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Original Paper

Monitoring of Asparaginase Activity and Asparagine Levels in Children on Different Asparaginase Preparations

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The antileukaemic enzyme L-asparaginase is used to achieve the greatest possible reduction in blood levels of the amino acid asparagine, an essential factor for the growth of leukaemic blasts. There are two main sources of the enzyme, *E. coli* and *Erwinia*. Faced with increasing reports of treatment complications, we established a programme to monitor enzyme activity and asparagine levels in serum, in children receiving treatment for acute lymphoblastic leukaemia (ALL) and non-Hodgkin's lymphoma (NHL). Trough asparagine and asparaginase levels were measured in 49 children on induction treatment with different *E. coli* preparations (Asparaginase medac™, Crasnitin™) and in 52 children on re-induction (Asparaginase medac™, Crasnitin™, and, in the event of allergic reactions, Erwinase™) just prior to each sequential application of 10000 U/m² of asparaginase. Measurements were made by an enzyme assay and an HPLC method. During induction, both *Escherichia coli* preparations induced the desired reduction in asparagine, but the asparaginase activity with Asparaginase medac™ was significantly higher than with Crasnitin™ (median of trough levels 475 versus 74 U/l). Under re-induction treatment (median, Asparaginase medac™ 528 U/l, Crasnitin™ 49 U/l, and Erwinase™ <20 U/l) complete asparagine depletion was recorded on day 3 in more than 90% of Asparaginase medac™ samples, more than 60% of Crasnitin™ samples and in 26% of Erwinase™ samples. The latter two groups included some children with unchanged asparagine levels and no measurable enzyme activity. Different asparaginase preparations are not readily interchangeable. When Asparaginase medac™ is used instead of Crasnitin™, an identical dose will be associated with significantly higher enzyme activity, well above the level required for complete asparagine depletion. Clinical studies will need to specify both the preparation and the dose to be used. When substitution of an alternative drug is mandatory owing to allergic reactions, monitoring is advisable. Copyright © 1996 Published by Elsevier Science Ltd

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INTRODUCTION

IN THE treatment of childhood lymphoblastic leukaemia, L-asparaginase is an essential element with proven therapeutic efficacy [1]. The mechanism of action is based on the fact that the growth of lymphoblastic blast cells depends on the external availability of the amino acid asparagine, since, in contrast to normal tissues, the malignant cells lack sufficient activity of the enzyme asparagine synthetase [2–5]. Therefore, the pharmacological aim of asparaginase treatment is the maximum reduction of asparagine concentration in patients' blood. Glutamine is a second substrate for asparaginase [6, 7] and changes in glutamine metabolism have been considered as contributing to asparaginase-associated side-effects [8, 9].

Asparaginase causes a wide spectrum of side-effects such as hepatic dysfunction, pancreatitis, hyperglycaemia and severe allergic reactions [3–5]. An increasing number of haemorrhagic and thrombotic events [10–14], in particular, have

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prompted renewed discussion on the relevance and optimum scheduling of L-asparaginase.

Biologically, the enzyme L-asparaginase is derived from either *Escherichia coli (E. coli)* [15] (Asparaginase medac™, Crasnitin™) or *Erwinia chrysantemi (Erwinia)* [16] (Erwinase™), each preparation having different chemical [3] and immunological [17] properties. *Erwinia* asparaginase is considered to be comparably less toxic [18], and is in many institutions, including our own, frequently employed in the event of allergic reactions to *E. coli* asparaginase [19]. Although *Erwinia* asparaginase has a shorter half-life than *E. coli* asparaginase [20], preparations are usually interchanged without modifying the dosage or the application interval. The well-established pharmacokinetic differences between *E. coli* and *Erwinia* asparaginase, associated with increasing reports of haemorrhagic and thrombotic events, prompted us to establish a monitoring programme in our own institution. Asparaginase activity was the primary parameter of this drug monitoring. Additional pharmacodynamic parameters measured included asparagine levels, as the main biochemical parameter of the desired therapeutic effect, and the aspartic acid concentration and levels of glutamine and glutamic acid, as parameters potentially associated with toxic side-effects.

PATIENTS AND METHODS

From August 1992, 56 children aged 1 month to 16 years (median 6 years, mean 7.2) received treatment according to the NHL-BFM 90 or ALL-BFM 90 treatment protocols for non-Hodgkin's lymphoma or acute lymphoblastic leukaemia, and underwent routine monitoring during induction (protocol I) or re-induction (protocol II). As a rule, therapy was started using the *E. coli* preparation. In the early 1990s, Crasnitin™ (Bayer AG, Leverkusen) was not commercially available in Germany, so most of these children received Asparaginase medac™ (Medac GmbH, Hamburg, produced by Kyowa Hakko, Japan). Erwinase™ (Speywood, London) was applied when allergic reactions to *E. coli* preparations were observed.

Induction

Eight 1–3 h infusions of 10000 U/m² L-asparaginase (L-ASP) were applied at 3 day intervals, starting on day 12 of therapy. The chemotherapeutic regime included prednisone (PRED) on days 1–36, weekly applications of vincristine (VCR 1.5 mg/m²) and daunorubicin (DNR 30 mg/m²) on days 8, 15, 22 and 29, as well as intrathecal methotrexate (MTX) on days 1, 15 and 29 (Figure 1). Asparaginase medac™ and Crasnitin™ were monitored with induction treatment in 39 and 10 children, respectively.

Re-induction

Asparaginase was applied at individual doses of 10000 U/m² on days 8, 11, 15 and 18, along with daily dexamethasone (DEXA 10 mg/m²), vincristine (1.5 mg/m²) and doxorubicin (ADR 30 mg/m²) on days 8, 15, 22 and 29 (Figure 1). Monitoring data were recorded in a total of 28 treatment courses with Asparaginase medac™, 8 with Crasnitin™ and 16 with Erwinase™ (10 children due to untoward reactions after Asparaginase medac™, 6 after allergic symptoms with Crasnitin™).

Sample collection

Parents and patients had consented to the procedure, and blood samples were withdrawn 1–2 h prior to each asparaginase infusion during the routine pretreatment laboratory tests. The interval between collection of the samples was 3 days, except on day 15 of re-induction therapy, when the interval was 4 days. Additional samples were available from blood sampling for clinical reasons, e.g. coagulation analysis.

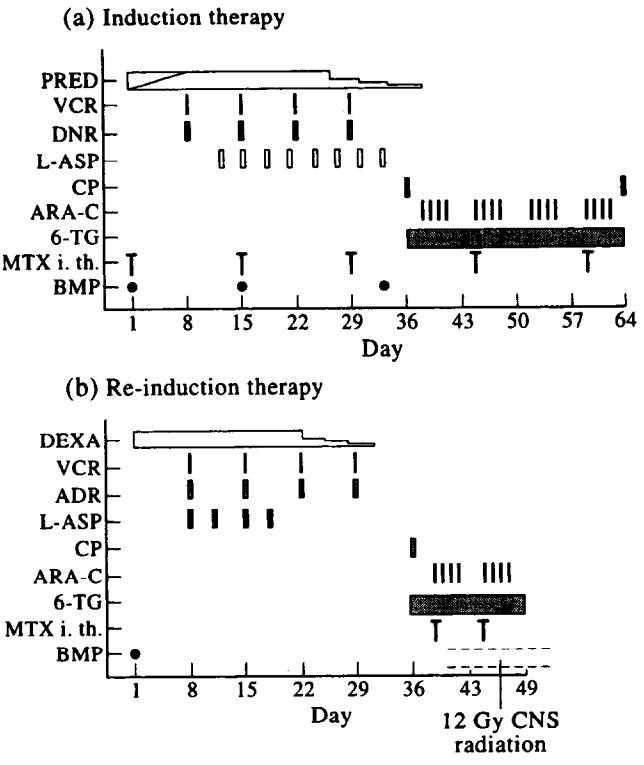

Figure 1. Treatment schedule for (a) induction and (b) re-induction therapy according to the ALL- and NHL-BFM-90 trial (BMP, bone marrow puncture; L-Asp, Asparaginase 10000 U/m²). PRED, prednisone; CP, cyclophosphamide; VCR, vincristine; MTX, methotrexate; i.th, intrathecal; DNR, daunorubicin; DEXA, dexamethasone; ADR, doxorubicin; 6-TG, 6-Thioguanine.

The samples were immediately centrifuged and divided in half. After extraction of the cellular components, one part (400 µl serum) was immediately deproteinised by adding 10% 100 µl sulphosalicylic acid (w/w) and deep-frozen (within 15–20 min after withdrawal) for storage until amino acid analysis. The other part was immediately frozen and used to determine the asparaginase activity. We decided not to use an asparaginase inhibitor, as the substance 5-diazo-4-oxo-L-norvaline (DONV) [21, 22] proved highly unstable in our tests and inhibitory activity was only seen at concentrations high enough to produce multiple major interference peaks in the HPLC chromatograms during the analytical procedure.

L-Asparaginase activity

A mixture of 100 µl serum and 400 µl 0.044 mM L-asparagine buffer solution was incubated at 37°C for exactly 45 min. After addition of 250 µl trichloroacetic acid 24.5% (w/w) and centrifugation, 250 µl of the supernatant was added to Nesslers solution (2000 µl water plus 250 µl Nesslers reagent) (Nesslers reagent A and B obtained from Merck, Darmstadt, Germany). The optical density at 450 nm was compared with an ammonium-sulphate–Nessler–standard curve, based on daily calibration at concentrations of 0.05, 0.1, 0.25, 0.5, 1.0 and 2.5 mM. The units of enzyme activity were defined as

micromoles of ammonia released per minute. Asparaginase standard curves between 10 and 10000 U/l in human standard-plasma resulted in an inter- and intra-assay reproducibility with a coefficient of variation of <15% down to 20 U/l. The detection limit, therefore, was 20 U/l. Because of spontaneous release of ammonia in plasma, lower activities could not be accurately quantified. Based on this assay, the asparaginase activity of the lots used in this study were comparable (about 10000 U/vial). Details of the asparaginase assay and the *in vitro* comparison of different asparaginase preparations have been previously published [23].

Amino acid analysis

Amino acid levels in the serum were measured using an RP-HPLC technique following precolumn derivation with *o*-phthaldialdehyde (OPA) and fluorescence detection according to Lenda and Svenneby [24]. Separation was made on a 250/8/4 ET Nucleosil 120-5-C18 column. The mobile phase was composed of: (a) 0.2 M potassium acetate pH 6.0, and (b) methanol (HPLC grade). A high pressure gradient (Equipment Pharmacia/LKB) served to adjust the composition of the mobile phase between 18 and 75% methanol at a flow rate of 1.3 ml/min (min:sec–min:sec/% methanol: 0:00–7:00/18%–7:05–16:00/20%–16:05–32:00/18%–32:10–37:30/75%–37:40–45:00/18%). Fluorescence detection (Shimazdu RF-530) was conducted at λ_{EM} = 450 nm and λ_{EX} = 340 nm.

The detection limit of the method was <0.1 μM, the limit of quantification was set to 0.1 μM. Daily calibration curves from 0.1–100 μM showed linearity >0.99 for asparagine (Asn), aspartic acid (Asp), glutamine (Gln, calibration linear up to 500 μM) and glutamic acid (Glu, calibration linear up to 200 μM). The coefficient of variation of approximately 5% (approximately 10% inter-assay and 4% intra-assay with 0.1 μM Asn) showed good reproducibility. The assay reproducibility for the other amino acids was <10% over the whole linear range.

Definitions

Trough levels are concentrations determined before application of asparaginase on the third day after the previous infusion (Figure 1). This is called day 3 (defining the day of application as day 0).

Under physiological conditions, asparagine levels are approximately 40–80 μM. The pretreatment asparagine values were within the normal range (children on Asparaginase medac™, median 61 μM; children on Crasnitin™, median 58 μM).

The depletion of asparagine was graded: complete depletion: ≤0.1; nearly complete depletion: >0.1–≤0.5 μM; moderate reduction: >0.5–≤1.0 μM; slight reduction: >1.0–≤40 μM; no reduction: >40 μM.

Significance was tested with the Mann–Whitney rank sum test (comparison of two groups) and with the Kruskal–Wallis One Way Analysis of Variance on Ranks (comparison of more than two groups), using Dunn's Method (both evaluations with Sigma Stat™ statistical software Version 1.02). The Wilcoxon signed rank test was used to test the changes in amino acid concentrations and Fisher's exact test was used for comparing the asparaginase effects in protocols I and II.

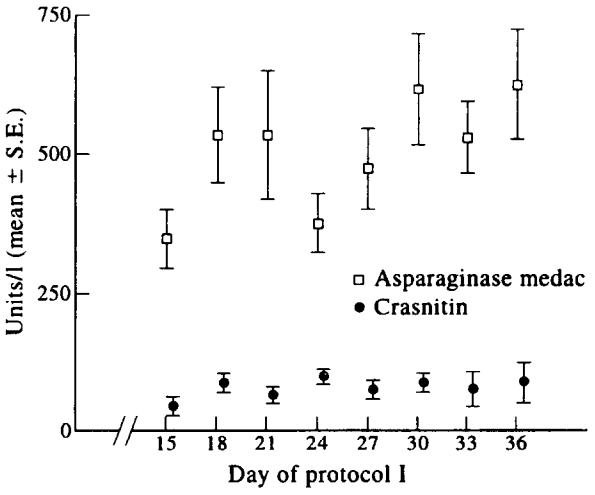

Figure 2. Asparaginase activity prior to each application in protocol I (4–27 observations per day, mean ± standard error of the mean).

RESULTS

Induction treatment (protocol I)

In the group of Asparaginase medac™-treated children, the asparaginase activity prior to each sequential application was (mean ± S.D.) 430 ± 300 U/l (median 475 U/l, 156 samples/39 children) and was thus significantly higher than in the Crasnitin™-treated children who had trough levels of 81 ± 51 U/l (median 74 U/l, 48 samples/10 patients). This difference was statistically significant ($P \leq 0.01$, Mann–Whitney Rank Sum Test) on all days with asparaginase application (Figure 2). The number of trough levels of L-asparagine below the limit of detection was higher in children receiving Asparaginase medac™, but in both groups, serum asparagine was completely or nearly completely depleted in all children (Table 1). Only one sample in the Asparaginase medac™ group showed a slightly higher L-asparagine concentration (1.08 μM). The recorded activity of these *E. coli* preparations

Table 1. Distribution of enzyme activities and L-asparagine levels in serum from patients under treatment according to protocol I of the ALL/NHL BFM 90 trial. Evaluation restricted to trough levels on the third day after asparaginase application

	Asparaginase medac™		Crasnitin™	
	% of samples (*n*)	No. of patients	% of samples (*n*)	No. of patients
Asparaginase activity				
≤20 U/l*	5.1(8)	8	10.4(5)	1
>20–≤100 U/l	3.2(5)	2	58.3(28)	10
>100 U/l	91.7(143)	39	31.3(15)	4
Total	100(156)	39	100(48)	10
L-Asparagine (μM)				
≤0.1*	87.6(148)	37	66.0(33)	9
>0.1–≤0.5	11.8(20)	13	34.0(17)	9
>0.5–≤1	—	—	—	—
>1	0.6(1)	1	—	—
Total	100(169)	39	100(50)	10

*Limit of detection.

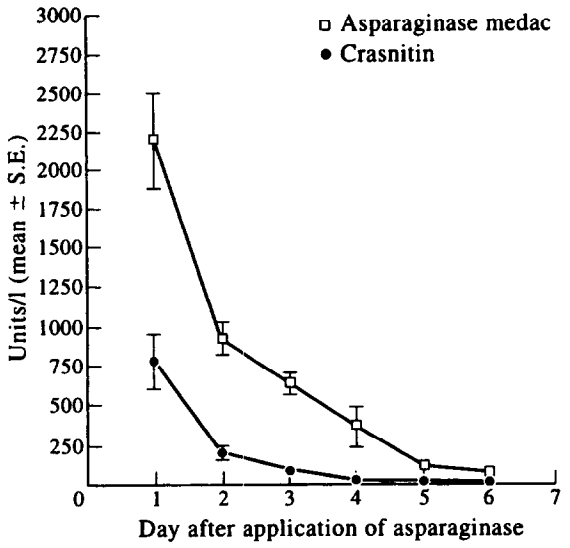

Figure 3. Activity values (means ± standard error of the mean) measured 1–6 days after infusion of Asparaginase medac™ or Crasnitin™.

1–6 days after application (Figure 3) showed that they have different half-lives and initial maximum levels.

Reinduction treatment (protocol II)

For both the *E. coli* preparations, trough levels of asparaginase activity (3-day values only) were comparable to the findings with induction treatment (Asparaginase medac™ 542 ± 243 U/l, median 528 U/l and Crasnitin™ 135 ± 135 U/l, median 49 U/l). In those children who received *Erwinia* asparaginase, the serum activity ranged around the limit of quantification (mean <20, median <20 U/l. 8 samples between 20 and 34 U/l, 16 samples <20 U/l) (Figure 4).

The differences in asparaginase activity were associated with differences in asparagine depletion. More than 90% of the samples on the third day after application of Asparaginase medac™ and more than 60% within the Crasnitin™ group showed complete depletion, while the proportion was only 26% after *Erwinia* asparaginase. The complete range of findings for the duration of the monitoring programme, including values of samples from days 1, 2 or 4 after asparaginase application in protocol II, is presented in Table 2.

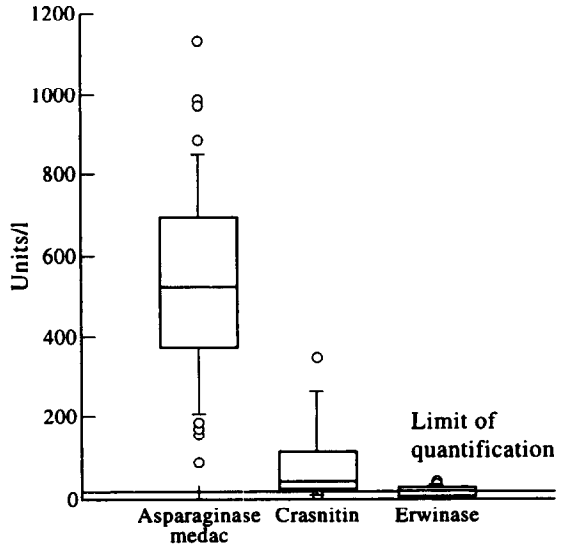

Figure 4. Asparaginase activity 3 days after application of 10000 U/m² in protocol II. (Plots the median, 10th, 25th, 75th, and 90th percentiles as vertical boxes with error bars and each outlier).

Focusing on day 3 after application of Crasnitin™, 5/48 evaluable samples in protocol I and 2/11 in protocol II showed enzyme activity ≤20 U/l ($P = 0.88$, Fisher's exact test). In addition, on day 3, 33/50 samples in protocol I and 7/11 samples in protocol II were completely depleted ($P = 0.7$). Comparing the preparation's ability to reduce asparagine levels to <0.5 μM, there was a significant difference between first and second exposure to Crasnitin™: 50/50 samples in protocol I versus 7/11 in protocol II showed nearly complete depletion ($P \leq 0.0001$).

Depletion time

Depletion times are a representation of the relationship between asparagine levels measured and the number of days from the previous asparaginase application (Figure 5). Approximately 11 days elapsed from the application of Asparaginase medac™ until recovery of the asparagine level, the corresponding time period after *Erwinia* asparaginase was only about 4 days. Depletion after Crasnitin™ can only be estimated due to the small number of samples, but seems to be comparable to the Asparaginase medac™ effect.

Changes in other amino acids

Table 3 shows the changes in the concentrations of aspartic acid, glutamic acid and glutamine on the first and third day after the previous asparaginase application. Aspartic acid was within the normal range, but was significantly elevated compared with the individual pretreatment values in each treatment group. Glutamine levels were markedly reduced by 75% one day after application of asparaginase medac™, and in single patients even on the third day levels <50 μM could be detected. L-glutamic acid was significantly enhanced on the first day after application and elevation persisted until day 3.

DISCUSSION

In the interpretation of these results with asparaginase, one important analytical aspect has to be discussed. *In vivo*, cleavage of the amino group from asparagine by the therapeutic enzyme asparaginase and the physiological asparagine synthesis represent dynamic homeostatic processes. *In vitro*, the catalytic effect of the enzyme on serum amino acids and mainly on asparagine may continue even after withdrawal of the sample. In our tests, DONV [21] showed inhibitory activity only at high concentrations (approximately 40 mM) and, due to impurities and rapid decomposition in aqueous solution, it produced unacceptable interferences in the analytical procedure. Similar problems have been reported by Gentili and colleagues [25]. We, therefore, decided to minimise the problem of ongoing asparaginase activity by rapid deproteination of the samples. The asparagine levels measured in the absence of an enzyme inhibitor may thus be falsely low in some instances, but do represent the lowest *in vivo* level. The asparaginase activity can be interpreted independently of these considerations.

The finding of an approximately 6-fold higher biological trough activity in the plasma with Asparaginase medac™ compared to Crasnitin™ appears surprising since both preparations are derived from *E. coli*. However, they not only have

Table 2. Distribution of enzyme activities and L-asparagine levels in serum from patients under treatment according to protocol II of the ALL/NHL BFM 90 trial, including all values of samples from days 1, 2, 3 and 4 after asparaginase application

	Asparaginase medac™		Crasnitin™		Erwinase™	
	% of samples (*n*)	No. of patients	% of samples (*n*)	No. of patients	% of samples (*n*)	No. of patients
Asparaginase activity						
≤20 U/l	—	—	33.3(7)	3	68.3(28)	16
>20–≤100 U/l	5.2(4)	4	38.1(8)	5	24.4(10)	6
>100 U/l	94.8(73)	25	28.6(6)	4	7.3(3)	3
Total	100(77)	27	100(21)	8	100(41)	16
L-Asparagine (μM)						
≤0.1	90.7(78)	27	57.1(12)	6	22.4(11)	8
>0.1–≤0.5	9.3(8)	3	9.5(2)	2	30.6(15)	12
>0.5–≤1	—	—	4.8(1)	1	18.4(9)	7
>1	—	—	28.6(6)	2	28.6(14)	7
Total	100(86)	27	100(21)	8	100(49)	16

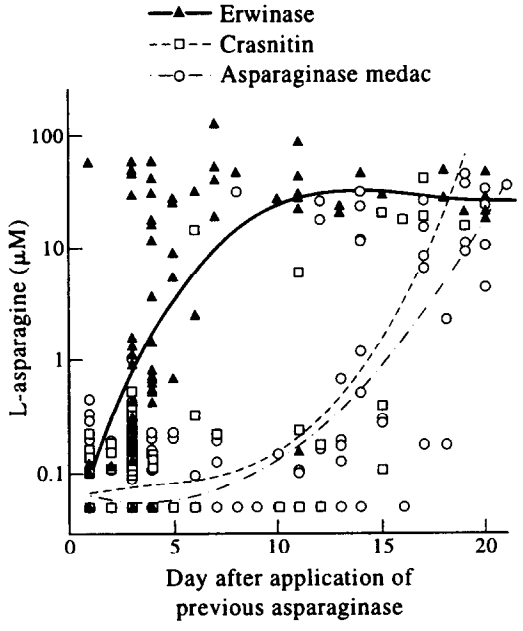

Figure 5. All asparagine levels plotted by day after previous asparaginase application. Samples from children with more than 4 standard deviations higher levels than the respective day-group were excluded from this evaluation due to the postulated influence of individual parameters. (Lines were fitted by 3rd order regression analysis with Sigma Plot 3.0.)

different half-lives, but also different initial maximum levels (Figure 3). A review of the literature, shows that this finding is not as surprising as it seems. Biological differences in different *E. coli* strains and mutants have been described [3, 5]. Comparable differences in peak and trough levels between the *E. coli*-derived asparaginase preparations, Elspar™ (Merck Sharp and Dohme) and Crasnitin™ have been reported by Schwartz and colleagues [26], but Asparaginase medac™ was not included in their investigation.

In the current drug monitoring programme, both *E. coli* preparations were found to induce almost complete depletion of serum asparagine concentrations. The significantly higher asparaginase activities in children on Asparaginase medac™, therefore, were not necessary for the desired pharmacodynamic effect.

The increase in glutamic acid up to values well above the normal range and the decrease in glutamine levels was most notable in the Asparaginase medac™ group (see Table 3). This may be interpreted as reflecting the application of a disproportionately high activity, or dosage, as complete depletion was achieved by much lower asparaginase activity produced with an identical dose of Crasnitin™ (Figure 2, Tables 1 and 2). Nevertheless, Crasnitin™ and even Erwinase™ produced measurable effects on aspartic acid, glutamic acid and glutamine. The effects on these amino acids were greater on day one than day three after the previous application. Before the subsequent application, the values were still higher than the pretreatment values, but reached the upper limit of the normal range (Table 3).

Previously, a randomised clinical trial showed a higher rate of thrombotic events as well as significantly stronger alterations of the coagulatory and fibrinolytic system in patients on Asparaginase medac™ compared to patients on Crasnitin™ [27]. The significant variations of the glutamic acid levels should be considered in this respect, as this amino acid is known to exert neuro- and endothelial toxicity [28].

The fact that practically no activity was measured before each sequential application in those children under re-induction treatment who received the *Erwinia* preparation was unexpected. The effect on the asparagine level was not observed in almost 30% of samples from 7 out of 16 children (Table 2). Considering that only children who had allergic reactions to either the *E. coli* asparaginase preparations were given Erwinase™ (5/10 with Crasnitin™, 10/39 with Asparaginase medac™), a cross-reaction with *E. coli* asparaginase antibodies and an associated inactivation cannot be excluded. Children receiving Erwinase™ as a second exposure represents a highly selected subgroup which is not strictly comparable to the other two. The Erwinase™ half-life, however, is known to be approximately 0.6 days and, therefore, clearly shorter than the *E. coli* asparaginase half-life of about 1.3 days [20]. Both facts may explain some of the observed differences. Recently, Dibenedetto and colleagues [29] reported disappointing asparagine depletion in the cerebrospinal fluid (CSF) with Erwinase™. Complete depletion in the CSF was seen 3 days after the application in only 60–70% of children, and 5 days after the previous application in approximately 30%. Depletion in the CSF was only achieved after the serum

Table 3. Glutamine, glutamic acid and aspartic acid 1 and 3 days after previous asparaginase application

Amino acid	Pretreatment level Median (μM)	Day after asparaginase application	Asparaginase medac™ Median (μM) (range)	Asparaginase medac™ Courses/ patients	Crasnitin™ Median (μM) (range)	Crasnitin™ Courses/ patients	Erwinase™ Median (μM) (range)	Erwinase™ Courses/ patients
L-Aspartic acid	8	1	24* (4–121)	41/24	13† (9–24)	11/9	23$^{n/a}$ (9–23)	4/4
(normal: 4–22 μM)		3	18* (4–128)	222/40	16* (5–65)	61/15	34* (5–98)	27/16
L-Glutamic acid	65	1	230* (114–337)	41/24	135* (70–215)	11/9	207$^{n/a}$ (76–267)	4/4
(normal: 18–65 μM)		3	142* (32–323)	222/40	81* (22–225)	61/15	80* (37–186)	27/16
L-Glutamine	452	1	110* (0–439)	41/24	337† (244–504)	11/9	207$^{n/a}$ (13–491)	4/4
(normal: 457–738 μM)		3	377* (0.7–732)	222/40	370† (0.1–783)	61/15	466^{ns} (281–611)	27/16

*Significant difference from pretreatment levels, $P \leq 0.001$, †$P \leq 0.05$; ns, not significant (Wilcoxon signed rank test); n/a, statistical analysis not applied.

asparagine had been depleted [30]; these observations are thus in good agreement with our data reported here. In addition, they also correspond to observations of a rapid loss of serum activity of *Erwinia* asparaginase after repeat application when used as front-line therapy [31]. Irrespective of any possible explanation, the treatment goal of asparagine depletion as the basis for the antileukaemic effect is not achieved in a substantial number of children under re-induction treatment. Apart from the reduced intensity of depletion, the duration of the treatment effect differs significantly between children on *E. coli* and those on *Erwinia* preparations (Figure 5). Whereas the treatment effect after Asparaginase medac™ lasts for almost 2 weeks from the last application, the asparagine levels after Erwinase™ recover within 3–4 days. The treatment intensity of the re-induction therapy with Asparaginase medac™, considering both intensity and duration of asparagine depletion, thus exceeds the activity of four applications of Erwinase™.

With respect to Erwinase™, the 3–4 day interval of i.v. application of the re-induction schedule is too long. It has to be stressed that the asparaginase trough activities and the related L-asparagine levels depend upon the particular protocol followed. Other trials which prescribe i.m. application of Erwinase™ 3 times per week [18] provide entirely and basically different pharmacological conditions which preclude any deductions from the presented data.

The ALL/NHL-BFM 90 treatment protocols, as with many other trials, leaves it up to the physician in charge to choose the particular preparation to be used, and only defines the recommended dose. However, the reported pharmacological differences between different asparaginase preparations need to be translated into corresponding dosage recommendations. The logical consequence will be to opt for specification of the particular preparation to be used by all treatment protocols employing asparaginase.

The current recommended dose of 10000 U/m^2 every 3 days is based on optimisation studies by Nesbit and colleagues [32] who defined an optimum of 6000 to 12000 U/m^2 in dose escalation trials using the Bayer preparation. Riccardi and colleagues showed that activity values of about 100 U/l in plasma led to adequate depletion in the plasma and cerebrospinal fluid [30]. The clinical trials of the BFM group developed chemotherapy regimes based on this product, and the present study confirms the basic assumptions of the early pharmacological and clinical studies. The introduction of new preparations demands repeat studies to define optimal dose schedules, especially since even the definition of the asparaginase unit varies between products [23]. In order to account for the higher activity of Asparaginase medac™, we have therefore started to reduce the dose of this preparation in a systematic dose-finding study.

During re-induction therapy, two children on Crasnitin™ and another two on Erwinase™ did not show any substantial effect. The two children receiving the *E. coli* preparation had not shown allergic reactions. Nevertheless, on the third day after asparaginase, they had practically normal L-asparagine concentrations and may thus be termed biochemical treatment failures. For children such as these an alternative product should be substituted, or they might at least be spared the potential risk of an anaphylactic reaction from the continued enzyme therapy.

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