine, 5 of normal epithelium, 10 of clear urine from patients with bladder tumors and 5 of bladder tumor tissues. To detect a band of β-G by the azo-coupling method the β-G activity of the specimen must be more than 2000 Fishman units.

Either on cellulose acetate electrophoresis or disc electrophoresis two kinds of electrophoretic pattern of β-glucuronidase were recognised, one which disclosed a single band at the site corresponding to γ-globulin region and another which disclosed two bands, a band at the site corresponding to the γ-globulin region and a band at the site corresponding to the β-globulin region. In all specimens which showed the latter pattern of β-glucuronidase, a band at the site corresponding to γ-globulin region was more intensive than the band at the site corresponding to the β-globulin region. Two of 10 urine specimens from normal subjects and 6 of 10 urine specimens from patients with bladder tumor showed this electrophoretic pattern of -glucuronidase consisting of two bands. (Fig. 3, 4) There couldn't be observed such a case which showed no band or a single band at the site corresponding to β-globulin region.

The β-glucuronidase isozymes of tissues gave similar results to those of urine. Namely, most normal tissues gave a single band of β-glucuronidase in the γ-globulin region on either cellulose acetate electrophoresis or disc electrophoresis. However, 4 of 10 tumor tissues gave two bands of β-glucuronidase, an intensive band at the site corresponding to the γ-globulin region and a weaker band at the site corresponding to the β-globulin region (Fig. 5, 6).

Discussion

The lactic dehyrogenase isozyme pattern of normal urine is known to show a marked preponderance of LDH_1 and LDH_2 and the pattern has been found to change in various disease as described below. However, urinary LDH isozymes have so far only been used in the diagnosis of renal diseases (1, 6, 19).

In 1965, Gelderman et al. (10) investigated the urinary LDH isozymes of 6 patients with bladder tumors and found that LDH_5 only increased in urine specimens which were contaminated with leucocytes, irrespective of the disease. They also reported that extracts of bladder tumors did not show an increase in LDH_5. Therefore, the increase in LDH_5 seemed to be derived mainly from contaminating leucocytes in the urine, not from malignant tissues. Consequently they concluded that the urinary LDH_5 fraction was of little value for early diagnosis of bladder tumors.

However, in this work we detected a marked increase of LDH_5 in urine specimens from patients with bladder tumors, which did not contain sufficient leucocytes to cause an increase of LDH_5. It is still unknown whether an increase of urinary LDH_5 is due entirely to malignant tissues but a significant preponderance of LDH_5 over LDH_1 was found in these specimens. These results are a strong indication that the pattern of urinary LDH isozymes is of diagnostic value. However, in its application care must be taken not to make erroneous diagnosis due to the presence of leucocytes or of erythrocytes.

In 1955 Boyland et al. (2) first reported that carcinoma of the bladder is associated with an increased urinary level of β-G and this has been confirmed by others. (16, 20). Moreover in 1953 Smith and Mills (21) first reported data suggesting the complexity of β-G, although studies on β-G isozymes have only recently been reported. In 1967 Plapp and Cole (18) separated 5 or 7 fractions of β-G by DEAE-cellulose chromatography. They found that these fractions had similar protein structures, but differed slightly in their carbohydrate contents and perhaps in their contents of amido-groups or other unidentified components. Yamamura et al. (23) separated 4 or 5 isozymes of β-G by starch gel electrophoresis. These isozymes migrated in positions corresponding to those of albumin, γ-globulin, β-globulin and α-globulin, respectively. But there have been no clinical reports on multiple forms of β-G.

In the present work multiple forms of β-G were separated as distinct visible bands, both by disc electrophoresis and by cellulose acetate electrophoresis. It is still uncertain whether these bands represented isozymes of β-G. Two other lysosomal enzymes, acid phosphatase and β-galactosidase, have been separated into similar bands by disc electrophoresis at pH 4.3 and 9.2 respectively (4, 14, 24).

Lancker and Lentz (13) obtained a single band of β-G from a rat liver homogenate by electrophoresis. This band was at the same distance from the origin as the weak band found in our study by disc electrophoresis. It is interesting that we also separated another weaker band from specimens of urine and tumor tissue from patients with bladder tumors, but not from other specimens. The biological significance of these multiple forms of β-G is still unknown (5). The present data are insufficient to demonstrate a specific change in isozymes in malignancy, but suggest that studies on urinary isozymes might be of value in the early diagnosis of

cases of bladder tumor and in follow-up studies on these cases. Studies are required on whether the appearance or increase of this second band in the urine of patients with bladder tumors represents enzyme derived from the tumor cells themselves or from the surrounding epithelium. These studies should be made in combination with histochemical analysis and results should be relevant to the multicentric theory of the histogenesis of urinary bladder cancer. Results should also show whether urinary isozymes are of value in estimating the degree of malignancy of bladder tumors. Moreover, as pointed out by Coodley (6), a critical analysis of information on isozymes should include a comprehensive survey of related enzymes, so studies are also required on the urinary isozyme patterns of various other enzymes, such as alkaline phosphatase, acid phosphatase and aldolase.

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