Pediat. Res. 6: 797–801 (1972)

Amino acid metabolism leukocyte
ammonia liver
glutaminase urea cycle

Leukocyte and Liver Glutaminase in Lysinuric Protein Intolerance

O. SIMELL[26], J. PERHEENTUPA, AND J. K. VISAKORPI

University of Helsinki Children's Hospital, Helsinki, Finland

Extract

Leukocyte glutaminase *I* activity was studied in 17 patients with lysinuric protein intolerance (LPI, familial protein intolerance) and 21 controls. The values were logarithmically distributed, the mean (95% confidence interval) being 9.1 (1.5–54.3) for the patients and 15.4 (2.3–104.4) for the controls, in nanomoles of ammonia per 10^6 leukocytes and 30 min. The difference is significant at $P < 0.05$. Only two of the LPI patients had a value below the range of the controls, and five others actually had a value above the mean of the controls.

Liver glutaminase *I* activity was measured in two of the LPI patients and in seven controls. The activity found in the patients was clearly higher than in the controls. We have disproved the speculation of a deficiency of liver glutaminase in LPI, as a basic derangement in LPI, common to both basic chemical disturbances of LPI, the renal leakage of basic amino acids and the delayed urea synthesis.

Speculation

Patients with LPI have decreased plasma levels of arginine, ornithine, and lysine. Arginine and ornithine supplement accelerates their slow urea synthesis. The possibility that this deficiency of arginine and ornithine is further exaggerated through a defect of their transport to the site of urea production in the parenchymal cells of liver is currently under investigation.

Introduction

Lysinuric protein intolerance is a disorder of amino acid and ammonia metabolism, characterized by failure to thrive, diarrhea, and vomiting in the infant fed cow's milk, and later by severe aversion to protein-rich food [8, 9, 17]. The patients show growth retardation, enlargement of the liver and often of the spleen, neutropenia and sometimes thrombocytopenia, and increased urinary excretion of the basic amino acids, particularly lysine. Amino nitrogen intake is followed by hyperammonemia, and the production of urea is slow. The disease is recessively inherited [14].

So far, we have seen 18 cases of this disease in Finland, and 1 patient, a Finnish immigrant, has been described from Sweden [6, 12]. A similar urinary amino acid pattern has been described in a few other patients [3, 10, 15, 22], in whom LPI can neither be excluded nor confirmed on the data presented. Especially two Japanese patients [15] are in our opinion likely cases of LPI.

Studies of the pathogenetic mechanism of LPI have not revealed the basic derangement. Recently, Malmquist *et al.* [12] have suggested a deficiency of the activity of glutaminase *I* in the liver and kidneys to be the primary defect of LPI. Measured on three occasions, the glutaminase activity in the leukocytes of

their one patient was lower than in any of their 10 controls.

To test this hypothesis, we have measured the activity of glutaminase *I* in the leukocytes of 17 of our patients and in the liver tissue of 2 of them. We were unable to confirm the hypothesis of the Swedish workers.

Material and Methods

Patients

The leukocyte glutaminase activity was determined in 17 of our patients, aged 0.5–26 years. Eleven of them have been reported earlier [8, 9, 17]. The remaining six have typical plasma and urinary amino acid patterns and show the clinical and test findings characteristic of LPI. The controls were healthy members of the laboratory personnel (6), children with different neurologic disturbances (7), diabetes mellitus (4), Basedow's disease (1), rheumatoid monarthritis (1), a newborn with hyperbilirubinemia, and a patient with classical cystinuria. The age distribution of the controls was similar to that of the patients. Liver tissue was assayed in two of the patients, 4.5 and 16.5 years old, and in seven controls. The controls are presented in the legend to Figure 2.

Leukocyte Assay

The method of Skoog and Beck [21] was used for the separation of leukocytes. Twenty milliliters of heparin blood were drawn and mixed immediately with 40.0 ml 3.00% dextran (mean mol wt, 210,000 or 150,000) in physiologic saline. The suspension was allowed to stand at 4° for 30 min. The supernatant containing most of the leukocytes with some erythrocytes was centrifuged at low speed, and the sedimented cells were exposed to hypotonicity and mixing for 30 sec to destroy the erythrocytes. After restoring the isotonicity, the cells were centrifuged and resuspended in 2.00 ml physiologic saline and, if excessive red coloring was present, washed once more with saline. The cells of the final leukocyte suspension were ruptured by sixfold freezing and thawing, using a methanol-Dry Ice bath, and the lysate was used immediately for assay of the enzyme activity. The number of leukocytes was counted in both the blood specimen and the final cell suspension. In the suspension it varied between 4,000 and 40,000, and between 4,300 and 23,900 cells/mm^3, for the patients and controls, respectively. The glutaminase activity was measured by the method of Sayre and Roberts [19], modified as follows. A mixture of equal volumes of 0.4 M sodium phosphate, pH 8.0, and 0.1 M boric acid, pH 8.1, buffers was used. Crystalline L-glutamine was added at 0.025 M to the buffer mixture immediately before the enzyme assay. This use of borate and fresh glutamine solution greatly diminished spontaneous release of ammonia. Leukocyte lysate, 1.00 ml, was incubated with 1.00 ml of glutamine-buffer for 1 hr. Samples, 0.200 ml each, were taken at zero time and at 15-min intervals for measurement of ammonia [18]. Ammonia liberation proceeded linearly during the incubation. As a blank a mixture of glutamine buffer and saline, 1.00 ml of each, was used.

The activity of glutaminase was expressed as nanomoles of ammonia liberated per 10^6 leukocytes during 30-min incubation at 37°, corrected by subtracting the blank. Incubation of leukocyte lysate plus buffer without glutamine never produced detectable amounts of ammonia. Heating the lysate at 100° for 30 min destroyed the enzyme activity. The initial ammonia concentration varied from nondetectable to 0.37 mM.

In the control incubations with saline the increase in ammonia concentration was always below 0.2 mM and was similar in the samples from the patients and the controls.

To determine the precision of the leukocyte assay, the whole procedure starting from the blood sample was performed in duplicate on two controls. The duplicate results for the glutaminase *I* activity were 39.0 and 32.3 for one and 9.2 and 9.4 for the other, in nanomoles of NH_3 formed per 10^6 leukocytes and 30 min.

Liver Glutaminase

From the patients, liver tissue specimens were obtained by percutaneous puncture with a Menghini needle. From the controls, the specimens were taken at laparotomy. The specimens were immediately put in ice-cold phosphate buffer and homogenized in a Potter-Elvehjelm homogenizer in an ice bath. The assay procedure was the same as for the leukocytes, except that the phosphate buffer was used alone. The enzyme activity was expressed relative to the protein concentration of the homogenate [11].

Results

The glutaminase *I* activity of leukocytes showed a large variation both in the controls and in the patients (Fig. 1). The values are logarithmically distributed, the mean (95% confidence interval) being 9.1 (1.5–54.3) for the patients and 15.4 (2.3–104.4) for the controls, in nanomoles of ammonia per 10^6 leukocytes and 30 min.

The difference is significant at $P < 0.05$. However, it cannot reflect a basic defect of LPI, as only two of the patients had values below the range of the controls, and five others actually had values above the mean of the controls.

The leukocyte glutaminase activity had no apparent correlation to the subject's age or leukocyte count in either of the groups, to the severity of the manifestation of the disease, or to the duration of the arginine-HCl treatment [9] of the patients.

One of the controls had a blood sample assayed twice, and one of the patients three times at intervals of a few days. The values found in the control were 104.1 and 9.6, and in the patient 10.4, 58.1, and 14.8, in nanomoles of ammonia per 10^6 cells and 30 min.

Any conclusion made about liver glutaminase activity on the basis of the activity in leukocytes alone appears adventurous in view of this degree of fluctuation in the latter. Therefore, direct determinations were obtained of the activity of glutaminase *I* in the liver. The findings are given in Figure 2. The activity found in the patients was clearly higher than in any of the controls, instead of being decreased. Though the controls were not normal subjects, most of them were in normal state of nutrition, and their selection does not seem to be biased. The increase in liver glutaminase *I* activity in the LPI patients thus appears significant. In any case, the hypothesis of a defect of this enzyme as the cause of the disease can definitely be discarded.

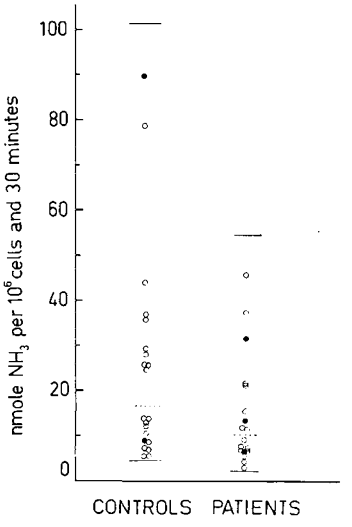

Fig. 1. Leukocyte glutaminase *I* activity in controls and patients with lysinuric protein intolerance. The log scale means and 95% confidence intervals are indicated. The *solid dots* indicate values from the same persons at intervals of a few days.

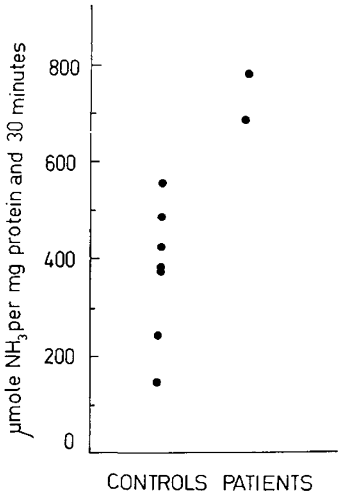

Fig. 2. Glutaminase *I* activity in liver tissue of controls and patients with lysinuric protein intolerance. The controls are, in descending order: *1*) 14-month-old infant with esophageal atresia, with normal nutrition maintained through a gastrostoma. *2*) 5-month-old infant, preagonal for cerebellar teratoma and serving as a kidney donor, normal nutrition maintained by nasogastric tube. *3*) 5-year-old boy with portal hypertension of unknown cause, normal liver histology. *4*) 15-year-old boy suffering from progressive myoclonus epilepsy, with characteristic cellular inclusion bodies in the liver, and hyperammonemic response to iv L-alanine load [9]. *5*) 2-month-old infant with pyloric stenosis. *6*) 58-year-old woman with stenosis of Oddi's sphincter. *7*) 51-year-old woman with gastric ulcus. The patients were girls, aged 16 and 4 years.

Discussion

An acceptable hypothesis of the basic defect of LPI has to account for two seemingly unrelated abnormalities: impaired synthesis of urea in the liver and decreased reabsorption of the basic amino acids in the kidney tubuli. A defect of the enzymes of the urea cycle or of carbamyl phosphate synthetase could not readily be explained as the basis of the increased aminoaciduria. Normal activity of these enzymes has in fact been demonstrated, at least under optimal conditions *in vitro* [7].

Malmquist *et al.* [12] recently proposed an anomaly of liver and kidney glutaminase for the basic defect in LPI and later extended their interesting speculation to explain the tubular dysfunction [13]. The defect would directly impair the urea synthesis, as glutamine is believed to be a mediator of ammonia from transaminations to the synthesis of carbamyl phosphate. Accumulation of glutamine would be associated with deple-

tion of glutamic acid and α-ketoglutarate. Malmquist *et al.* [13] suggested that α-ketoglutarate is necessary for the tubular reabsorption of lysine, which would involve temporary coupling of these two molecules to form saccharopine.

We have disproved the speculation of a deficiency of liver glutaminase in LPI. The activity of this enzyme has not been measured in kidney tissue, but a marked deficiency is unlikely in view of the fact that LPI patients have no apparent defect in urinary excretion of ammonia or in the capacity to maintain acid-base balance. Furthermore, a defect in kidney glutaminase alone could not explain the impaired urea production.

We have confirmed the finding that the leukocyte glutaminase activity is decreased in LPI. However, the difference is only statistical. The enzyme is known to be highly inducible by protein supplement in the liver and kidneys of animals [1, 5]. A patient who was protein-intolerant because of defect of liver lysine:NAD-oxidoreductase was found to have decreased activity of hepatic glutaminase *I* [2]. Celiac disease and other affections of the small intestine provide an example of secondary glutaminase deficiency of another tissue, intestinal mucosa [4]. We believe that the leukocytes of LPI patients present an example of secondary depression of glutaminase *I* in still another tissue.

The increase in glutaminase *I* activity in the patient's liver may be a significant finding. Experiments have been performed in rats to elucidate the mechanism of this activation of glutaminase [20]. Low protein intake brought about a clear decrease in the liver glutaminase *I* activity. Simultaneous treatment with ammonium acetate for 1 week did not alter the activity. Thus, the increase in the enzyme in the LPI patients is presumably not a direct consequence of the hyperammonemia occurring in them. A probable metabolic feature of the LPI is general activation of transamination reactions as reflected in increased plasma alanine concentration [9, 13]. The increase in glutaminase activity may be a part of the metabolic arrangement required for the transamination activity [20].

The acceleration of urea synthesis in LPI patients after an arginine or ornithine supplement has been well documented [9]. The hypothesis of defective glutaminase in liver fails to account for this phenomenon.

Renal leakage of arginine in addition to lysine and decreased plasma levels of both of them plus ornithine have been demonstrated in LPI [16]. We are currently exploring the possibility that this deficiency of arginine and ornithine is further exaggerated at the site of urea production in the liver cells, through a defect in the transport of these amino acids which is present in the liver cells in addition to the kidney tubuli.

Summary

We have seen 18 cases of lysinuric protein intolerance. The link between the two characteristic chemical features of this disease, postprandial hyperammonemia and defective renal reabsorption of basic amino acids, is unknown.

Recently, deficiency of glutaminase in the liver and kidney has been proposed to be the single defect basic to both these disturbances. This hypothesis was based on measurement of glutaminase activity in the leukocytes of one patient. We have measured the glutaminase activity in the leukocytes of 17 patients with LPI, and in the liver tissue of 2 of them. Statistically, the patients had slightly lower leukocyte glutaminase activity than the controls, but only two of the patients had a value below the range of the controls and five others had a value above the mean of the controls. In the liver, the activity of this enzyme was clearly higher in the patients than in the controls.

References and Notes

1. BEATON, J. R.: The relation of dietary protein level to liver enzyme activities in cold exposed rats. Can. J. Biochem., *41:* 1871 (1963).
2. COLOMBO, J. P., BÜRGI, W., RICHTERICH, R., AND ROSSI, E.: Congenital lysine intolerance with periodic ammonia intoxication: a defect in L-lysine degradation. Metabolism, *16:* 910 (1967).
3. FLEMING, W. H., AVERY, G. B., MORGAN, R. I., AND CONE, T. E.: Gastrointestinal malabsorption associated with cystinuria; report of a case in a negro. Pediatrics, *32:* 358 (1963).
4. GELFAND, M. D., SPIRO, H. M., AND HERSKOVIC, T.: Small intestine glutaminase deficiency in celiac disease. Amer. J. Dig. Dis., *13:* 638 (1968).
5. GOLDSTEIN, L., AND KENSLER, C. J.: Factors which affect the activity of glutaminase *I* in the guinea pig kidney. J. Biol. Chem., *235:* 1086 (1960).
6. JAGENBURG, R., LINDSTEDT, G., AND MALMQUIST, J.: Familjär (?) proteinintolerans med hyperammoniemi. Läkartidningen, *67:* 5255 (1970).
7. KEKOMÄKI, M., RÄIHÄ, N. C., AND PERHEENTUPA, J.: Enzymes of urea synthesis in familial protein intolerance with deficient transport of basic amino acids. Acta Paediat. Scand., *56:* 631 (1967).
8. KEKOMÄKI, M., TOIVAKKA, E., HÄKKINEN, V., AND SALASPURO, M.: Familial protein intolerance with deficient transport of basic amino acids, report on an adult patient with chronic hyperammonemia. Acta Med. Scand., *183:* 357 (1968).
9. KEKOMÄKI, M., VISAKORPI, J. K., PERHEENTUPA, J., AND SAXÉN, L.: Familial protein intolerance with deficient transport of basic amino acids; an analysis of 10 patients. Acta Paediat. Scand., *56:* 617 (1967).

10. KIRKMAN, H. N., AND KIESEL, J. L.: Congenital hyperammonemia. Pediat. Res., *3:* 358 (1969).
11. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., AND RANDALL, R. J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem., *193:* 265 (1951).
12. MALMQUIST, J., AND HETTER, B.: Leucocyte glutaminase in familial protein intolerance. Lancet, *ii:* 129 (1970).
13. MALMQUIST, J., JAGENBURG, R., AND LINDSTEDT, G.: Familial protein intolerance; possible nature of enzyme defect. New Engl. J. Med., *284:* 997 (1971).
14. NORIO, R., PERHEENTUPA, J., KEKOMÄKI, M., AND VISAKORPI, J. K.: Lysinuric protein intolerance, an autosomal recessive disease; a genetic study of 10 Finnish families. Clin. Genet., *2:* 214 (1971).
15. OYANAGI, K., MIURA, R., AND YAMANOUCHI, T.: Congenital lysinuria: a new inherited transport disorder of dibasic amino acids. J. Pediat., *77:* 259 (1970).
16. PERHEENTUPA, J., KEKOMÄKI, M., AND VISAKORPI, J. K.: Studies on amino-nitrogen metabolism in familial protein intolerance. Pediat. Res., *4:* 209 (1970).
17. PERHEENTUPA, J., AND VISAKORPI, J. K.: Protein intolerance with deficient transport of basic amino acids; another inborn error of metabolism. Lancet, *ii:* 813 (1965).
18. RAHIALA, E.-L., AND KEKOMÄKI, M. P.: A simplified one-stage cation exchange technique for the determination of whole blood ammonium. Clin. Chim. Acta, *30:* 761 (1970).
19. SAYRE, F. W., AND ROBERTS, E.: Preparation and some properties of a phosphate-activated glutaminase from kidneys. J. Biol. Chem., *233:* 1128 (1958).
20. SIMELL, O.: Effect of protein intake and ammonium acetate treatment on rat liver glutaminase *I* activity. In preparation.
21. SKOOG, W. A., AND BECK, W. S.: Studies on the fibrinogen, dextran, and phytohemagglutinin methods of isolating leukocytes. Blood, *11:* 436 (1956).
22. WHELAN, D. T., AND SCRIVER, C. R.: Hyperdibasicaminoaciduria: an inherited disorder of amino acid transport. Pediat. Res., *2:* 525 (1968).
23. All procedures have been performed in accordance with the provisions set forth in the Declaration of Helsinki.
24. The skillful technical assistance of Mrs. Maisa Taalikka is acknowledged.
25. Supported by the National Research Council for Medical Sciences, and the Foundation for Pediatric Research, Finland.
26. Requests for reprints should be addressed to: OLLI SIMELL, M.D., University of Helsinki Children's Hospital, 00290 Helsinki 29, Finland.
27. Accepted for publication June 13, 1972.

 Printed in U.S.A.