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ORIGINAL ARTICLE

**Masahito Tsurusawa · Motoaki Chin · Asayuki Iwai
Keiko Nomura · Hideaki Maeba · Takashi Taga
Takeshi Higa · Tomoko Kuno · Toshinori Hori
Akiko Muto · Miyo Yamagata**

L-Asparagine depletion levels and L-asparaginase activity in plasma of children with acute lymphoblastic leukemia under asparaginase treatment

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Abstract *Purpose*: To determine the minimum levels of L-asparaginase (ASNase) activity necessary to maintain L-asparagine (Asn) depletion under ASNase treatment in acute lymphoblastic leukemia (ALL). *Methods*: We measured ASNase activity using an enzyme coupling method with a limit of detection of 2 U/l and examined the relationship between ASNase activity and Asn levels in blood samples from 14 children with ALL. *Results*: In all but one patient showing high ASNase antibody titers, minimum ASNase activity to maintain Asn depletion levels below the limit of detection (40 ng/ml) ranged from 6 to 180 U/l with a median value of 16 U/l. In 11 patients, the enzyme activity corresponding to minimum detectable Asn levels ranged from 2 to 32 U/l with a median value of 6.5 U/l. Patients with an ASNase activity of 2 U/l or an undetectable activity (<2 U/l) had nearly normal Asn levels: 4140 ± 1161 ng/ml at 2 U/l and 7235 ± 3107 ng/ml at <2 U/l (mean $\pm$ SD). Statistical analysis showed that ASNase activity in the range of 2–32 U/l was inversely correlated with Asn levels ($r = -0.803$, $P = 0.001$). *Conclusion*: These results show that Asn levels are strongly correlated with plasma ASNase activity even at low enzyme activities (<50 U/l) and that this sensitive ASNase assay can be used to estimate plasma Asn depletion levels.

M. Tsurusawa (✉) · M. Chin · A. Iwai · K. Nomura
H. Maeba · T. Taga · T. Higa · T. Kuno · T. Hori · A. Muto
Japanese Children's Cancer and Leukemia Study Group (JCCLSG) Central Office, Department of Pediatrics, Faculty of Medicine, Aichi Medical University, 480-1195 Aichi, Japan
E-mail: tsuru@aichi-med-u.ac.jp
Tel.: +81-56162-3311
Fax: +81-56162-2886

M. Yamagata
Pharmaceutical Research Institute Branch Office, Kyowa Hakko Kogyo Co. Ltd., 411-8731 Shizuoka, Japan

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Abbreviations *ALL* Acute lymphoblastic leukemia · *Asn* Asparagine · *ASNase* Asparaginase · *SSA* Sulfosalicylic acid

Introduction

The antileukemic effect of L-asparaginase (ASNase), an important component of therapy for acute lymphoblastic leukemia (ALL), is believed to result from the inhibition of protein synthesis in leukemic cells that do not express a sufficient level of asparagine synthetase to synthesize asparagine (Asn) [7, 11, 13]. Since it is assumed that the pharmacologic effect of ASNase depends on the depletion of Asn from the circulating pool of amino acids, determination of the degree and duration of Asn depletion from blood is necessary to monitor the efficacy of the enzyme [12]. However, routine monitoring of Asn levels is still a laborious task in clinical practice because the accurate measurement of plasma Asn levels under ASNase treatment requires the rapid inhibition of persistent ASNase in the blood samples [3], for which a deproteinization procedure using sulfosalicylic acid (SSA) is currently employed [8]. It is desirable to estimate Asn depletion levels by measurement of the ASNase activity, but available pharmacokinetic data on ASNase treatment have not defined the minimum levels of ASNase activity required to hydrolyze Asn in vivo and ex vivo [1, 2, 5, 6, 15, 16, 20].

In this study, we measured ASNase activity using an enzyme coupling method with a lower limit of detection of 2 U/l in 14 children with ALL. Asn levels were also measured in two blood samples with or without deproteinization by SSA. The results indicate that Asn levels are strongly correlated with plasma ASNase activity even at low enzyme activities (<50 U/l).

Materials and methods

Patients

Entered into this study were 12 children with newly diagnosed ALL and 2 with relapsed ALL. Newly diagnosed patients were treated with the Japanese Children's Cancer and Leukemia Study Group (JCCLSG) ALL-2000 protocols. The Regional Ethics Committee approved the study protocol. Verbal and written information about the study was given to the parents and written informed consent was obtained. If appropriate, informed consent was also obtained from the child. The patients were stratified to standard-risk or high-risk groups based on age and leukocyte counts at diagnosis [19]. The four-drug regimen (vincristine + prednisolone + ASNase + Adriamycin) was employed as induction therapy for the newly diagnosed patients. In this regimen, nine doses of ASNase (Kyowa Hakko, Japan) at 2000 U/m^2 were given intramuscularly three times a week starting on day 9. After remission had been achieved, the patients received intensified ASNase treatment: standard-risk patients received two weekly doses of ASNase at 2000 U/m^2 every 6 weeks for 6–18 weeks and high-risk patients received one dose of ASNase at 6000 U/m^2 weekly for 6–11 weeks. One of the patients with recurrent ALL was treated with the high-risk ALL-2000 protocol and the other patient was treated according to the ALL-REZ BFM protocol [10].

Sample collection

Blood samples were obtained when the last dose of intensified ASNase treatment was administered in the JCCLSG protocol or when the last dose of ASNase was administered in course R1 of the BFM protocol. Blood samples for ASNase activity and Asn level measurements were collected on day 0 (just before administration of the last dose) and every 2 to 3 days for 2 weeks as part of routine laboratory testing. ASNase antibodies were also measured in samples on day 0. Samples were placed in heparinized tubes and centrifuged at −4°C. The plasma was then divided into three parts: one was deproteinized by adding an equal volume of 10% (w/v) SSA, the second was immediately frozen for Asn determination, and the third was frozen for measurements of ASNase activity and antibodies.

Measurement of ASNase levels

A series of enzyme reactions are triggered when ASNase catalyzes the substrate L-asparagine to produce L-aspartate in the presence of 2-oxoglutarate, NADH, and the conjugating enzymes L-glutamic oxaloacetic transaminase (GOT) and L-malate dehydrogenase (MDH). Through these reactions, NADH is oxidized and the absorbance of the reaction solution is decreased. We measured the ASNase levels using this series of reactions [4]. To the patient's plasma in a 96-well plate was added a mixed reagent solution of 2-oxoglutarate, NADH, GOT and MDH. The plate was allowed to stand at 37°C and, after the addition of L-asparagine solution to each well, placed in a plate reader and reacted at 37°C for 5 or 45 min to measure the decrease in absorbance at 340 nm. The same procedure was applied to a standard solution of known ASNase level (phosphate buffer solution containing BSA) to produce a calibration curve. To correct for the effect of L-aspartate contained in the patient's plasma, the same procedure was carried out simultaneously by adding phosphate buffer solution instead of L-asparagine solution continuously to obtain a blank correction. The determination limit of this method was 2 U/l.

Measurement of the L-asparagine level

The patient's plasma was mixed with an equal volume of SSA under ice cooling. The mixture was centrifuged and the supernatant was used as the sample solution. A given volume of the sample solution was automatically injected into an amino acid analysis system. The amino acid analysis was performed by RP-HPLC using precolumn derivation with *o*-phthalaldehyde and subsequent fluorescence detection according to the method of Yasui [21]. The lower determination limit of this method was 40 ng/ml.

Measurement of anti-ASNase IgG and IgE antibody titers

Anti-ASNase IgG antibody and IgE antibody titers in patients' samples were measured by the ELISA methods described by Tsukimoto et al. [18] and by Takatsuka et al. [17], respectively.

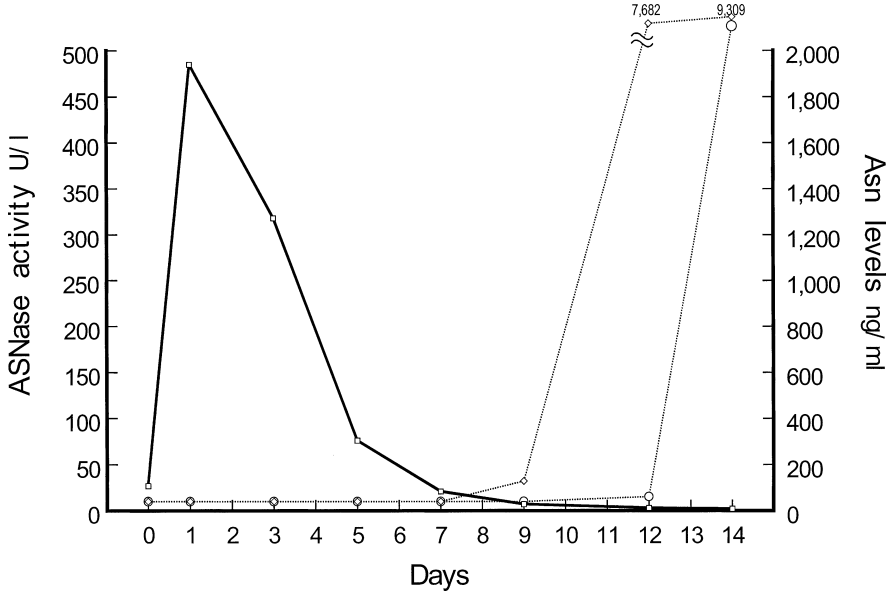

Fig. 1 Plasma ASNase activity and Asn levels after the last injection of L-asparaginase at 6000 U/m^2 in patient 11. Blood samples on day 0 were obtained just before the administration of ASNase (□ trough ASNase activity, ◇ Asn levels in plasma with SSA, ○ Asn levels in plasma without SSA)

Statistics

The correlation between Asn levels after deproteinization with SSA and plasma ASNase activities was assessed by Spearman's rank correlation test. SPSS statistical analysis software (SPSS 9.0 J) was used for all computations.

Results and discussion

The mean (±SD) baseline level of Asn in plasma obtained from 11 children with ALL before ASNase treatment was 7045±1785 ng/ml. In the 14 patients studied here, Asn levels were strongly correlated with plasma ASNase activity. Figure 1 shows a representative patient (patient 11, Table 2), in whom Asn levels in the deproteinized samples were below the level of detection (<40 ng/ml) until day 7 (ASNase activity 21 U/l), rose to 129 ng/ml on day 9 (ASNase activity 7 U/l), increased dramatically to 7682 ng/ml on day 12 (ASNase activity 3 U/l) and then increased further to 9309 ng/ml on day 14 (ASNase activity <2 U/l). In this patient, Asn levels were significantly lower in untreated plasma samples than in deproteinized samples from day 9 to day 14. These results show that very small amounts of residual ASNase (2–7 U/l) in the plasma sample can hydrolyze Asn before measurement [3].

Tables 1 and 2 show plasma Asn levels, ASNase activity and antibody levels in 14 patients. In all patients except one (patient 14 with a high antibody titer), minimum ASNase activity to maintain Asn depletion levels below the limit of detection ranged from 6 to 180 U/l with a median value of 16 U/l. This finding is in accordance with data reported by other investigators [1, 2, 5, 6, 16] and strongly suggests that the recommended plasma level of 100 U/l to secure Asn depletion is not required in all patients. In 11 of these 13 patients, the enzyme activity corresponding to minimum detectable Asn levels ranged from 2 to 32 U/l with a median of 6.5 U/l. In the other two patients (patients 9 and 13), Asn depletion (<40 ng/ml) persisted for the observation

Table 1 Plasma ASNase activities and Asn levels in children with ALL treated with 2000 U/m^2 ASNase. Some Asn levels and ASNase activities after day 1 are not shown in the table because they were less important (*ND* not detected)

Patient number	Day	Asn (ng/ml)		ASNase (U/l) enzyme coupling	Antibodies (U/ml)	
		Plasma	Plasma + SSA		IgG	IgE
1	0	ND	ND	82	ND	ND
	1	ND	ND	178		
	3	ND	ND	58		
	6	ND	210	9		
	8	4,830	5,600	2		
	10	5,580	5,660	<2		
2	0	ND	ND	11	ND	ND
	1	ND	ND	123		
	7	ND	ND	14		
	10	ND	1,360	4		
	14	6,430	6,510	3		
3	0	ND	ND	19	3	8
	1	ND	ND	218		
	7	ND	ND	17		
	10	ND	860	6		
	13	80	1,650	3		
	14	2,100	3,610	2		
	16	3,670	3,930	2		
4	0	6,013	6,122	<2	6	324
	1	ND	ND	12		
	3	216	2,622	2		
	5	5,851	5,871	<2		
5	0	14,930	15,750	<2	3	283
	1	ND	ND	344		
	3	ND	ND	169		
	5	ND	90	32		
	7	8,270	8,120	<2		
6	0	ND	ND	107	ND	ND
	1	ND	ND	180		
	5	ND	42	19		
	7	4,000	4,940	2		
	9	7,310	6,520	<2		
7	0	ND	ND	222	ND	ND
	3	ND	ND	163		
	7	ND	ND	15		
	10	ND	98	3		
	12	4,720	4,980	<2		
8	0	ND	ND	60	ND	ND
	1	ND	ND	246		
	5	ND	ND	17		
	7	ND	2,070	4		
	9	7,000	7,720	<2		
9	0	ND	ND	243	ND	ND
	1	ND	ND	378		
	12	ND	ND	6		

Table 2 Plasma ASNase activities and Asn levels in children treated with 6000 U/m^2 (patients 10, 11, 12 and 14) or 10,000 U/m^2 (patient 13) ASNase. Patients 13 and 14 relapsed. Some data for Asn levels and ASNase activities after day 1 are not shown in the table because they were less important (*ND* not detected)

Patient number	Day	Asn (ng/ml)		ASNase (U/l) enzyme coupling	Antibodies (U/ml)	
		Plasma	Plasma + SSA		IgG	IgE
10	0	ND	ND	36	2	23
	1	ND	ND	482		
	7	ND	ND	37		
	10	ND	60	10		
	12	170	1220	7		
	14	1600	2120	4		
11	0	ND	ND	27	4	61
	1	ND	ND	485		
	7	ND	ND	21		
	9	ND	129	7		
	12	61	7682	3		
	14	2107	9309	<2		
12	0	ND	ND	140	ND	ND
	1	ND	ND	933		
	2	ND	ND	964		
	11	100	ND	14		
	13	ND	1050	6		
13	0	ND	ND	6500	ND	ND
	1	ND	ND	3430		
	10	ND	ND	36		
	13	ND	ND	12		
	14	ND	ND	9		
14	0	5850	6310	<2	1360	11,200
	1	6010	6080	<2		
	16	4900	4870	<2		

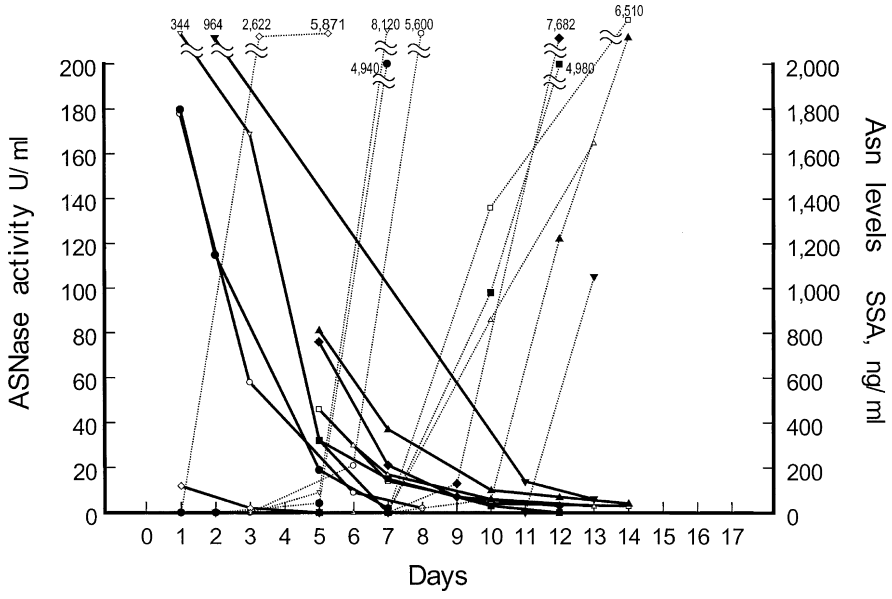

Fig. 2 Plasma ASNase activity and Asn levels after the last injection of ASNase in ten children with ALL. Each patient shows a inverse correlation between trough ASNase activity (*solid lines*) and Asn levels in plasma with SSA (*dashed lines*) (○ patient 1, □ patient 2, △ patient 3, ◇ patient 4, ▽ patient 5, ● patient 6, ■ patient 7, ▲ patient 9, ♦ patient 10, ▼ patient 11)

period (12 or 14 days). Patients with an ASNase activity of 2 U/l or an undetectable activity (<2 U/l) had nearly normal Asn levels: 4140 ± 1161 ng/ml at 2 U/l and 7235 ± 3107 ng/ml at <2 U/l (mean $\pm$ SD). The Asn levels (range 42–6510 ng/ml) after deproteinization with SSA and plasma ASNase activities (range 2–32 U/l) in 14 samples obtained from 11 patients were significantly inversely correlated by Spearman's rank correlation test ($r = -0.803$, $P = 0.001$). This inverse correlation between Asn levels and plasma ASNase activities for individual patients is shown in Fig. 2. Thus, our assay system showed that Asn levels are strongly correlated with plasma ASNase activity in the very low range of 2–32 U/l and that the detection limit is sensitive enough to estimate the asparagine depletion levels under ASNase treatment in plasma of children with ALL.

In one patient who had a relapse (patient 14) and high ASNase antibody titers, ASN activity was undetectable and Asn levels remained almost at baseline for 16 days after administration. Two other patients with high antibody levels (patients 4 and 5) also showed a rapid decline in ASNase activity and a very short duration of Asn depletion. These results suggest that "silent inactivation" by neutralizing antibodies reduces the therapeutic effect of ASNase [4, 9, 14] and that determination of antibody levels coupled with a sensitive ASNase assay is more important in monitoring the efficacy of ASNase treatment.

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