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Intracellular Enzymes in Serum, Lymph and Urine After Renal Ischaemia

G. Szabo, Z. Magyar

National Institute of Traumatology, Budapest, Hungary

Summary

Urine, renal and thoracic duct lymph were collected in dogs before and after clamping both renal arteries. After 30 minutes of renal ischaemia there was a marked increase in concentration of cytoplasmic enzymes in renal lymph and urine. Less prominent rises also were observed in plasma and thoracic duct lymph.

After 2 hours of ischaemia in addition to further increase in concentration of cytoplasmic enzymes a rise in activities of mitochondrial (GPT, alc. phosphatase, arylamidase) and lysosomal (acid phosphatase) enzymes was observed in urine and renal lymph. In some animals a significant arterial-renal venous difference was found in LDH concentration, and in renal lymph the LDH isoenzyme pattern shifted towards more slowly migrating M subunits.

There was marked parallelism between enzyme concentrations in renal lymph and urine.

It is concluded, that determination of intracellular enzymes in renal lymph and urine affords a method to assess severity of ischaemic cell damage in the kidney.

The effects of temporary interruption or curtailment of blood flow to the kidney is of great interest because of the relationship between renal ischaemia (e.g., in shock) and acute renal insufficiency. More recent technical difficulties encountered at renal transplantation attest to the practical importance of this relationship.

Blood flow to the kidney is rapid. To circumvent excessive dilution in the blood stream of the substances under study, we investigated changes in renal lymph and urine rather than renal venous blood. Determination of intracellular enzymes in lymph draining injured tissue allows assessment of extent of cellular damage (20). After mild injury, only intracellular enzymes, free in cytoplasm, increase in lymph. With greater injury, there is an additional increase in enzymes associated with certain intracellular organelles (21). Moreover, in various renal diseases, there is a more or less specific pattern of urinary enzyme excretion (1, 3, 8, 11).

The present report describes biochemical changes in serum, urine and renal lymph after temporary occlusion of the renal artery.

Methods

General

Experiments were performed on dogs anesthetized with pentobarbitone sodium (30 mg/kg). Both kidneys were exposed after a transabdominal midline incision. A lymph vessel was cannulated with polyethylene tubing in the left renal hilum. Plastic catheters were introduced into both ureters. The thoracic duct was cannulated on the left side of the neck. In the first group of animals, both renal arteries and veins were clamped for 30 minutes. In the second group, renal blood flow was interrupted for two hours.

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Urine, renal and thoracic duct lymph samples were collected before renal ischaemia and after removal of clamps. Duration of collection was one hour. Arterial and renal venous blood samples were taken before obstruction of the renal circulation, immediately after its restoration and two hours later.

Biochemical Methods

Lactic acid dehydrogenase (LDH) in lymph, serum and urine samples was determined by the U.V. method of *Wroblevsky and La Due* (37) using the Biochemica Test combination of C.F. Boehringer & Söhne, Mannheim. The results are expressed as mU/ml sample = μmol transformed substrate, per minute, per ml sample at 25°C and calculated in the following way: ΔE/min x 5053 = mU/ml.

Lactate dehydrogenase isoenzymes were separated electrophoretically (35) in 1% agarose medium and sodium barbiturate – HCL buffer (pH 8.6; ionic strength 0.05) on microscopic slides at 7 V/cm. The slides were developed according to *Van der Helm* (34) and the percentage of isoenzyme fractions were determined with a Zeiss densitometer.

From the distribution of isoenzymes the percentage ratio of H and M subunits were calculated according to the formula H% = LDH-5 + 0.75 LDH-4 + 0.5 LDH-3 + 0.25 LDH-2.

Maleic acid dehydrogenase (MDH) was estimated according to the recommendations of *Bergmeyer and Berndt* (5) using the Biochemica Test Combination of C.F. Boehringer & Söhne. The results were calculated in the following way: ΔE/min at 340 mμ x 5212 = mU/ml sample.

Glutamic acid dehydrogenase (GDH) was determined by the UV method as described by *E. and F.W. Schmidt* (31), the results were calculated with the formula ΔE/min at 340 x 803 = mU/ml sample.

Glutamic-oxaloacetic acid transaminase (GOT) was determined by the method of *Reitman and Frankel* (28). The number of international mU/ml was read from a calibration curve.

Glutamic-pyruvic acid transaminase (GPT) was determined with the Biochemica Test Combination of C.F. Boehringer & Söhne which employs the U.V. method of *Wroblevsky and La Due* (38).

The results were calculated in the following way: ΔE/min at 340 mμ x 1185 = mU/ml.

Leucin arylamidase (AA) was determined by a modification of original method of *Bratton and Marshal* (7). The results were calculated from a calibration curve and expressed as mU/ml of the sample.

Acid phosphatase (acP) was determined with the method of *Richterich* et al. (29) and the results were calculated as follows: Extinction at 405 mμ x 101 = mU/ml.

Alkaline phosphatase (alc.P) was determined with the Biochemica Test Combination of Boehringer & Söhne. The results are calculated according the formula: Ext. at 405 mμ x 200 = mU/ml of the sample.

For the enzyme reactions all urine samples were previously centrifuged and dialysed for 4 hours. Unfortunately in most experiments it was impossible to collect enough post-ischaemic renal lymph and urine. Therefore in some experiments only a few selected enzyme assays were performed and in the experiments with 2 hours ischaemia the determination of some enzymes was omitted.

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Total protein concentration in serum and lymph was determined by the biuret method of *Kingsley* (18), creatinine chromogen concentration in serum and urine with the picric acid method (27). Potassium in lymph was measured by flame photometry with a Spectromom 401 flame photometer.

Results

Interruption of blood flow to the kidneys for 30 minutes produced marked acute changes in renal function. In the immediate postischaemic period, urine flow dropped from 0.226 ± 0.056 ml/min to 0.036 ± 0.003 with corresponding changes in glomerular filtration rate (endogenous creatinine clearance) (Table 1).

After 30 minutes, ischaemic damage to renal tissue was mild. The most prominent change was a rise of LDH and MDH activities. LDH levels were significantly increased in renal lymph and urine as well as in circulating plasma and thoracic duct lymph. The changes of MDH activity were more or less similar. Furthermore, in renal lymph and urine, a significant increase of GOT-activity was observed (Table 1).

There was no alteration in serum, lymphatic and urinary levels of GDH, GPT, AA, alkaline and acid phosphatase.

Fractional distribution of LDH-isoenzymes in postischaemic serum and lymph as compared to control values were not significantly changed (Table 3).

After two hours of renal ischaemia, the immediate changes in urine flow and glomerular filtration rate were similar to those observed after 30 minutes. Besides an increase of LDH and GOT activities in urine, circulating plasma, renal and thoracic duct lymph, there was also a significant difference in LDH activity between renal venous and arterial blood plasma. Interruption of renal blood flow for 2 hours raised the level also of all other enzymes studied (GOT, GPT, AA alkaline and acid phosphatase) in renal lymph and urine. In thoracic duct lymph, a significant increase of alkaline phosphatase activities was observed (Table 2). As revealed by electrophoretic separation of LDH-isoenzymes, 2 hours of ischaemia led in renal lymph to a significant decrease of the percentage of H subunits and of the H/M ratio (Table 3).

Discussion

During severe acute injuries affecting various tissues, intracellular enzymes escape into circulation blood. During some acute renal diseases, there is an increase of urinary enzyme excretion (3, 4, 5, 30, 36). Moreover, localization and distribution of various enzymes and isoenzymes in different parts of the nephron suggest that the enzymes under study are mostly contained in tubular cells (10, 12, 22, 23).

A basic assumption in the present study is that severity and localization of tissue damage may be assessed by analysis of enzymes escaping from injured cells. A mild injury is characterized only by escape of enzymes present in cytoplasm. This derangement may result from increased membrane permeability or by some metabolic or excretory pathway (19). On the other hand, if severe injury completes the breakdown of damaged cells, it is anticipated that enzymes associated with cell membranes and organelles leak into body fluids (32). This means also that in these two types of injury, the expected differences in biochemical composition of body fluids are both quantitative and qualitative (20).

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Table 1. Effect of 30 minutes renal ischaemia on enzyme concentrations (mU/ml)

		n	A_1	V_1	A_2	V_2	A_3	V_3	D_1	D_2	R_1	R_2	U_1	U_2
LDH	$\bar{M}$	9	75,2	109,9	93,3	108,2	148,5$^+$	163,2	44,2	162,4$^+$	70,6	526,2^x	48,7	562,8^x
	±SEM		20,0	23,7	21,3	17,0	16,0	14,5	9,7	44,7	19,6	41,2	4,0	26,2
MDH	$\bar{M}$	11	64,7	89,1	84,7	109,3$^+$	146,3$^+$	134,5	90,0	169,9$^+$	132,2	748,6$^+$	59,0	752,3^x
	±SEM		13,4	20,3	12,0	25,6	32,1	47,6	19,0	34,4	29,2	68,6	7,6	59,6
GDH	$\bar{M}$	5	3,4	3,0	3,1	2,7	2,6	2,7	3,0	3,3	2,7	3,3	1,7	3,7
	±SEM		0,5	0,5	0,5	0,4	0,7	0,6	1,1	0,6	0,5	0,6	1,0	1,2
GOT	$\bar{M}$	12	8,5	6,7	10,0	8,9	8,1	7,4	8,3	11,0	4,6	11,7$^+$	6,2	19,4^x
	±SEM		1,6	1,5	1,7	1,0	1,0	1,1	1,2	0,9	1,0	2,8	1,2	2,6
GPT	$\bar{M}$	8	4,1	4,6	6,3	6,1	6,7	6,8	4,6	5,2	4,2	4,4	2,5	2,4
	±SEM		0,8	0,8	0,6	0,7	0,7	0,8	1,3	1,2	1,1	1,3	0,9	0,5
AA	$\bar{M}$	9	9,4	9,8	8,3	8,8	8,8	8,8	8,4	7,6	3,6	3,5	2,1	2,2
	±SEM		1,1	1,3	1,6	1,3	1,2	1,2	1,3	1,2	1,1	1,1	0,2	0,8
AlcP	$\bar{M}$	13	27,7	25,9	31,4	22,5	25,4	22,8	24,2	19,6	10,1	13,4	18,4	54,2
	±SEM		4,6	5,1	3,8	3,6	4,3	4,5	3,5	3,4	2,6	3,0	3,7	14,2
AcP	$\bar{M}$	9	4,1	4,7	6,1	6,4	6,2	6,6	3,4	4,7	0,6	2,1	1,7	1,5
	±SEM		0,6	0,2	0,4	0,4	0,4	0,5	0,9	0,9	0,4	0,8	0,4	0,4
Protg%	$\bar{M}$	9	5,58	5,68	5,72	5,71	5,65	5,65	3,94	4,01	3,27	3,33		
	±SEM		0,17	0,15	0,15	0,14	0,18	0,14	0,14	0,18	0,10	0,12		
Diuresis ml/min	$\bar{M}$												0,226	0,036
	±SEM	13											0,056	0,003
C_k ml/min	$\bar{M}$												25,5	5,0
	±SEM	13											4,2	1,7
K meq/lit.	$\bar{M}$		4,46				4,82		3,89	3,92	4,53	4,33		
	±SEM	13	0,41				0,39		0,38	0,30	0,12	0,14		

A_1, A_2, A_3: activities in arterial bl. serum before the clamping of the renal artery, immediately after and 2 hours after the release of the clamp.
V_1, V_2, V_3: activities in renal venous bl. serum.
D_1, D_2: activities in thoracic duct lymph before and after renal ischaemia.
Activities in renal lymph (R_1, R_2) and in urine samples (U_1, U_2) collected before and after renal ischaemia.
+ = Significant difference between the means of control (preischaemic) and postischaemic values ($p < 0.05$).
x = Significant difference between pre- and postischaemic values ($p < 0.01$-0.001).
□= Significant arterio-venous difference ($p < 0.01$).

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Table 2. Effect of 2 hours renal ischaemia on enzyme concentrations.

		n	A_1	V_1	A_2	V_2	A_3	V_3	D_1	D_2	R_1	R_2	U_1	U_2
LDH	$\bar{M}$	13	46,5	99,2	70,1	130,8	107,2^x	241,4$^\square$	36,1	223,9	60,3	1273,0^x	69,8	652,5^x
	±SEM		5,9	6,9	10,8	16,5	10,1	40,5	5,2	37,8	15,4	70,3	14,1	43,1
GOT	$\bar{M}$	9	6,3	6,7	12,0	12,4	20,4^x	25,4	6,5	17,0$^+$	6,2	36,8^x	6,9	32,9
	±SEM		2,1	1,9	2,5	2,5	3,5	2,6	2,5	3,6	1,6	7,3	1,9	5,8
GPT	$\bar{M}$	12	6,6	5,5	7,8	8,1	8,7	10,0	5,0	5,1	5,7	11,0^x	0,5	4,4$^+$
	±SEM		1,6	0,9	1,4	1,2	1,5	1,6	0,9	1,2	1,2	2,3	0,3	1,4
AA	$\bar{M}$	13	9,2	9,4	10,6	11,7	10,7	11,7	7,7	8,9	4,1	7,7	3,5	11,2^x
	±SEM		0,5	0,6	0,9	0,9	0,9	0,8	0,7	1,0	0,8	1,8	0,6	1,6
AlcP	$\bar{M}$	13	17,3	17,7	20,7	21,7	22,3	23,7	12,7	25,0$^+$	5,8	16,2$^+$	23,3	75,5^x
	±SEM		3,5	4,7	5,0	3,2	3,5	3,3	2,1	5,2	2,1	8,7	10,5	24,5
AcP	$\bar{M}$	13	3,8	3,8	5,3	7,0	5,3	7,1	3,1	4,1	1,0	4,5	5,3	11,8$^+$
	±SEM		0,7	0,5	0,5	1,1	0,7	0,9	0,5	0,5	0,4	1,0	1,3	1,4
Protg%	$\bar{M}$	13	6,35	6,22	6,30	6,29	6,28	6,18	4,67	4,77	4,21	4,71		
	±SEM		0,08	0,05	0,06	0,07	0,06	0,05	0,20	0,19	0,20	0,17		
Diuresis	$\bar{M}$	13											0,335	0,045
ml/min													0,065	0,010
C_k	$\bar{M}$	12											42,6	2,2
ml/min	±SEM												7,3	1,1

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Table 3. The effect of 30 minutes and 2 hours renal ischaemia on the percentual ratio of LDH-isoenzyme fractions.

30 minutes		Control								After ischaemia							
		LDH-1	LDH-2	LDH-3	LDH-4	LDH-5	H	M	H/M	LDH-1	LDH-2	LDH-3	LDH-4	LDH-5	H	M	H/M
Serum+ %*	M	37,7	18,3	24,4	8,2	11,4	65,7	34,3	2,07	40,6	19,1	27,2	5,4	7,7	72,0	28,1	2,79
	±SEM	6,0	2,3	4,5	1,2	2,4	3,6	3,6	0,32	3,9	2,2	3,1	0,6	1,4	2,5	2,5	0,93
Thoracic duct %	$\bar{M}$	23,4	24,5	35,6	7,2	8,5	61,0	38,9	1,63	22,6	25,7	36,3	7,2	7,9	61,9	38,1	1,74
	±SEM	4,5	2,6	5,5	1,4	0,8	2,2	2,2	0,16	8,8	3,5	5,1	1,1	1,5	2,5	2,5	0,23
Renal lymph %	$\bar{M}$	34,7	21,2	26,8	5,8	5,6	71,1	28,8	2,59	36,5	25,3	26,4	6,4	5,2	70,3	29,6	2,52
	±SEM	5,8	2,3	2,4	1,2	1,3	2,2	2,3	0,26	3,3	1,7	2,0	1,4	0,3	2,2	2,2	0,37

2 hours		Control								After ischaemia							
		LDH-1	LDH-2	LDH-3	LDH-4	LDH-5	H	M	H/M	LDH-1	LDH-2	LDH-3	LDH-4	LDH-5	H	M	H/M
Serum %+	$\bar{M}$	35,9	31,5	18,9	6,5	7,1	70,5	29,4	2,62	35,3	23,9	24,6	7,3	8,8	67,0	32,9	2,06
	±SEM	4,6	6,1	3,1	1,2	1,8	2,7	2,7	0,38	2,8	2,2	2,8	0,7	1,0	1,2	1,2	0,11
Thoracic duct %	$\bar{M}$	27,1	23,3	37,3	7,9	4,5	65,4	34,5	1,91	29,8	20,8	29,4	9,7	10,2	59,7	35,9	1,82
	±SEM	3,0	1,9	4,9	1,2	1,3	1,2	1,2	0,11	2,6	2,5	1,4	1,2	2,8	4,5	2,7	0,19
Renal lymph %	$\bar{M}$	48,9	25,3	16,6	4,6	4,9	75,8	24,1	3,53	31,7	23,6	21,7	10,8	12,0	63,0	36,9	1,87x
	±SEM	5,5	2,3	2,9	0,8	1,4	3,0	3,0	0,53	2,8	3,3	3,7	1,7	4,3	3,6	3,6	0,31

+Before ischaemia: arterial bl. serum.
After Ischaemia: renal venous blood drawn 2 hours after the release of the clamp.
xSignificant difference between the pre- and postischaemia values ($p < 0{,}01$)

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Assessment of biochemical changes in blood plasma and urine as a measure of renal injury has disadvantages. The rate of blood flow in the kidney relative to organ weight is high and hence enzymes escaping from cells into blood plasma are markedly diluted. Enzyme studies in urine may be inconclusive because of dependence of enzyme excretion on renal function and the presence of cells and enzyme inhibitors in urine. On the other hand lymph is a part of extracellular fluid readily accessible for collection and study. Moreover, enzymes and other constituents of damaged cells escape directly into extracellular fluid and thus into lymph.

In the present investigations, enzyme activities were measured also in urine, thoracic duct lymph, arterial and renal venous blood plasma. These enzymes were selected not only on methodologic grounds but primarily on the basis of subcellular localisation.

Lactate dehydrogenase in kidney is in highest concentration in the proximal convolution (22, 23). In the human kidney LDH-1 predominates in the proximal nephron; more distally there is a shift towards slowly migrating isoenzyme fractions (4, 10, 12). The isoenzyme pattern in urine, derives from contributions from different part of the nephron. Therefore, urine isoenzyme patterns take an intermediate position. However, as in homogenates of whole kidney, the H subunits strongly predominate (33). A preponderance of H subunits (H/M = 2.59 and 3.53) was observed also in normal renal lymph.

Increased urinary LDH excretion occurs in various kidney diseases and experimental renal lesions (e.g. shock, renal infarction in dog and temporary occlusion of renal artery in dogs and rabbits) (2, 6, 17). In the present investigation increased urinary enzyme activity was observed after only 30 minutes. Similar (about 10 fold) increases were observed also after 2 hours ischaemia. In both groups there was also a significant increase of LDH activity in thoracic duct lymph and arterial blood plasma. High LDH values in thoracic duct lymph derive from renal lymph. It should be noted that both renal arteries were clamped and only one of several renal hilar lymphatics was cannulated. The rise of plasma LDH-concentration is especially significant. During thoracic duct cannulation the major connection between lymphatic and venous system was severed. This observation suggests a direct access route of this protein to the blood stream. This postulate is corroborated by significant arterial-renal venous difference in plasma LDH concentration. Similar observations were made in rabbits after 45 to 300 minutes clamping of the renal artery (17) and in dogs after 3 1/2 hours limb ischaemia (33). The highest increases of LDH activity were observed in renal lymph. Moreover, after 2 hours of ischaemia lymphatic enzymatic activity was more than twice the level after 30 minutes of ischaemia.

A significant shift in the LDH-isoenzyme pattern occurred only in renal lymph after 2 hours of ischaemia. There was also an increase in the percentage ratio of M subunits (i.e., H/M ratio decreased from 3.53 to 1.87). This finding suggests damage predominantly of the lower portions of the tubules, i.e., high lymphatic LDH derives from damaged distal nephrons. However, as H and M subunit activity depend on oxygen supply to cells this explanation may not be complete.

Maleate-dehydrogenase – an enzyme of the citric acid cycle, is associated primarily with the matrix of the mitochondria, but is also in cytoplasm. There are differences between mitochondrial and cytoplasmic MDH (9) and in certain organs their ratio may differ. In the heart for instance nearly all cellular MDH is localized in cytoplasm. With severe myocardial damage mitochondrial MDH may increase outside cells (32). In the present

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investigation changes of MDH concentration in plasma, urine and lymph roughly paraleled those of LDH. Accordingly, no additional information was gained from study of this enzyme.
Glutamic-oxaloacetic acid transaminase is an ubiquitous cytoplasmic enzyme. High urinary GOT excretions develop in shock and after clamping renal arteries (2, 17). Moreover, a significant arterial-renal venous concentration gradient occurs after total renal ischaemia (16). In the present investigation GOT activities increased in renal lymph and urine after mild injury. More severe injury also produced a rise in plasma and thoracic duct lymph. Interpretation of these observations is similar to that of LDH.
Glutamic-pyruvic acid transaminase in many animal tissues is not confined to the mitochondrial matrix. However, no data was uncovered concerning subcellular localisation of this enzyme in the dog's kidney. In the present investigation as noted previously (21), this enzyme did not escape after mild injury.

The highest GPT activities were found in the proximal convolutions (10). Increased excretion occurs in urine of patients in shock (2). In rabbits after total renal vascular occlusion urinary GPT concentration – in contrast to LDH, GOT and aldalose activities – only minimally increased (17). In the present experiments – GPT initially almost undetectable in urine, rose after 2 hours of renal ischaemia while its concentration in renal lymph doubled.
Glutamic acid dehydrogenase can be considered a "pure" mitochondrial enzyme (15). Its levels are fairly high in cells of tubules (10). Mild anoxic injury to the kidney produces neither a change of GDH concentration in urine or in renal lymph.
Amino acid arylamidase is localised histochemically mostly in brush borders of cells lining proximal tubules (3, 24). In contrast to leucyl-aminopeptidase (LAP) arylamidase is absent from cytoplasm. Rather, it localizes to the microsomal fraction of kidney homogenates (25, 26). In the rat, various lesions of the tubules including haemorrhagic shock, produce increased AA excretion (4). Increased AA activity in urine develops in conjunction with acute renal failure (13).

In the present experiments increased AA activity was detected in urine and renal lymph after 2 hours of renal ischaemia.
Alcaline phosphatase is localised in the kidney predominantly in cells of the convolutions (10). This enzyme binds to mitochondrial membranes. Increased urinary excretion develops in various acute renal diseases including tubular necrosis and renal infarction (36). Haemorrhagic shock in rats, acute and chronic renal ischaemia in dogs, also leads to increased alc-P-activity in urine (4). In the present experiment an insignificant increase of urinary alc-P activity after mild injury is contrast to a significant rise in urine, renal and thoracic duct lymph after more severe damage.
Acid phosphatase (ac.P.) is a lysosomal enzyme. Its escape from cells denotes severe cell breakdown. As expected, mild injury had no effect on ac.P. concentrations in the examined fluids. More severe injury resulted in a moderate increase of activity in renal lymph and urine.
Potassium concentrations in lymph did not change after 30 minutes of ischaemia. However, this finding does not necessarily imply a reluctance for K^+ to leave cells after injury (20). Perhaps it is explicable by a rapid interchange of electrolytes between extracellular fluid and blood plasma.

The near constancy of protein concentrations in lymph after injury signifies that changes of enzyme activity per unit volume are accompanied by parallel changes of activity per

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gram protein. In the present experiments the drop in urine flow parallels a similar decrease of glomerular filtration rate. This observation renders it unlikely that increased urinary enzyme activity is simply a consequence of the concentration of the filtered enzymes during tubular water absorption. Furthermore, a nearly perfect parallelism exists between changes in urinary and renal lymph enzyme activities. The values in both fluids are occasionally almost numerically identical. Thus, there seems a close relationship between changes in urine and renal tissue fluid in severe acute anoxic renal damage. Biochemical analysis of urine in similar clinical conditions (shock, allograft rejection, renal infarction) may be of diagnostic value in the early assessment of extent and severity of renal cell damage.

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Doctor Gyorgy Szabo, National Institute of Traumatology, Mezo Imre ut 17. Budapest, VIII. Hungary

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Lymphography in Mediastinal Lymph Node Hyperplasia

– Report of Two Cases –

M. Nishimine, M. Sako and A. Kubota, S. Okada

Department of Radiology and Division of Pathology, Department of Clinical Laboratory, Kobe University School of Medicine, Kusunoki-Cho, Ikuta-Ku, Kobe, Japan

Summary

In two cases of mediastinal lymph node hyperplasia lymphography was performed in an attempt to evaluate the retroperitoneal lymphatic system. Since the lymphograms show the non-specific pattern of lymphoid hyperplasia, they do not allow a differential diagnosis, but suggest that the disease is more general than hitherto suspected.

Mediastinal lymph node hyperplasia has been regarded as a benign tumor-like mass of unknown etiology, which histologically and radiologically resembles thymoma. Since *Castleman* (1, 2) first described this rare and specific entity in 1954, only about 50 cases have been reported in the literature, eight of them in our country (3, 4, 5).

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