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Changes of Amino Acid Serum Levels in Pediatric Patients with Higher-risk Acute Lymphoblastic Leukemia (CCG-1961)

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Abstract. *Background: Deamination of asparagine (Asn) and glutamine (Gln) by asparaginases (ASNase) is associated with good prognosis in acute lymphoblastic leukemia (ALL). Chemotherapy drugs used for ALL may accelerate catabolism of other amino acids (AA). Materials and Methods: We studied ASNase activity and changes of Asn, Gln, serine (Ser), threonine (Thr), histidine (His), proline (Pro) and arginine (Arg) levels and sought relationships in sera from 73 pediatric ALL patients, who received ASNase-containing chemotherapy. Results: Asparaginase activity averaged 0.4±0.34 IU/ml (mean±SDEV) in all specimens. All AA decreased after treatment, ranging from 18.6%-82.6% of control. Asparaginase activity of 0.7 IU/ml provided 90% Asn and Gln deamination. The data were dichotomized in subsets of low ASNase (range 0.02-0.39 IU/ml, mean=0.17±0.09 IU/ml) and high ASNase (range 0.4-1.69 IU/ml and mean=0.72±0.32 IU/ml). Asparagine and Gln % deamination values were correlated with ASNase activity (p=0.0002 and p=0.0001). Similarly, decreases of Arg and Ser levels were also correlated, p=0.0009 and p=0.032, respectively. In the high ASNase subset, a 39% decrease of Arg and 26% of Ser was obtained. Low ASNase activity was correlated with lower Asn and Gln % deamination and with moderate decrease of Ser (14.6%) and Arg (19.6%). Threonine, Pro and His also decreased, but no correlations were obtained with ASNase activity. Conclusion: Asparagine, Gln and five other AA declined during ASNase treatment. Asparagine and Gln % deamination values are highly correlated with serum ASNase activity. Asparaginase may indirectly cause moderate depletion of serum Arg and Ser levels, providing an enhancement in leukemia blasts apoptosis. Toxicity from the ASNase and other drugs could enhance the decrease of AA serum levels. Further studies are needed to verify these findings and their potential clinical importance in the treatment of ALL patients.*

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Asparaginase (ASNase) is an important drug for treatment of childhood acute lymphoblastic leukemia (ALL) (1, 2). Its ability to catalyze asparagine (Asn) and glutamine (Gln) deamination in the central circulation makes it specifically useful in ALL, because most lymphoblasts cannot effectively synthesize Asn and they undergo apoptosis (1, 2). Glutamine can be used as an amino-group donor by the mammalian enzyme asparagine synthetase (AS) to synthesize Asn de novo, primarily in the liver, and release it in the circulation. Asparagine deamination alone may be insufficient to induce apoptosis in T-lymphoblasts *in vitro* (3).

In addition to ASNase-induced deamination, malnutrition and altered metabolism of all other nutrients are most probably related to leukemia and chemotherapeutic stress, which causes imbalance of metabolism in favor of catabolic processes (4, 5). The mechanisms of tumor-induced malnutrition were investigated in pediatric oncology and include increased catabolism by tumor growth and decreased nutrient intake secondary to anorexia, emesis, diarrhea and occasional intestinal obstruction (4-6). Alterations in amino acid (AA) and protein metabolism with negative nitrogen balance are measurable in cancer-bearing adults and children even before the onset of anorexia or weight loss (6).

A series of cancer-related cytokines and tumor-derived growth factors can mobilize fatty acids and AA from

adipose tissue and skeletal muscle (5). An increased glucose requirement by many tumors produces an increased metabolic demand on the liver, resulting in increased energy expenditure (5) and accelerated gluconeogenesis. In addition, the imbalanced AA nutrition has been proposed as a potential mechanism of enhancing the cytotoxic effects of other chemotherapeutic agents in leukemia (2, 7-9).

Asparaginase treatment is lethal to T-lymphoblasts and possibly other leukemic cells, which may lack ability to up-regulate asparagine synthetase (AS), thus depending on an external supply of Asn from extra-cellular sources (1,2,10). Therefore, Asn, which is non-essential to normal cells, is conditionally essential to the AS-deficient cells, which undergo apoptosis, primarily because of the inhibition of protein biosynthesis (1-3).

Asparaginase has a unique spectrum of host toxicity dependent on its ability to cause immediate cessation of protein synthesis, since virtually all proteins contain Asn and Gln (2,10). The changes that affect the proteins of the coagulation system have considerable clinical importance because they may induce bleeding and thromboembolic events and may be associated with life-threatening complications when the central nervous system is involved (10-12). Other organ systems potentially affected by relevant functional disorders are the pancreas and the liver (10-12). A recent study that evaluated liver biopsy specimens, serum liver function tests and lipid profiles found that none of the 27 pediatric ALL patients had entirely normal liver histology during modern intensified therapy (11). Fatty infiltration was detected in 25 out of 27 patients (93%) and siderosis in 19 out of 27 (70%); 14 out of 27 (52%) had both. Serum liver enzymes reach fairly high levels (11). In addition to the therapeutic deamination by ASNase, chemotherapy impairs liver function, most probably causing reduced capabilities to maintain homeostasis of alerted serum AA levels (10-12).

In view of the accepted ASNase mode of action, the highest prognostic pharmacologic marker of ASNase therapy could be Asn and Gln deamination. These were predicted by the ASNase enzymatic activity in newly diagnosed patients (1). Possible changes of serum levels of other AA may contribute to the antileukemic or adverse effects of ASNase treatment. We report here the results from the pharmacodynamic (PD) analyses of the database of monitored ASNase activity and Asn, Gln and five other AA levels in sera from 73 HR ALL patients, which could elucidate useful information on ASNase mechanism of action.

Materials and Methods

Patients, treatment protocol and serum specimens. Newly diagnosed ALL patients with Rome/NCI higher risk features were entered in the ASNase biology study from CCG-1961, the randomization arms and treatment regimens of which are described elsewhere (13). Before and after ASNase administration, serum specimens were collected from each patient during the various phases of therapy. Samples were shipped on dry ice and stored at -80˚C to prevent *ex vivo* AA deamination, as reported previously (1). Multiple serum samples were assayed and decipherable data was gathered from 1001 patients. The specimens from 73 randomly selected antibody-negative patients' sera (n=93 pairs) were analyzed for ASNase enzymatic activity and AA concentrations. The demographic and clinical characteristics at diagnosis of the randomly selected 73 patients for this study are super-imposable with similar characteristics of all 1001 patients included in ASNase biology studies (13).

Determination ASNase enzymatic activity and amino acid levels. Asparaginase activity was measured by the ammonia nesslerization method (1, 13), and AA concentrations by precolumn derivatization and reversed-phase high-performance liquid chromatography (HPLC), as reported (1). In addition to Asn and Gln, five other glucogenic AA were chosen for investigation, based on preliminary observed changes of their serum levels during ALL treatment. Namely, three essential AA: threonine (Thr), histidine (His) and arginine (Arg) and two nonessential AA: serine (Ser) and proline (Pro), in addition to Asn and Gln, were examined. For all AA peaks area under the curve (AUC) was gathered from the HPLC-linked MAXIMA computer program. The corrected absolute concentrations were calculated for Asn and Gln and the other AA serum levels changes were calculated by comparison of the before and after ASNase treatment values of the AUC_{AA}/AUC_{IS} ratio and expressed as the percentage of control.

Statistical methods. Amino acid deamination (decrease) was expressed as a ratio of post- to pretreatment (post-Tx, pre-Tx) percentage of control according to the equation:
% AA Deamination (Decrease) = [(AA pre-Tx – AA post-Tx) / AA pre-Tx] x 100%.
The average ± standard deviation (SD) (or standard error of means [SEM]) values were calculated for the whole database. Further subdivision of these data was done by averaging various concentration ranges of ASNase enzymatic activity and the commensurate values for serum AA levels changes, expressed as percentage of control. Unpaired, two-sample, unequal variance *t*-tests were used for statistical comparison of data series from various subgroups. Serum levels of AA were defined as unchanged if the range of change was less than 10% of the control.

Results

Patient characteristics. Demographic and clinical characteristics at diagnosis of our 73 randomly selected subjects are super-imposable with similar characteristics of all 1001 HR ALL patients from CCG-1961 included in the ASNase biology studies (13). There were one Down's syndrome and one Ph+ patient and another with unbalanced t(1:19) translocation. Institutional immunophenotypes for CD2, CD7, CD10 and CD19 were ≥ 30% in 15.5%, 22.2%, 81.4% and 84.3% of cases, respectively. Central review of

Table I. *A. Two sub-groups by < or ≥ 0.4 IU/ml ASNase activity and commensurate % of changes (or deamination) of the serum AA levels.*

ASNase, IU/ml	% of Changes of the AA levels (Mean±SD)					% Deamination (Mean±SD)		# samples
	Ser	Thr†	His†	Pro	Arg†	Asn	Gln	
0.17±0.09	14.64±36.63	17.12±37.61	31.06±25.99	19.71±34.19	19.61±34.08	78.41±18.04	66.94±29.54	N=55
0.72±0.32	26.04±21.79	20.62±48.12	28.19±49.72	20.46±77.98	39.12±22.84	88.63±8.10	86.82±19.60	N=38
*p**	0.032	0.35	0.37	0.48	0.0009	0.0002	0.0001	N=93

† Essential AA. * Non-paired *t*-test evaluations.

Table I. *B. Sub-groups according to narrower ranges of ASNase activity and commensurate % of changes (or deamination) of the AA levels (Mean±SEM).*

N	ASNase, IU/ml	Ser % of Change	Thr % of Change	His % of Change	Pro % of Change	Arg % of Change	Gln Deam%	Asn Deam%
14	0.07±0.01	9.04±8.96	20.10±12.4	36.29±6.11	9.77±11.9	18.06±7.64	60.59±8.29	74.19±4.63
21	0.15±0.01	6.87±9.71	10.67±8.24	29.07±5.77	24.53±7.03	22.79±7.59	68.67±5.82	77.17±4.05
14	0.24±0.01	22.22±4.11	16.83±9.05	23.51±8.18	16.74±7.52	14.48±12.5	62.46±9.52	80.26±5.39
6	0.34±0.01	37.24±17.8	33.38±8.58	43.44±6.68	32.90±10.2	22.31±5.39	86.17±5.09	88.28±4.41
8	0.43±0.01	17.76±7.76	15.62±7.84	44.88±10.1	49.21±8.85	37.39±8.73	77.67±8.47	84.25±4.36
9	0.55±0.01	26.88±10	3.77±27.6	28.71±7.8	8.39±40	46.70±6.74	87.75±8.97	89.12±1.42
6	0.64±0.01	29.38±8.4	23.51±19.4	0.84±48.7	20.42±22.2	36.17±4.75	78.67±7.78	89.53±2.52
9	0.79±0.03	35.20±3.94	19.78±7.61	31.34±4.35	1.42±31.5	32.47±9.17	92.16±2.67	91.94±2.26
6	1.36±0.08	18.73±8.28	50.94±11.1	27.78±8.18	28.79±16	41.31±11.4	97.76±0.94	87.88±3.62

ploidy classification determined normal ploidy in 24.4%, hypoploidy in 11.1%, pseudoploidy in 31.1% and hyperploidy in 33.3% of 45 patients. Lymphadenopathy was observed in 55.6% of 72 cases (moderate in 38.9% and marked in 16.7%). There was moderate and marked spleenomegaly in 52.8% and 11.1% of 72 patients, respectively. Massive hepatomegaly was present in 9.1% and moderate in 53.0% of 66 patients. Large mediastinal mass was observed in 9.6%. The average Hb at diagnosis in 73 patients was 8.52±2.81 g/dl.

Asparaginase activity and changes in serum levels of AA. The post-treatment ASNase levels were 0.4±0.34 IU/ml (mean±SD, n=73 patients/ 93 serum pairs), ranging from 0.02 IU/ml to 1.69 IU/ml. Glutamic acid serum levels were mostly increased and aspartic acid levels were variable. Serum levels of the other seven AA were generally decreased. As expected, the ASNase-targeted substrates decreased dramatically, from average pre-Tx serum levels of 38.9±16.2 μM (mean±SDEV) for Asn and 340.2±143.8 μM for Gln, to post-Tx serum levels of 6.3±7.1 μM and 87.9±134.9 μM, respectively. Asparaginase activity predicted Asn and Gln % deamination. On average, ASNase activity of 0.7 IU/ml provided ≥90% deamination of Asn and Gln. Among other AA, His and Arg serum levels were decreased at ~30% of control, whereas Ser, Thr and Pro serum levels were decreased at ~20%.

Correlations of the changes of the serum AA and ASNase activity levels produced 2 subgroups of data based on the ASNase level: 0.02-0.39 and 0.4-1.69 IU/ml ranges. The commensurate AA values were averaged (Table IA). Asparagine and Gln % deamination values correlated highly with serum ASNase activity in these patients, $p=0.0002$ and $p=0.0001$, respectively. Serine and Arg level changes were correlated with serum ASNase activity levels, as evidenced by p values of 0.032 and 0.009, respectively. The serum levels of His, Thr and Pro were generally decreased in the post-treatment samples. However, no association was found between serum ASNase activity and Thr, His and Pro. This was clear when the values were compared between the two subsets obtained from the higher or lower than 0.4 IU/ml ASNase activity. *P* values from *t*-tests for Thr, His and Pro were not significant (Table IA).

Table IB shows the analysis of the total database conducted according to narrower concentration ranges of ASNase activity. Commensurate values for AA levels

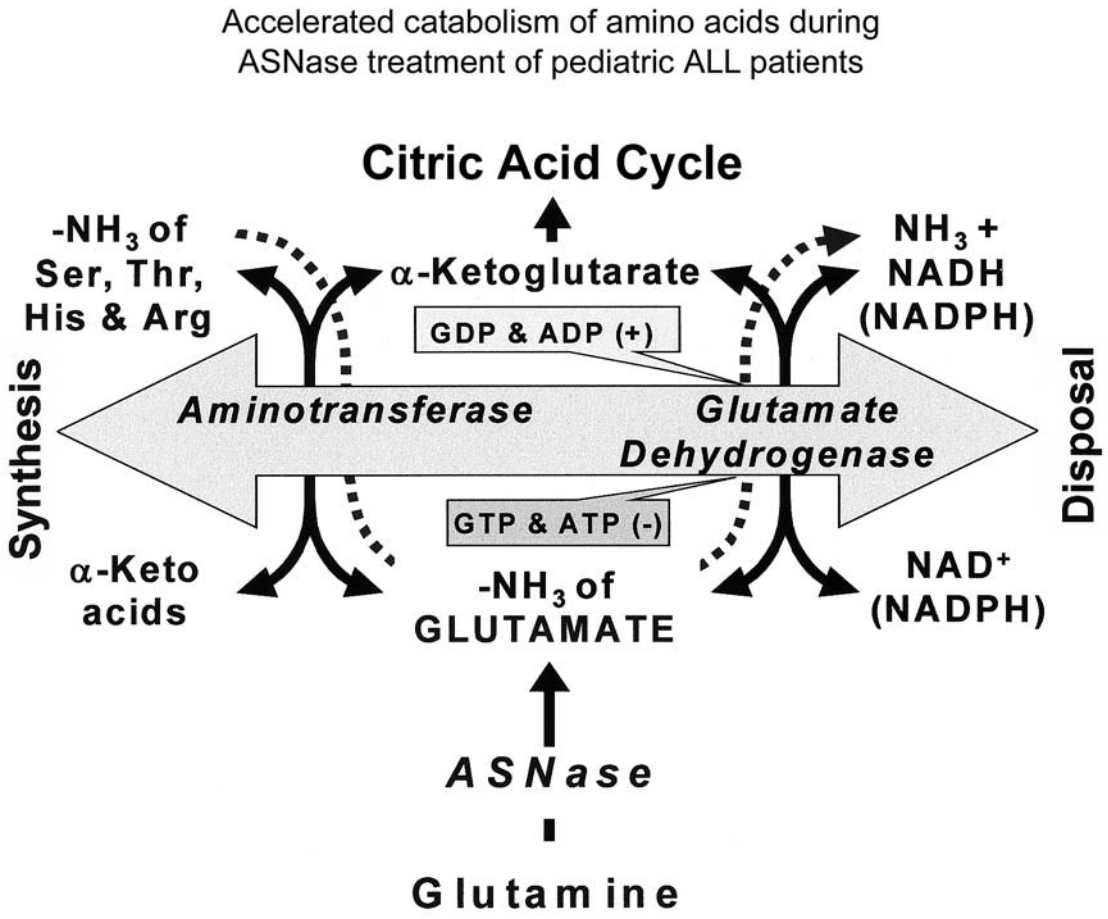

Figure 1. *Biochemical relationship between ASNase treatment and amino acids degradation are shown. The diagram represents the bi-directional reactions chain for synthesis or disposal of amino acids. The direction of these reactions depends on the relative concentrations of glutamate, a-ketoglutarate, NH_3 and the ratio of oxidized to reduced coenzymes. During ASNase treatment high glutamate levels are accumulated. At increased liver glutamate levels, the reaction proceeds in the direction of amino acid degradation and the formation of NH_3 (dotted line). Low energy states in ALL patents will accentuate this shift, since GDP and ADP are allosteric activators of glutamate dehydrogenase. a-Ketoglutarate is used in the Krebs cycle to meet the increased energy demands of patients stressed by ALL and chemotherapy.*

changes as % of control *vs.* average ASNase activity values are presented. The best-fit lines were obtained from PD analyses, which indicated the enzymatic nature of the relationships between ASNase activity and % deamination of Asn and Gln. The patterns of the curves for Arg, Ser, His and Pro also were consistent with results of the analyses presented in Table IA. Detailed analyses (Table IB) demonstrated that, in the subgroup with the highest average ASNase levels (1.36 IU/ml), there is 50.9% decrease of serum Thr levels. However, in general, no correlation was found ($p=0.35$, Table IA).

A significant proportion of serum specimens decreased with mild (10-25%) or moderate (25-75%) range levels for all AA studied. However, a profound decrease (>75% of control) was deduced significantly only for Asn and Gln, 72 (77%) and 62 (67%) out of 93 samples, respectively. In contrast, only 1-4% of specimens had profound declines of other AA (Ser, Thr, His, Pro and Arg) levels after ASNase treatment.

Since Gln is involved in the biosynthesis of multiple AA, an attempt to correlate Gln deamination (and the parallel increase of Glutamate levels) with the catabolic changes of other AA levels was made. When His and Pro were plotted *vs.* Gln % deamination, a distinct saturable zero-order biochemical pattern was obtained. The parabolic plots between Gln % deamination and Ser, Thr and Arg indicated that higher Gln depletion might be linked with decrease of Ser, Thr and Arg in serum.

Discussion

Asparaginase is among the most important drugs used for ALL treatment (1, 2, 13). In separate relapsed ALL studies, mean ASNase concentrations of 0.75 IU/ml (14) and depletion of Asn (<3 μM) and Gln (<127 μM) (15) were correlated with improved treatment outcome. Our PD analyses produced ASNase enzyme-substrate relationships for Asn and Gln deamination in HR ALL patients, similar to standard-risk ALL patients (1). Asparaginase < 0.4 IU/ml activity provided sub-optimal Asn and Gln deamination (Table IA) and ASNase activity 0.75 IU/ml provided ≥ 90% deamination of Asn and Gln. Thus, ASNase significantly contributes to remission induction in ALL patients by deaminating the Asn and Gln. To explore possible broader-spectrum effects of ASNase on AA homeostasis, we evaluated the serum level changes of five other AA.

Serum levels of AA are affected by nutrient intake and tissue uptake, but elimination and liver efflux normally maintain homeostasis (16). In stressed ALL patients, catecholamines, hormones, cytokines and drugs may shift the metabolic and AA balance toward catabolism (4-6, 17). Blood ammonia is increased by ASNase therapy and azotaemia was reported in 83% of patients (10). Subsequent NH_3 elimination may result in negative nitrogen balance (6, 10, 16, 18). Evaluation of blood and urine biochemical parameters showed significant nitrogen losses in leukemia patients (17). Asparaginase may contribute to sustaining this negative nitrogen balance.

The results of AA serum changes in 73 pediatric HR ALL patients showed that, along with Asn and Gln, at least five other AA decreased during ASNase-containing treatment (Tables IA and IB). This decline may mainly reflect the enhanced negative nitrogen balance and utilization of AA for Glu detoxification (Figure 1) (4-6, 16-18). Increased NH_3 and Glu levels may be partially detoxified by synthesis of Gln in the kidneys and by liver ureagenesis (16). Larger Glu amounts accumulated during ASNase treatment can be detoxified *via* α-ketoglutarate. High Glu levels direct this bi-directional reaction to the α-AA degradation side (16). Negative nitrogen balance in the form of Glu detoxification-NH_3 loss continuously utilizes other α-AAs in these reactions, decreasing AA levels.

Hepatotoxicity by ASNase may reduce the liver input and *de novo* biosynthesis of AAs and further impair homeostasis (10-12). In addition, disease-related factors might enhance utilization of AA in the Krebs cycle and gluconeogenesis from AA, because of increased caloric demands (4-6, 16) and facilitate glycolysis during leukemia treatment. Gut toxicity can impair AA absorption (19) and renal toxicity may decrease re-absorption or increase excretion of AA. Methotrexate-induced folate deficiency may be partially responsible for His depletion (20). Recovery from anemic states will widely utilize glycine (Gly), as a major precursor for *de novo* heme biosynthesis. That can decrease Ser, since it is converted to Gly, which is accelerated by low hemoglobine (Hb) (16); and the mean Hb at diagnosis in these patients was 8.52 g/dl. High Glu levels inhibit the His or Pro to Glu conversions (16) and the Glu➡Pro synthesis will be enhanced, which explains the pattern of His/Pro decrease (Table I). Arginine decrease at higher ASNase levels (Table IA) may be important, since Arg deaminase has a potent antileukemic effect (9). Lastly, imbalance of AA generally may enhance the anti-leukemic cytotoxicity of other drugs (2, 7-9).

We conclude that diminished serum levels of Ser, Thr, His, Pro and Arg are mainly sustained by the biochemical changes related to ASNase action and negative nitrogen balance in ALL patients. Under toxic treatment conditions, the liver is unable to completely restore the alerted AA homeostasis in serum. Along with the established role of Asn and Gln in leukemia cell kill, the decrease of other AA may contribute to the anti-leukemic or toxic effects of combination treatment and need to be elucidated further.

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