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Deamination of Glutamine is a Prerequisite for Optimal Asparagine Deamination by Asparaginases *In Vivo* (CCG-1961)

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Abstract. *Background: Glutamine (Gln) deamination by asparaginase (ASNase) appears to contribute in the decrease of serum asparagine (Asn) levels and enhance leukemic cell apoptosis. The pharmacodynamic (PD) rationale is based on the role of Gln as the main amino group donor for Asn synthesis from aspartate by the enzyme asparagine synthetase (AS). Materials and Methods: Relationships between ASNase enzymatic activity and Asn or Gln levels were examined in 274 pairs of pre- and post-ASNase serum specimens from 200 high-risk acute lymphoblastic leukemia (ALL) patients from the Children's Cancer Group (CCG-1961). Data were analyzed according to a novel PD model based on previous best-fit projections (NONMEM) from the CCG-1962 standard-risk ALL study. Results: The PD results from high-risk and standard-risk ALL patients were superimposable. The percentages of Asn and Gln deamination were predicted by ASNase activity in patients' sera. Pharmacodynamic analyses strongly suggested that >90% deamination of Gln must occur before optimal Asn deamination takes place in vivo. Asparaginase activity ≥ 0.4 IU/ml yielded mean Gln and Asn % deamination values of 90%. Lower ASNase concentrations yielded lower Gln or Asn % deamination. This ASNase concentration coincides with the in vitro determined IC_{50} value on CEM/0 human T-lymphoblastic leukemia cells. Conclusion: Asparaginase activity of ≥ 0.4 IU/ml provided optimal Asn and Gln deamination in high-risk ALL patients. Deamination of Gln correlates with enhanced serum Asn deamination in vivo. Therefore, deamination of Gln may enhance the antileukemic effect of ASNase.*

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The unique ability of asparaginase (ASNase) to catalyze Asn and Gln deamination in blood makes it useful in treating acute lymphoblastic leukemia (ALL), since ALL lymphoblasts cannot synthesize Asn *de novo*, thus leading to cellular apoptosis (1-5). ASNase preparations used in the United States include native *E. coli* ASNase and its pegylated formulation (PEG-ASNase) (1-3, 6). A randomized comparison of E. coli ASNase *vs.* PEG-ASNase for childhood standard-risk ALL (CCG-1962) showed rapid clearance of lymphoblasts from days 7 and 14 bone morrow aspirates in patients on the PEG-ASNase arm. This was supported by the PK fact that ASNase activity, determined as area under the time curve (AUC), was prolonged in patients treated with PEG-ASNase as compared with the native enzyme (1). Abshire *et al.* found a correlation between PEG-ASNase activity and 2nd remission rate in relapsed ALL patients, with an average concentration of 0.75 IU/ml in the complete response (CR) group *vs.* 0.45 IU/ml in non-responders (6). These and other studies suggested that ASNase activity of > 0.1 IU/ml may be necessary to achieve appreciable serum Asn depletion in the majority of patients (1, 6-10). Therefore, a more precise definition of ASNase PK-PD effective parameters is needed.

There is significant interpatient variability of ASNase, Asn and Gln concentrations in serum of standard-risk (SR) ALL patients after treatment (1, 11-13). When ASNase enzymatic activity is present (1, 14), the highest prognostic PD marker of drug efficacy is the sustained Asn deamination, which may be further enhanced by Gln deamination (12). Furthermore, Gln depletion most probably is an important contributing element in the antileukemic effect of ASNase, because Gln has many important roles in cellular metabolism and protein biosynthesis (15). Although Gln has been classified as a nonessential amino acid, in major trauma, surgery, sepsis,

bone marrow transplantation, intense chemotherapy and radiotherapy, when its consumption exceeds its biosynthesis, it becomes a conditionally essential amino acid (16).

Asparaginase-refractory T-cell lines up-regulated the low level of AS activity in monoclonal lines in response to ASNase treatment (17). In addition, the impact of AS on drug resistance to ASNase is associated with post-ASNase treatment (Tx) augmentation of AS expression. The attempts to explore relationships between ASNase sensitivity and AS mRNA expression *in vitro* may be of limited usefulness due to the *in vivo* importance of Asn input from the hepatic circulation (4, 12, 18, 19). Based on this information, we hypothesized that ASNase-induced Gln deamination contributes to the optimal deamination of Asn in patients' serum and that the glutaminase activity of the ASNase formulation may have a significant therapeutic contribution in ALL (1, 4, 12, 18, 19, 21).

We report here the results from the PD studies in high-risk (HR) ALL patients from CCG-1961. These investigations evaluated the pattern of amino acid (AA) deamination in correlation with the enzymatic activity in HR ALL patients, compared these results from HR with SR ALL patients, defined the pharmacodynamically optimal ASNase concentrations and explored the possible contribution of Gln deamination to sustained Asn deamination.

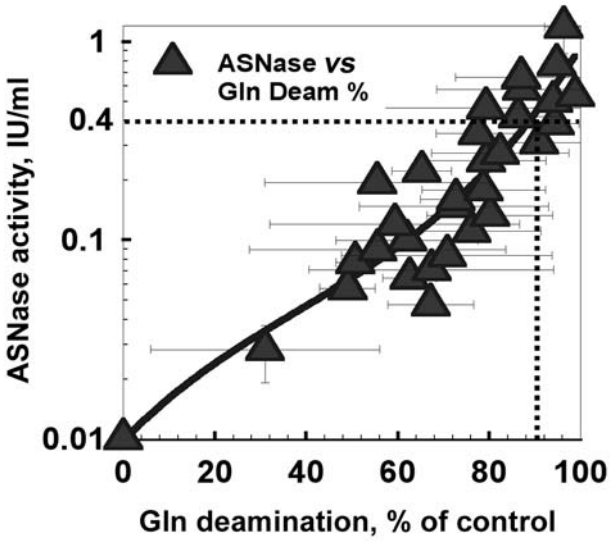


Figure 1. *Relationship between ASNase activity and Gln concentrations in serum from 200 HR ALL patients expressed as percent of control. The average % deamination of Gln was predicted by the ASNase activity based on the best-fit model obtained by the NONMEM program in these patients. ASNase levels ≥0.4 IU/ml provided ≥90% Gln % deamination in serum.*

Materials and Methods

Patients and CCG-1961 protocol. From November 1996 to May 2002, patients with newly diagnosed ALL and Rome/NCI high-risk features were entered in the randomized prospective ASNase biology study of CCG-1961. Details of the CCG-1961 randomization arms and treatment regimens are described elsewhere (14).

Serum specimens. Pre-ASNase (control) and post-ASNase serum specimens from 1001 patients were collected per CCG-B951 biology protocol, shipped on dry ice and stored at -80˚C, to prevent *ex vivo* AA deamination (1, 12, 14). Most HR ALL patients (60%) had developed neutralizing anti-ASNase Ab (14). A smaller % of patients (29%) had positive ASNase activity in serum that ranged from 0.015 to 1.89 IU/ml. Of those, Asn and Gln concentrations were measured at two log-different ASNase activity levels in 274 paired pre- and post-treatment serum samples from 200 patients.

Determination of ASNase enzymatic activity and amino acid levels in patients' sera. Asparaginase activity was measured by the nesslerization of ammonia method and the AA concentrations by precolumn derivatization and reversed-phase high-performance liquid chromatography (HPLC) (1, 12, 14).

Statistical methods. Asparagine and Gln deamination were expressed as % of post- to pre-treatment serum levels. The best-fit model for relationships between serum ASNase activity and AA deamination was derived from the nonlinear mixed effects model (NONMEM) analyses for SR ALL (CCG-1962) (1). These PD principles were used in this study to project the enzyme-substrate relationships. Further, considering that Gln is biochemically important in the *de novo* Asn biosynthesis, we stratified the database according to Gln % deamination ranges. These analyses produced subsets of patients with statistically identical average ASNase activities, while they had different ranges of Gln % deamination values in each set. Commensurate Asn deamination parameters within each of these subsets were also examined. Finally, nonpaired *t*-tests were used for statistical evaluations.

Results

Serum ASNase activity and amino acid deamination. Pharmacodynamic NONMEM projections of ASNase activity *vs.* Asn or Gln % deamination from the CCG-1962 SR ALL population were superimposable with analogous PD projections in 200 HR ALL patients with the utilization of the nearest-neighbor statistical method resulting in a series of 30 mean of means values (Figure 1). Furthermore, the dichotomization of the 274 serum pairs database according to ASNase activity ranges demonstrated that a better PD profile was obtained at ≥0.4 IU/ml ASNase concentrations for all averaged commensurate PD parameters of AA deamination ($p \leq 0.001$-0.02, non-paired *t*-tests, Table I). Asparaginase activity <0.4 IU/ml yielded a mean Gln % deamination of 68%±32%, whereas ASNase activity ≥0.4 IU/ml yielded a statistically different mean value of 91%±17.4% ($p<0.001$) (Figure 1). At lower ASNase concentrations (0.16±0.1 IU/ml, mean±SDEV) Asn was statistically better deaminated than Gln, 75% *vs.* 68%, respectively ($p=0.005$). However at 0.7±0.3 IU/ml ASNase activity levels, Asn and Gln % deamination values were >

90% with no statistical difference between these amino acids ($p=0.43$), (Table I).

Asparaginase activity of < 0.1 IU/ml provided insufficient depletion of Asn and Gln in the range of deamination at 0-60% of control. Therapeutically adequate Asn deamination (<3 μM) was observed in 24 out of 121 post-treatment samples with an average ASNase activity of 0.11 IU/ml. Therefore, a greater minimum ASNase concentration than 0.1 IU/ml is needed.

Effect of Gln deamination on Asn depletion. PD analyses suggested that >50% of Gln deamination might be necessary for optimal Asn deamination (≥90%). To investigate this finding the database was first stratified by ASNase activity in the ranges of 0.015-0.099, 0.1-0.249, 0.25-0.49 and 0.5-1.89 IU/ml with n=78, 79, 63 and 54 sample pairs, respectively (Figures 2A and 2B). Then, in each of the 4 groups, 3 subsets of data were identified with different Gln % deamination ranges (*e.g.*, 0-59.9%, 60-94.5% and 95-99.9%), each with statistically similar ASNase enzymatic activities ($p=0.11$ to $p=0.93$). These analyses demonstrated that post-treatment Asn levels decreased (Figure 2A) and the proportion of samples with <3 μM Asn (Figure 2B) improved with increased % Gln deamination, independent of ASNase activity in each subset of PD database. This finding strongly suggested that greater deamination of Gln is required for better/optimal Asn deamination under similar ASNase serum concentrations.

Table I. *Effect of lower (<0.4 IU/ml) or higher (>0.4 IU/ml) ASNase concentrations on Gln and Asn deamination (n=200 pts, 274 serum pairs).*

Sample subsets	ASNase conc. <0.4IU/ml	ASNase conc. ≥0.4IU/ml	*P* values from *t*-tests
n, serum pairs	196	78	
ASNase activity, IU/ml	0.16±0.1*	0.7±0.3*	0.002
Post-Tx Gln, μM	114±143*	26.2±51.2*	0.02
Gln % deamination	68.1±32.1*	90.9±17.4	<0.001
Post-Tx Asn, μM	9.98±10.6*	3.89±3.8*	0.02
Asn % deamination	74.8±23.3*	89.2±9.2*	0.002
Samples with <3μM Post-Tx Asn	53 (27%)	36 (46.2%)	
t-tests between Asn and Gln % deamination	$p=0.005$	$p=0.43$	

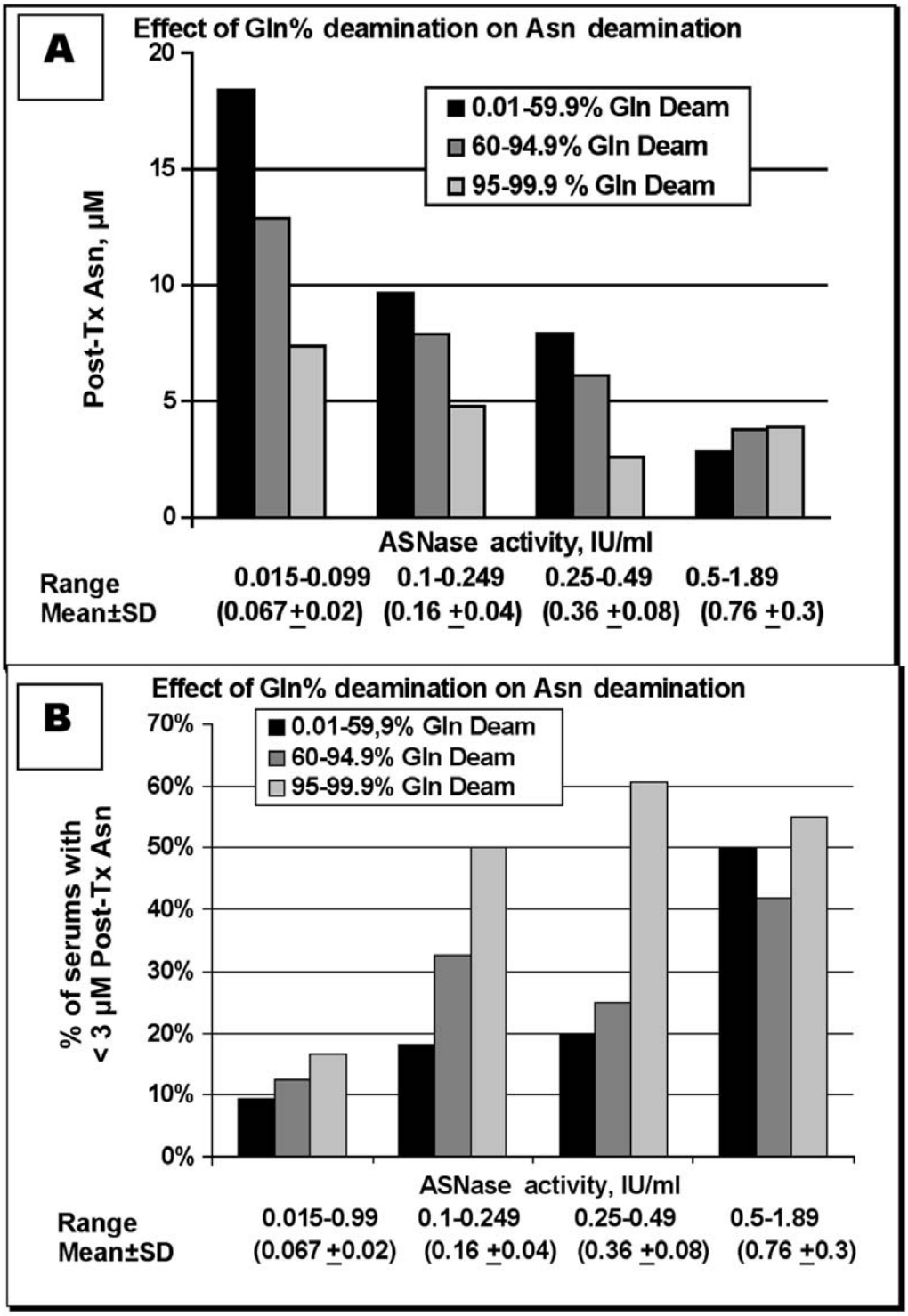
* (mean ±SD)

Discussion

Predictive PD parameters are required for optimal individualization of the pediatric ALL therapy. Asparagine and Gln % deamination were predicted by serum ASNase activity in SR ALL (1). The present study reveals similar relationships of ASNase activity *vs.* Asn or Gln % deamination in the sera of 200 HR ALL patients according to the best-fit population PK-PD model from the NONMEM projections from SR ALL patients (1). These correlations are independent of disease characteristics and were consistently shown in various data subsets from the CCG-1961 HR ALL study population. This fact validates the conclusions derived from these PD analyses. These findings can be applied to the ASNase PD in the pediatric ALL population and can be used for individualizing the treatment in a given patient with this class of antileukemic drug.

Asparaginase activity < 0.4 IU/ml provided insufficient deamination of Asn and Gln in the majority of the patients studied. In contrast, serum ASNase activity ≥ 0.4 IU/ml, with an average of 0.7 IU/ml, had an optimal PD effect, *e.g.*, ≥90% statistically non-different deamination of both AA (Table I, Figure 1). Boos *et al.* have reported Gln depletion by higher doses of native ASNase (11). Similarly, Abshire *et al.* reported high ASNase activity levels in 2nd CR patients with ALL, which averaged 0.75 IU/ml in the POG-9310 study (6). Furthermore, the deamination intensities of Asn and Gln in the CCG-1941 study of relapsed pediatric ALL were independently correlated with a superior therapeutic outcome (20). Thus, the optimal 14-day trough ASNase drug levels of 0.7-0.75 IU/ml are pharmacodynamically and therapeutically relevant and desired.

Glutamine deprivation may have an additional antileukemic effect and enhance the cytotoxicity of other antileukemic agents, because Gln is required for *de novo* purine and protein biosynthesis by leukemic cells (15, 16, 21, 22). Glutamine is the amino-group donor, for the *de novo* Asn biosynthesis catalyzed by mammalian AS (16, 23). This last supposition was proven by our PD analyses showing that profound Gln deamination significantly correlated with optimal Asn deamination (Figure 2A and 2B), thus enhancing the *in vivo* PD effectiveness of ASNase. That Gln deamination contributes importantly to the antileukemic effect of ASNase was supported further by the results of *in vitro* experiments of PEG-ASNase-induced cytotoxicity against CEM/0 leukemia cells (17, 24). Asparagine depletion alone is not correlated to leukemic cell kill, unless intense deamination of Gln is induced by ≥0.4 IU/ml ASNase concentrations (24). Specifically, at 0.75 IU/ml of ASNase activity, when ≥95% of Gln is deaminated, virtually all human leukemic cells (CEM/0) undergo apoptosis *in vitro*. These findings suggested that Gln deamination plays an important role in the antileukemic efficacy of ASNase, due to the inhibition of the *de novo* Asn biosynthesis by mammalian AS in leukemia cells *in vitro*, as it is also observed in liver cells *in vivo* (19).



Figures 2A and 2B. *The effect of Gln percent deamination against Asparaginase concentration and optimal Asn deamination post drug treatment is shown here. The total database from 200 patients was subdivided into 4 groups according to ASNase activity levels then it was stratified according to Gln deamination % ranges. Subsets with statistically non-different average ASNase activities, at the same time with different ranges of Gln % deamination values in each were created. The bars depict commensurate Asn deamination parameters within each Gln deamination subset in four groups according to ASNase activity. Figure 2A represents post-treatment serum Asn and Figure 2B demonstrates % of samples with <3µM post-treatment Asn in a given subset as parameters of effective Asn deamination. In any given ASNase activity range, Asn depletion is better with greater Gln % deamination; however, at higher ASNase activity levels, Gln deamination is less critical for Asn deamination, most likely due to diminishing effect.*

We recently have reported PD model-based simulations and comparisons of Asn deamination between the different clinically relevant regimens of Erwinase, *E. coli* ASNase, and PEG-ASNase allowing PK-PD equivalent dose-drug-regimen substitution while alternating the formulation (25). These studies demonstrated that an effective Asn deamination required sufficient ASNase 14-day trough concentrations, which achieved optimal Gln deamination. In summary, the effective deamination of Gln, which is induced by ASNase activity of ≥0.4 IU/ml, sustains optimal Asn depletion by depriving the AS of Gln for its Asn biosynthesis. Furthermore, Gln depletion enhances the anti-leukemic effect of ASNase therapy (24). Verifications of these PD studies in new ALL clinical trials are sought.

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