

CLINICAL TRIALS

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Tolerance Evaluation of L-asparaginase loaded in red blood cells

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Abstract *Objective:* A pilot clinical study was conducted to evaluate the toxicity of a single dose of L-asparaginase loaded in red blood cells (RBCs).

Methods: Thirteen patients received a single dose of L-asparaginase in the range 30–200 IU · kg⁻¹. The enzyme was loaded in one autologous blood unit using a lysis-resealing process. A control population of 33 patients receiving L-asparaginase intravenously were tested in parallel. IgG, IgM and IgE class anti-L-asparaginase antibodies were detected using specific radioimmunoassays.

Results: L-Asparaginase pharmacodynamic parameters may be greatly improved by administration of the drug after internalisation in RBCs as compared to intravenous injection of free drug. The drug elimination was prolonged and similar to that of circulating carrier. After one injection of 30 IU · kg⁻¹, plasma L-asparagine was eliminated in 10 days and this was extended to 50 days for 150–200 IU · kg⁻¹. The drug was well tolerated and only transient variations were observed for some of the biological parameters measured. We did not reach the maximum tolerable dose (MTD) of L-asparaginase loaded in RBCs. No significant clinical toxicity was detected. In particular, no immune adverse effects were observed.

Conclusion: This study opens new perspectives for the clinical utilisation of L-asparaginase. This mode of administration of the drug is able to improve pharmacodynamic parameters and enzymic efficacy and to increase the general tolerance of the treatment.

Key words L-Asparaginase, Acute lymphoblastic leukaemia, non-Hodgkin's lymphoma; red blood cells, tolerance study

Introduction

L-Asparaginase, the enzyme which converts L-asparagine to L-aspartic acid, has been widely included in clinical protocols for the treatment of acute lymphoblastic leukaemia (ALL) and non-Hodgkin's lymphoma (NHL) since 1967 (Tallal et al. 1970, Land et al. 1972). In childhood ALL, approximately 90–95% of children with newly diagnosed disease achieve complete remission when L-asparaginase is used in combination with vincristine and prednisone (Ortega et al. 1977).

The general toxicity of the drug is relatively high and results in adverse-effects including immediate hypersensitivity with anorexia, nausea and vomiting, hyperglycaemia, liver dysfunction, pancreatitis, coagulopathy and neurological disorders. All these toxic manifestations have been largely documented in the past and reviewed by various authors (Haskell et al. 1969; Oettgen et al. 1970; Land et al. 1972; Haskell 1981; Nesbit et al. 1981). However, the heterologous origin of the enzyme leads to an immune response. Anaphylactic reactions may be observed in up to 33% of patients (Fabry et al. 1985; Cheung et al. 1986).

In order to overcome such difficulties several authors have tried to use red blood cells loaded with L-asparaginase as a new mode of administration of the drug in monkeys (Updike and Wakamiya 1983;

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Updike 1985; Alpar and Lewis 1985). A considerably prolonged life span of the enzyme and a major reduction of cutaneous anaphylactic reactions in the guinea pig have been demonstrated. These very encouraging results led us to adapt to L-asparaginase the methodology we had previously developed for the internalisation of drugs in red blood cells (Kravtsoff et al. 1990, 1991). Our preliminary results (Kravtsoff et al. 1992) were similar to those of the previous authors.

Recently, we evaluated the pharmacokinetic and pharmacodynamic parameters of *Escherichia coli* L-asparaginase loaded in RBCs (Kravtsoff et al. 1996). The results showed a good correlation between the life spans of RBCs and L-asparaginase loaded in RBCs. The elimination of the enzyme was biphasic. A rapid partial clearance was observed during the first 24 h. Considerable heterogeneity occurred from one patient to another; the level of L-asparaginase circulating at 24 h represented 47 (13)% of the total injected dose. However, the RBCs circulating after 24 h had a normal in vivo life span [$t_{1/2} = 27$ (6) days], very similar to that of the remaining circulating L-asparaginase loaded in the RBC compartment ($t_{1/2} = 29$ (9) days).

The depletion of plasma L-asparagine was related to the dose of L-asparaginase injected or remaining in circulation after 24 h. The enzymic efficacy of L-asparaginase loaded in RBCs was considerably increased compared to the free enzyme injected intravenously. A single injection of $30 \text{ IU} \cdot \text{kg}^{-1}$ L-asparaginase loaded in RBCs eliminated plasma L-asparagine in 10 days. This value was increased up to 50 days for a dose of $150\text{--}200 \text{ IU} \cdot \text{kg}^{-1}$.

This clinical study was conducted in 13 patients to evaluate the toxicity of a single dose of L-asparaginase loaded in RBCs.

Patients and methods

Patients and evaluation

Patients treated with L-asparaginase loaded RBCs.

Thirteen patients, nine men and four women, were included in the study (Table 1). Written informed consent was obtained from all patients and the trial was approved by the Ethics Committee of the Regional University Hospital of Tours. The eligibility criteria were as follows: age from 15 to 79 years, World Health Organization (WHO) performance status ≤ 3 , estimated life expectancy ≥ 3 months, serum bilirubin, transaminases, amylase, glycaemia, prothrombin and creatinine levels less than 1.3 times those of controls and no antineoplastic treatment during the 4 weeks before entry. Only heavily pretreated and chemoresistant patients were included in the study.

Diagnosis was NHL in ten patients, chronic lymphocytic leukaemia (CLL) stage C according to Binet's classification in two patients and soft tissue sarcoma in one patient. All patients had previously received chemotherapy with or without radiotherapy before entry in the study. The number of chemotherapy regimens varied and comprised between two and seven, including anthracycline therapy in all except one. None had received L-asparaginase intravenously. Previous treatment included high-dose chemotherapy and autologous bone marrow transplantation in two patients and radiotherapy in five patients. Table 1 summarises the individual characteristics of the patients.

Pretreatment evaluation comprised complete history, physical examination and laboratory evaluation, including complete white blood cells and platelet counts, differential smear, total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, γ -glutamyl transferase, lactic dehydrogenase, glycaemia, amylasaemia, creatinine, fibrinaemia, prothrombin level, kaolin cephalin clotting time, fibrinogen thrombin time, antithrombin III level and complement level (CH_{50}). In addition, all patients underwent initial chest X-ray computed tomography and ECG. Liver computed tomography was performed in patients with measurable abnormalities. Follow-up assessment included complete white blood cell and platelet counts, liver and coagulation tests as previously described, glycaemia, amylasaemia, creatinine, lactic dehydrogenase, complement level, serum asparagine and plasma and erythrocytic L-asparaginase levels on days 2, 3, 6, 10, 28, 50 and 90.

Table 1 Diagnosis and pretreatment of patients entered in the study (NHL non-Hodgkin's lymphoma, CLL chronic lymphocytic leukaemia, C chemotherapy, R radiotherapy, G bone marrow graft)

Patient #	Age/sex	Diagnosis (stage)	Year since diagnosis	L-Asparaginase dose ($\text{IU} \cdot \text{kg}^{-1}$)	Prior regimen n/regimen
1	68/M	NHL (IV)	5.5	30	5/C
2	60/M	NHL (III)	3.5	30	3/C
3	76/M	NHL (III)	5.5	30	4/C
4	56/M	NHL (III)	2	60	4/C 1/R
5	57/M	CLL (B)	2.5	60	5/C
6	63/M	NHL (IV)	6	60	5/C
7	35/F	NHL (III)	2	100	3/C 2/R
8	52/F	NHL (IV)	7	100	7/C
9	16/M	Sarcoma	2.5	100	7/C 2/R 2/G
10	77/M	NHL (III)	6	150	6/C
11	52/M	CLL (B)	4	200	5/C
12	45/F	NHL (IV)	2	200	5/C 1/R 1/G
13	62/F	NHL (IV)	2	200	2/C 2/R

The erythrocytic and plasma L-asparaginase levels were determined using the method of Cooney et al. (1970). Erythrocytes were lysed by freezing and thawing and L-asparaginase measured in the lysate. The plasma L-asparaginase levels were measured by HPLC. Toxic effects were evaluated according to WHO criteria (WHO Publication No. 48, 1979).

Control population treated with intravenous L-asparaginase.

Thirtythree patients with ALL (71%), NHL (21%) or lymphosarcoma (8%) in the course of their therapy were treated for 5 days with daily intravenous administration of 1000 IU · kg⁻¹ L-asparaginase. These patients were considered as a control population and analysed for anti-L-asparaginase immune response and allergic reactions using the same methods as patients receiving L-asparaginase-loaded RBCs.

The differences in the distribution of the diagnoses between the two groups were due to the necessity of enrolling in the study patients without prior administration of L-asparaginase. New patients with ALL are included, in our institution, in a treatment protocol including L-asparaginase administration as first line, so that these patients could not be included in this trial when they relapsed or progressed.

Immunological studies

We developed specific radioimmunoassays for determination of immune response following L-asparaginase administration. The methods have been described in detail by Kravtsoff et al. (1992). IgG and IgM anti-L-asparaginase antibodies were determined using a solid-phase radioimmunoassay (RAST). The antibody titre was expressed in arbitrary units (AU) as:

antibody titre = (cpm with patient serum — cpm with normal standard serum)/cpm with normal standard serum

IgE antibodies directed against the enzyme were evaluated using a specific paper radioimmunosorbant test (PRIST). Results were expressed as above. Measurement of IgG, IgM and IgE anti L-asparaginase was performed on days 10, 28, 50 and 90.

Preparation of L-asparaginase-loaded RBCs

The procedure used to load *E. coli* L-asparaginase (Kidrolase, Roger Belon, France) into homologous RBCs has been previously described in detail (Kravtsoff et al. 1996).

Doses injected

In preliminary experiments (Kravtsoff et al. 1992) we observed that the lowest dose of circulating entrapped L-asparaginase able to eliminate plasma asparagine was close to 0.3 IU · ml⁻¹. This dose may be achieved by injecting about 30 IU · kg⁻¹ RBCs-entrapped enzyme. It was defined as our lowest starting dose. Patients received a single injection of homologous RBCs loaded with L-asparaginase. Doses were increased progressively. Three of them received a single dose of 30 IU · kg⁻¹, three others 60 IU · kg⁻¹, three 100 IU · kg⁻¹, one 150 IU · kg⁻¹ and the last three 200 IU · kg⁻¹ (Table 1)

Results

The dose given to the control group (free enzyme), 1000 IU · kg⁻¹ · 5 days, is considerably higher compared with the doses of experimental formulation but the

pharmacokinetics parameters are very different. Considering the elimination half-life of the free enzyme was 1.44 (0.35) day, according to Asselin et al. (1993), the mean area under the curve (AUC) was approximately 10 800 (3800) IU · kg⁻¹ · days for the controls. Using the pharmacokinetics parameters mentioned previously (Kravtsoff et al. 1996), we obtained for the patients receiving L-asparaginase loaded in RBCs the following relationship:

$$\text{AUC (IU} \cdot \text{kg} \cdot \text{days)} = 16.39 \cdot \text{injected dose (IU} \cdot \text{kg}^{-1}) + 344; (R^2 = 0.912)$$

For patients receiving 200 IU · kg⁻¹, AUC was 3326 (1072) IU · kg⁻¹ · days, which is about one-third of the value of the control group for an overall injected dose 25 times lower. The plasma L-asparaginase elimination is extended to 50 days. Table 2 summarises the abnormalities or variations in the biological parameters detected in this study according to WHO criteria (WHO 1979).

L-Asparaginase is known to have slight haematological toxicity. The two cases of anaemia (Table 2) existed before the treatment. In this study, no patient experienced granulocytopenia. However, one patient (#1) developed grade 3 thrombocytopenia after treatment. Two others had pre-existing grade 1 and grade 2 thrombocytopenia.

Patient #1 died from delayed acute pulmonary embolism after the treatment. He developed a grade 3 thrombocytopenia over 47 days and a transient reduction (up to 48%) of AT III over 11 days. However, before the injection, he had already presented dyspnoea, cardiac insufficiency and several episodes of pulmonary embolism. Except for this case, L-asparaginase loaded RBCs were well tolerated at all doses in this group of patients with advanced malignancies. No significant non-haematological intolerance reactions were observed (Table 2). Eight patients showed moderate and reversible modifications of coagulation parameters, particularly AT III activity, four patients presenting a decreased level and three an increased

Table 2 Biological abnormalities or modifications observed in 13 patients treated with L-asparaginase loaded in RBCs according to WHO criteria (WHO Publication No. 48, 1979). Values are presented in the text

Severity	n	Grade 1	Grade 2	Grade 3
Thrombocytopenia	3	1	1	1
Anaemia	2	—	1	1
Alkaline phosphatase	1	1	—	—
Creatinaemia	1	1	—	—
Allergic	None	—	—	—
Parameter variation		Mild	Moderate	Severe
Bilirubinaemia	1	1	—	—
Hyperamylasaemia	1	—	1	—
AT III increase	3	2	1	—
decrease	4	3	1	—
Complement (CH ₅₀)	1	—	1	—
AST	1	1	—	—

level. Patient #1 had AT III activity which decreased from 70% (day 0) to 48% (day 3), then increased slowly to 102% at day 28. In addition, this patient also had a low fibrinogen concentration between days 0 and 13 but the initial level was $2 \text{ g} \cdot \text{l}^{-1}$. Similarly, patient #5 presented a low AT III activity (58%) at day 7 and day 50 but the initial level was already reduced at 70%. Only one patient had transient hyperamylasaemia. This patient, treated with $200 \text{ IU} \cdot \text{kg}^{-1}$, showed an increase in amylase between days 20 and 36 with a maximum at $236 \text{ IU} \cdot \text{l}^{-1}$ (day 28); he had already had a diabetic period 2 years before treatment. Patient #9 presented increased alkaline phosphatase between days 2 and 90 [mean level $205 (33) \text{ IU} \cdot \text{l}^{-1}$] but the initial value was $149 \text{ IU} \cdot \text{l}^{-1}$.

No modification of fibrinogen or complement factor levels occurred, except for one case of a decrease in CH_{50} value between days 6 and 70 with a minimal value of 15 units at day 70 following an initial value of 70 units. These modifications were associated with a decrease in complement factor C_4 during the same period with an initial value of $100 \mu\text{g} \cdot \text{ml}^{-1}$ and a final value of $20 \mu\text{g} \cdot \text{ml}^{-1}$. No significant variations were observed for the other factors.

Anti-L-asparaginase antibody frequencies and titres are presented in Table 3 (Kravtsoff et al. 1992). Antibody frequency, after L-asparaginase-loaded RBC injection, was not significantly different from those observed after intravenous administration of L-asparaginase in the control population, i.e. 38% ($n = 13$) and 30% ($n = 33$), respectively ($P = \text{NS}$). Antibody titres were identical in both overall populations. However, titres increased in control patients with allergic reactions as compared to those without reactions ($P < 0.001$). All the anti-L-asparaginase antibodies detected were IgG or IgM; none belonged to the IgE class. As a major result of this study, a single injection of L-asparaginase loaded in RBCs did not induce in the 13 patients studied any of the anaphylactic intolerance reactions which are frequently encountered using another mode of administration of the drug. In the control population receiving L-asparaginase intravenously, we observed nine allergic reactions (27%, $P < 0.05$), similarly to other authors.

Table 3 Immune response and allergic reactions in patients receiving L-asparaginase intravenously (control population) or loaded in RBCs. Antibody titre in arbitrary units is defined as: (cpm with patient serum – cpm with normal standard serum)/cpm with normal standard serum

	<i>n</i>	Antibody titre (arbitrary units)	Antibody frequency	%
Control population				
Overall population	33	4.1 (7.4)	10	30
With allergic reaction	9	12.2 (7.9)	9	27
Without allergic reaction	24	0.5 (1.0)	1	3
L-Asparaginase RBC population				
With allergic reaction	0	–	0	0
Without allergic reaction	13	4.1 (5.7)	5	38

Discussion

Toxicity of L-asparaginase loaded in RBCs compared favourably with previous data published for native or polyethylene glycol-L-asparaginase (Ettinger et al. 1995; Asselin et al. 1995). In this trial we did not reach the maximum tolerable dose (MTD) of L-asparaginase internalised in RBCs. The escalation dose was stopped as it would have been necessary to process two blood units instead of a single one. The maximum dose we used ($200 \text{ IU} \cdot \text{kg}^{-1}$) was sufficient to obtain a sustained elimination of plasma L-asparagine, which is the main biological objective of the administration of this drug. It might be possible to increase the volume of RBCs infused to increase the total injected dose. However, repeated administration (every 2–3 months) of a new RBC preparation will give a result similar to that of an increased overall injected dose. Furthermore, it may be assumed that the major reduction in the total injected dose of internalised L-asparaginase to obtain significant sustained elimination of plasma L-asparagine and the slow elimination of the carrier by the reticuloendothelial system would have favoured a reduction in the most common types of toxic effects of the free enzyme. This may explain why the general tolerance of the drug during this trial was satisfactory and why we observed only limited and transient variations in the parameters evaluated.

The adverse effect observed for patient #1 seems not to have been related to the treatment as he had had previous episodes of pulmonary embolism. The dose of internalised L-asparaginase he received was also very low ($30 \text{ IU} \cdot \text{kg}^{-1}$); however, delayed aggravation of the patient's status due to a toxic effect of the treatment cannot be strictly ruled out as the thrombotic effect of L-asparaginase has been well documented in the past (Miniero et al. 1986; Cappellato et al. 1986; Vicarioto et al. 1986).

Most current protocols using L-asparaginase include repeated administration of the enzyme, frequently at a dose of $1000 \text{ IU} \cdot \text{kg}^{-1}$. Consequences include the formation of anti-L-asparaginase antibodies of various classes inducing frequent hypersensitivity reactions in up to 30% of patients (Fabry et al. 1985; Nai-Kong et al. 1986). We observed a similar value (27%) in our control population.

Although obtained on a limited population (13 patients), in spite of sustained circulating internalised L-asparaginase, we observed encouraging results in terms of immune tolerance, as compared to the control population receiving L-asparaginase intravenously ($P < 0.05$). The immunogenicity of the enzyme remained unmodified by the mode of administration used. The formation of IgG or IgM anti-L-asparaginase antibodies was not suppressed and antibody titres and frequencies were similar to those observed after intravenous L-asparaginase administration. The differences in the distribution of the diagnoses in the two

groups of patients did not apparently modify the immune response to L-asparaginase administration.

However, the circulating antibodies did not elicit the expected adverse reactions. This may be explained by the fact that the internalised enzyme was protected by the RBC membrane and was no longer accessible to circulating antibodies. The enzyme was progressively eliminated by the reticuloendothelial system and the free enzyme in the plasma remained undetectable (Kravtsoff et al. 1996). The anaphylaxis related to L-asparaginase was demonstrated by Fabry et al. (1985) to be essentially a complement-mediated phenomenon. We did not observe significant modifications of complement factors, which is in favour of the absence of circulating antigen-antibody complexes in our patients. It was shown that IgE antibodies could be formed against L-asparaginase (Cheung et al. 1986). We did not detect formation of such IgE antibodies, using this mode of administration. However, in this trial, the patients received only one injection of L-asparaginase-loaded RBCs. The immune tolerance could be different after repeated injections of the internalised drug and this still has to be evaluated in future experiments using high doses of L-asparaginase-loaded RBCs. If necessary, it would be possible to overcome adverse effects by eliminating the drug carrier using RBC exchange transfusion.

This study opens new perspectives for the utilisation of L-asparaginase. This mode of administration of the drug is able to improve pharmacodynamic parameters and enzymic efficacy and to increase the general tolerance of the treatment. Such prolonged depletion of plasma asparagine associated with L-asparaginase-loaded RBCs may favour tumour cell elimination. This would also considerably modify the existing protocols, particularly by reducing the hospitalisation period which is currently necessary for the repeated intravenous administration of the drug. A phase II trial is warranted to evaluate the potential clinical efficacy of this mode of administration as compared to classical intravenous administration of L-asparaginase.

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