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Silent hypersensitivity to *Escherichia coli* asparaginase in children with acute lymphoblastic leukemia

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Abstract

This prospective study aimed to assess the incidence of silent hypersensitivity to *Escherichia coli* asparaginase in the treatment of acute lymphoblastic leukemia (ALL). Thirty-three children with newly diagnosed ALL were included in the study and treated according to the FRALLE 2000 protocol. The 'A group' ($n=18$) differed from the 'B-T group' ($n=15$) by a less intensive chemotherapy, the absence of concurrent prednisone therapy, and different asparaginase administration modalities during the second intensification. Asparagine, asparaginase activity, and anti-asparaginase antibodies were measured in each phase before the next injection of asparaginase. Eighteen percent of children presented a silent hypersensitivity. Most of them were in the 'B-T group' ($p=0.07$), and maintained low antibody titers throughout the treatment. Clinical hypersensitivity was statistically more frequent in group A ($p=0.002$), and allergy occurred mainly during the second intensification when antibody concentrations were significantly increased. We did not find any significant difference between asparaginase activity or asparagine depletion between the silent hypersensitivity and clinical allergy groups. In all, the results of this study suggest that chemotherapy and corticosteroid therapy associated with asparaginase treatment can lower antibody production and contribute to maintaining a silent hypersensitivity state.

Keywords: Allergy and hypersensitivity, the humoral immune response, lymphoid leukemia

Introduction

L-asparaginase is a major component of the treatment of childhood acute lymphoblastic leukemia (ALL). Its biological activity relies on its ability to hydrolyze asparagine into aspartate and ammonia, and thus deplete the environment of an amino acid necessary for the proliferation of leukemic cells [1,2]. Leukemic cells that are unable to synthesize asparagine because of an asparagine synthetase deficiency undergo apoptosis [3]. Nevertheless, this protein synthesized from bacterial sources presents several side effects, including the production of specific antibodies. The

incidence of antibody production ranges from 0 to 70%, but may vary depending on the different enzymatic preparations (native or pegylated *Escherichia coli* asparaginase, *Erwinia* asparaginase), the site of administration (intramuscular or intravenous), and the type of concurrent treatments [4–10]. Production of immunoglobulin G (IgG) has been involved in allergic manifestations, and in the decrease of L-asparaginase activity, which may represent a mechanism of chemotherapy resistance [9–13]. In addition, antibody production without clinical allergy, also known as the 'silent hypersensitivity' phenomenon, has been described with variable

incidences ranging from 5 to 46%. It has been suggested that these silent antibodies could also increase asparaginase clearance and might be associated with a poor outcome [5,8–10,14]. However, their immunological characteristics and involvement in perturbation of asparaginase function have not been totally explored. This prospective study aimed to assess the incidence of 'silent hypersensitivity' and its impact on outcome in a single-center series of 33 children treated for ALL.

Patients and methods

Patients and treatment schedules

Between February 2003 and December 2005, 39 children diagnosed with ALL were treated in the Onco-Hematology Unit of the University Hospital of Nantes according to the FRALLE 2000 protocol (full details can be accessed at <http://www.e-cancer.fr/les-essais-cliniques/registre-des-essais-cliniques?task=detailEtude&idFiche=410>). Patients were eligible if they were newly diagnosed with ALL (except FAB [French–American–British] L3 ALL). Parental consent was obtained in all cases. The study was performed according to institutional guidelines and was approved by the institutional review board. Six children were excluded from the study because they proceeded to bone marrow transplant ($n = 3$), were lost to follow-up ($n = 1$), had severe pancreatitis ($n = 1$), or because of technical impossibilities regarding sample analyses ($n = 1$). Finally, 33 children were included in the study. Children were assigned to different risk groups in accordance with the criteria listed in Table I. Treatment comprised 4 weeks of induction, 12 weeks of consolidation, 8 weeks of a first intensification, 8 weeks of interphase, and 8 weeks of a second intensification. The multiagent treatment regimen is described in Table II. *E. coli* asparaginase (Kidrolase®; OPi EUSA-Pharma,

Limonest, France) was administered intramuscularly three times weekly during induction, first intensification, and second intensification, with different doses in arms A and B-T.

In patients with platelet counts $< 50\,000/\mu\text{L}$, L-asparaginase was given intravenously. Patients developing allergy were switched to *Erwinia* asparaginase ($n = 2$), polyethylene glycol (PEG)-asparaginase ($n = 1$), or complete discontinuation of asparaginase therapy ($n = 10$).

Sample collection

Blood samples were collected approximately 2 h before each L-asparaginase injection. Samples were immediately frozen after centrifugation. Serum was stored at -80°C for later analysis. Multiple blood samples were obtained for the determination of anti-asparaginase antibodies, asparaginase activity, and asparagine. For consolidation and interphase periods in which no L-asparaginase was administered, only one measurement was carried out for the determination of antibody titer. Asparaginase activity or asparagine depletion was not measured during the second intensification.

Determination of anti-asparaginase antibodies

The usual enzyme-linked immunosorbent assay (ELISA) for measurement of anti-asparaginase antibody was developed by Wang *et al.* [15]. The antibody reference pool was established by the selection of plasma samples from patients with ALL who had allergic reactions. In order to elaborate a standard curve without using patient serum as a reference, we developed a method using a rabbit polyclonal antibody anti-asparaginase solution as the positive antibody control serum. Assay development and validation steps are described below.

Table I. Criteria for eligibility in the FRALLE 2000 protocol.

FRALLE protocol	Criteria for eligibility
FRALLE A	Newly diagnosed B ALL, presenting these characteristics: Ages > 1 and < 10 years old No criteria for FRALLE B and T
FRALLE B	Newly diagnosed B ALL, presenting one of these characteristics: Age ≥ 10 years old Central nervous system leukemia White blood cell count at diagnosis $\geq 50\,000/\text{mm}^3$ Genetic analysis: $t(9;22)$, $t(4;11)$, hypodiploidy ≥ 44 Expression of BCR-ABL or MLL-AF4 fusion protein Rearrangement of MLL detected by Southern blot or by FISH for CD10–
FRALLE T	Newly diagnosed T ALL, in children from 1 to 20 years old, whatever the white blood cell count

ALL, acute lymphoblastic leukemia; FISH, fluorescence *in situ* hybridization.

Table II. Multi-agent therapy in A1, B1, and T1 groups of the FRALLE 2000 protocol.

Phase, drug, dose (per day)	FRALLE A (days)	FRALLE B (days)	FRALLE T (days)
Induction			
Prednisone 60 mg/m ² PO or IV	1–7	1–7	1–7
Prednisone 40 mg/m ² PO or IV		8–28	8–28
Dexamethasone 6 mg/m ² PO or IV	8–28		
Vincristine 1.5 mg/m ² IV	8, 15, 22, 29	8, 15, 22, 29	8, 15, 22, 29
Daunorubicin 40 mg/m ² IV	22, 29*	8, 15, 22	8, 9, 10, 15
L-asparaginase 6000 IU/m ² IM or IV	10, 12, 14, 17, 19, 21, 24, 26, 29	8, 10, 12, 15, 17, 19, 22, 24, 26	8, 10, 12, 15, 17, 19, 22, 24, 26
IT methotrexate, cytarabine, methylprednisolone		8, 15	8, 15
IT methotrexate	1, 14	1	1
Cyclophosphamide 1000 mg/m ² IV			8
Consolidation			
Thioguanine 60 mg/m ² PO		1–21	1–21
Etoposide 150 mg/m ² IV		1, 8, 15	
Cytarabine 60 mg/m ² SC		1, 2, 8, 9, 15, 16	1, 2, 8, 9, 15, 16
Vincristine 1.5 mg/m ² IV	1, 8, 29, 36, 57, 64	29, 43	29, 43
Prednisone 40 mg/m ² PO		29–35	29–35
Dexamethasone 6 mg/m ² PO	1–15, 29–33, 57–61		
6-Mercaptopurine 50 mg/m ² PO	1–77	29–49	29–49
Methotrexate 25 mg/m ² PO	8, 15, 22, 36, 43, 50, 64, 71, 78	36	36
Methotrexate 5000 mg/m ² IV		29, 43	29, 43
Cyclophosphamide 1000 mg/m ² IV			1, 15
IT methotrexate	2, 30, 58		
IT methotrexate, cytarabine, methylprednisolone		1, 29, 43	1, 15, 29, 43
Intensification 1			
Dexamethasone 10 mg/m ² PO or IV	1–14	1–14	1–14
Vindesine 3 mg/m ² IV	1, 8, 15	1, 8, 15	1, 8, 15
Adriamycin 25 mg/m ² IV	1, 8, 15	1, 8, 15	1, 8, 15
L-asparaginase 6000 IU/m ² IM or IV	3, 5, 8, 10, 12, 15	3, 5, 8, 10, 12, 15	3, 5, 8, 10, 12, 15
Thioguanine 60 mg/m ² PO	29–49	29–49	29–49
Etoposide 150 mg/m ² IV	29, 36, 43	29, 36, 43	29, 36, 43
Cytarabine 60 mg/m ² SC	29, 30, 36, 37, 43, 44	29, 30, 36, 37, 43, 44	29, 30, 36, 37, 43, 44
IT methotrexate	3, 30		
IT methotrexate, cytarabine, corticoid		1, 29	1, 29
Interphase			
Vincristine 1.5 mg/m ² IV	1, 29	1, 15, 29, 43	1, 15, 29, 43
Dexamethasone 6 mg/m ² PO	1–5, 29–33		
Prednisone 40 mg/m ² PO		1–7, 29–35	1–7, 29–35
Mercaptopurine 50 mg/m ² PO	1–49	1–49	1–49
Methotrexate 25 mg/m ² PO	8, 15, 22, 36, 43, 50	8, 15, 22, 36, 43	8, 22, 36
Methotrexate 5000 mg/m ² IV		1, 29	1, 15, 29, 43
IT methotrexate	2, 30		
Intensification 2			
Prednisone 40 mg/m ² PO		1–14	1–14
Vincristine 1.5 mg/m ² IV	1, 10, 20, 30	1, 8, 15	1, 8, 15
Daunorubicin 30 mg/m ² IV		1, 8, 15	1, 8, 15
Methotrexate 100 mg/m ² IV	1, 10, 20, 30		
L-asparaginase 6000 IU/m ² IM or IV		3, 5, 8, 10, 12, 15	4, 6, 8, 10, 12, 15
L-asparaginase 20 000 IU/m ² IM or IV	2, 11, 21, 31		
Thioguanine 60 mg/m ² PO		29–49	29–49
Cyclophosphamide 1000 mg/m ² IV		29	29
Cytarabine 60 mg/m ² SC		29, 30, 36, 37, 43, 44	29, 30, 36, 37, 43, 44
IT methotrexate, cytarabine, methylprednisolone		1, 29	1, 29
IT methotrexate	2		

*Administration of daunorubicin at day 22 and 29 was determined by randomization.
PO, per os; IV, intravenous; IM, intramuscular; SC, subcutaneous; IT, intrathecal.

Assay design and development. Anti-asparaginase antibody levels were measured by ELISA. Briefly, *E. coli* asparaginase (Kidrolase®; OPi EUSA-Pharma, Limonest, France) was coated in the wells using microtiter plates (Nunc, Denmark) at a concentration of 4 IU/mL in 0.05 M potassium phosphate buffer at a pH of 7.4 and a volume of 100 µL per well. After overnight incubation at +4°C and two wash cycles, 250 µL per well of the saturation buffer (phosphate buffered saline [PBS] supplemented with 5% bovine serum albumin) was added to all wells for 2 h at room temperature. After two wash cycles, the plates were then stored at -20°C for up to 2 months with good stability.

In the present study, as the volume of positive serum from treated patients was a limiting factor, a rabbit polyclonal antibody against asparaginase (Biodesign) was used at different concentrations (from 250 to 1.95 ng/mL). The rabbit polyclonal antibody anti-asparaginase was incubated for 1 h at 37°C in darkness, and then detected with a secondary polyclonal sheep anti-rabbit IgG horseradish peroxidase (HRP) conjugate (Serotec) at a concentration of 67 ng/mL in a volume of 100 µL per well for 1 h at 37°C in darkness. This method allows quantification of patient samples. The advantages of this commercial positive control are its availability through (1) assay development and (2) duration of the study, and its use in (3) testing samples from different independent clinical trials.

The positive anti-asparaginase antibody control, normal human serum (NHS) negative control, and patient serum samples at different dilutions were added and incubated for 1 h at 37°C in darkness, after which a polyclonal goat anti-human IgG, IgA, IgM horseradish peroxidase conjugate (Serotec) was added and incubated at a concentration of 20 ng/mL in a volume of 100 µL per well for 1 h at 37°C in darkness. After a final washing step, 100 µL per well of substrate tetramethylbenzidine (TMB) was added and the enzymatic reaction was stopped by the addition of 100 µL of 1 M sulfuric acid after 10 min of incubation. Absorbances were measured at 450 nm. When optical density (OD) readings of positive and negative controls were in the acceptable range, the antibody levels of patient samples were determined by extrapolating OD values to rabbit anti-asparaginase concentrations using the standard curve. The level of antibody immune response was analyzed during the time course and by comparison between samples.

Assay optimization involved the study of various parameters such as sample dilution, incubation time, and the choice and concentration of secondary reagent. The selection of optimal conditions was based on the degree of non-specific binding and the signal obtained for a pool of positive samples.

Assay validation. Assay validation included determination of the cut-off point, and study of matrix interference, specificity, precision, stability, and linearity. Positive samples were obtained from patients treated with Kidrolase® and before pre-administration values (negative samples). A pool of serum (Blood Center, Besançon, France) was used as a negative control (QC-) for the validation. Positive samples were pooled in order to obtain a high QC (quality control), which was diluted 10- and 50-fold in the pool of negative samples (QC-) in order to obtain a mid-QC and low QC, respectively. The cut-off point was determined after measuring the variability of 30 individual negative samples which were assayed, and no antibody detection was observed. The rabbit polyclonal anti-asparaginase used for the standard curve was diluted in a cohort of healthy donor samples, showing a linearity $r^2 \geq 0.98$, with a sensitivity > 1.95 ng/mL. Precision was studied by calculating intra-day ($n = 3$) values of the three QCs, high (8%), mid- (2%), and low (9%). Precision was also studied by calculating inter-day variability ($n = 3$) of the three QCs, high (8%), mid- (11%), and low (15%). Through competition between positive pooled human serum and the rabbit polyclonal anti-asparaginase, the specificity of ELISA detection was demonstrated. Matrix effects were determined by studying the variability of negative samples spiked with the high QC at a 2–16-fold dilution. Stability was assessed as variability of the absorbance of the high QC, which was tested after incubation for 24 h at -20°C, and three freeze-thaw cycles after 1 month and 6 months at -20°C.

Determination of asparaginase activity

Measures of asparaginase activity were performed using the automated kinetic enzymatic method described by Orsonneau *et al.* [16]. First, the elimination of endogenous ammonia was achieved by the addition of α -ketoglutarate and nicotinamide adenine dinucleotide diphosphate (NADPH₂) (reagent 1) and glutamate dehydrogenase and NADPH (reagent 2) to the plasma sample (Roche Diagnostics, Meylan). L-asparagine was then added to the sample to a final concentration of 40 mmol/L, which generated the production of ammonia through hydrolysis by the L-asparaginase contained in the plasma. The kinetics of ammonia production were evaluated by the decrease of optical density at 340 nm, and finally allowed the determination of asparaginase activity.

Determination of asparagine levels

Asparagine levels were determined by high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS). Deproteinization was carried out by the addition of 300 µL of sulfosalicylic

acid (concentration: 53.3 g/L) to 100 μ L of patient serum. After homogenization and then centrifugation (10 min, 4000 rpm), the supernatant was removed and then subjected to derivatization using the AccQ-Tag method. Briefly, 10 μ L of the deproteinized serum was mixed with 70 μ L of borate buffer, and then added to 20 μ L of ACQ reagent (obtained by diluting 1 mL of an acetonitrile solution with the reagent powder, then shaking and heating at 50°C for 5 min). Ten microliters of this derivative solution were injected into the capillary HPLC-MS/MS system for analysis. The HPLC system, equipped with an analytical column (Atlantis dC18; 150 mm $\times$ 2.1 mm, 3.0 μ m), was coupled with a mass spectrometer (Quattro Micro[®]; Waters Corporation). Chromatography was performed at 35°C with a flow rate of 0.3 mL/min. For the quantitative analysis of L-asparagine in multiple reaction monitoring (MRM) mode, we studied the transition (m/z) 303.1 > 171.1. Cone voltage was set at 25 V, and energy collision was 20 eV. The limit of detection was 0.066 μ M and the limit of quantification was 0.175 μ M.

Statistical analysis

Associations between asparaginase activity and asparagine levels and groups were analyzed with linear mixed models. Associations between immunization groups and treatment groups were performed using the Fisher test. Comparisons of antibody concentrations were determined with the non-parametric Mann-Whitney test. p -Values less than 0.05 indicated statistical significance. The receiver operating characteristic (ROC) curve was elaborated, analyzing the elevation of antibody concentrations between the first and the last measure in the second intensification for each patient.

Results

Thirty-three children (21 boys and 12 girls) with a median age of 5.9 (range, 1–17) years were included. At diagnosis, five children presented a white blood cell count > 50 000/ μ L and none had central nervous system (CNS) disease or testicular involvement. The 28 children with B-lineage ALL were treated in the standard-risk protocol FRALLE A ($n=18$) or in the high-risk protocol FRALLE B ($n=10$). Patients with T-lineage ALL were treated in FRALLE T protocol ($n=5$). The high-risk B protocol was close to the FRALLE T regimen regarding asparaginase administration modalities and concomitant chemotherapy. Thus, FRALLE B and T groups were combined for the purpose of this analysis.

Antibodies

Positivity of antibody titers was defined as concentrations greater than 1.5 μ g/mL. In the whole series, 45.4% (95% confidence interval [CI] 29–62%; $n=15$) presented an allergic manifestation and were designated as the ‘clinical allergy’ group. IgG anti-asparaginase was detected in 14 of these patients. For 11 of these 14 patients, antibodies were detected before the allergy appeared, spreading from the fourth dose in induction to the third dose in the second intensification. We identified two peaks of incidence in the clinical allergy group: the first at the end of induction ($n=8/14$), and the second during the second intensification ($n=5/14$).

Six patients (18.2%; 95% CI 8–34%) exhibited IgG production without clinical allergy, and were designated as the ‘silent hypersensitivity’ group. For this group, antibodies appeared regularly in each phase of treatment without any peak of incidence (Figures 1 and 2).

Considering the amount of antibody, we found similar concentrations between the two groups during the four first phases ($p=NS$). In contrast, there was an important increase of IgG titers during the second intensification of the clinical allergy group, whereas the silent immunization group did not show any increase (mean concentrations: 217.47 and 3.82 ng/mL, respectively; $p=0.0001$) (Figure 1). Of the 33 patients enrolled in this study, no allergic reaction or antibody production occurred for 12 patients (36.4%; 95% CI 21–53%) (Figure 3).

Allergy

Allergic clinical manifestations, classified from mild symptoms (urticaria, pain at injection site, localized erythema) to anaphylactic shock, mostly occurred during the second intensification ($n=13/15$). Only two patients experienced hypersensitivity in the first intensification. Of the three patients who underwent a change of asparaginase formulation, one had a new allergy. The alternative asparaginase formulations that were used included polyethylene glycol *E. coli* asparaginase ($n=1$) and *Erwinia chrysanthemi* asparaginase ($n=2$). For 13 patients, antibodies were detected before ($n=11$) or simultaneously ($n=2$) with allergic manifestations. For one patient, despite a severe allergy just after asparaginase injection, antibodies could not be detected.

A significant increase of clinical allergy was found in the FRALLE A group compared to the FRALLE B and T groups (72% vs. 13%, respectively; $p=0.002$). The incidence of silent hypersensitivity was more important in the B-T group ($n=5/6$) than in the A group ($n=1/6$) ($p=0.07$). Although the antibody

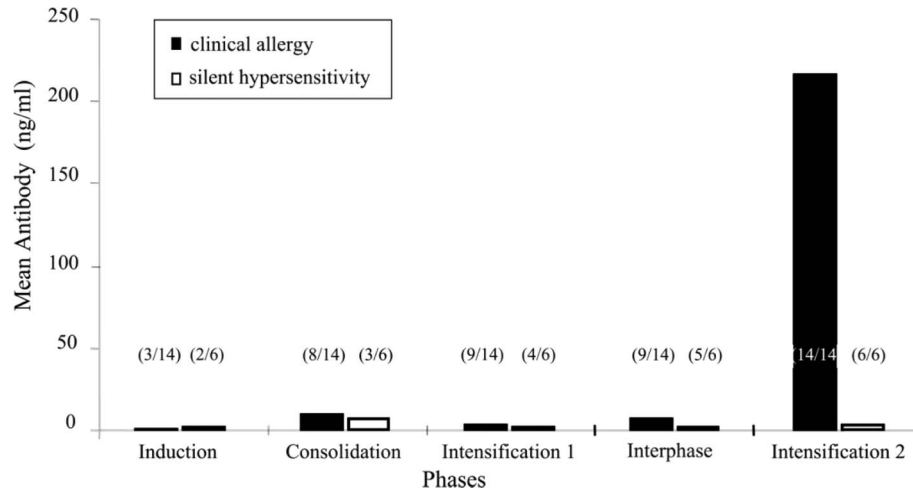


Figure 1. Mean anti-asparaginase antibody concentrations in patients with clinical allergy and silent hypersensitivity in the five phases of treatment. Numbers in parentheses indicate the cumulative number of patients with positive antibody out of the total number of patients presenting a clinical allergy (above black columns) or a silent hypersensitivity (above white columns).

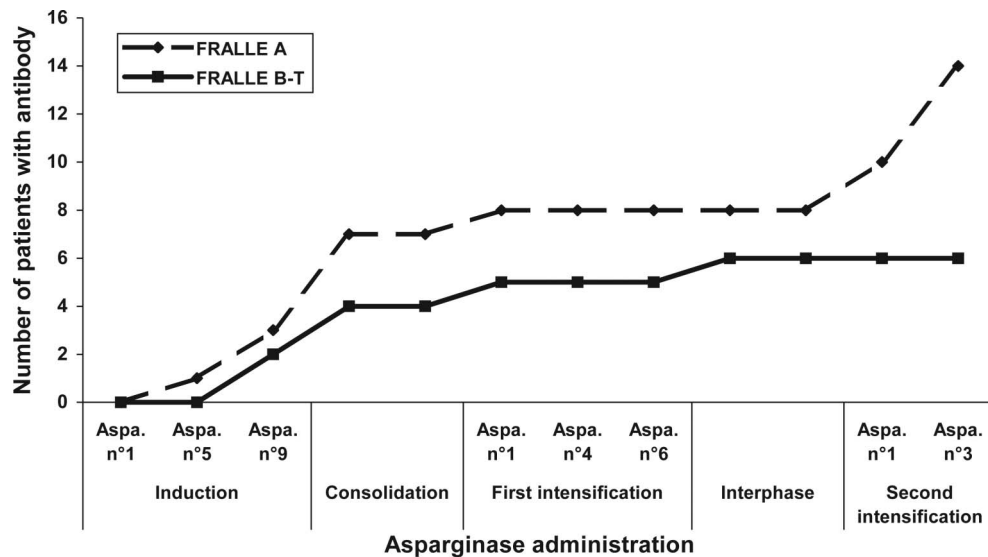


Figure 2. Occurrence of anti-asparaginase antibodies in the FRALLE A and B-T groups in the different phases of therapy. Anti-asparaginase antibodies, asparagine concentrations, and asparaginase activity were measured just before the administration of L-asparaginase, three times during induction and the first intensification, and twice during the second intensification.

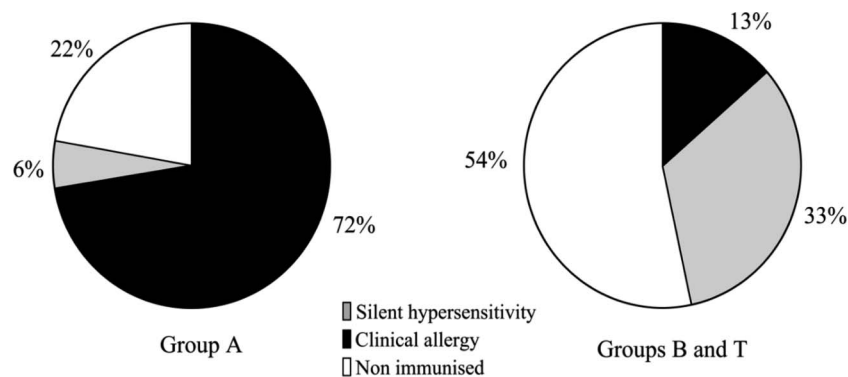


Figure 3. Distribution of non-immunized patients, patients with clinical allergy, and patients with silent hypersensitivity in A group and B-T group.

titers were not statistically different in the first four phases, there was a significant increase in group A during second intensification compared to B and T groups, with mean concentrations of 200.7 and 3.1 ng/mL, respectively ($p = 0.0001$). The increase of IgG anti-asparaginase antibodies in the second intensification could predict the probability of an allergic reaction. Actually, the ROC curve area was 0.89 ± 0.079 . With a cut-off value of 2.9 ng/mL, the sensitivity was 92% and the specificity was 98%.

Asparaginase activity

Enzyme activity (measured just before the next administration of L-asparaginase) during induction and the first intensification averaged 602 ± 298 IU/L with a median of 535 IU/L, in 302 samples from 30 patients (median per patient = 11). In most cases, L-asparaginase activity reached a level of at least 100 IU/L and was 3–10-fold higher than the minimal recommended threshold (Table III). There was no significant difference between L-asparaginase activity in the three immunization groups ($p = 0.7$), or within the different phases of each group ($p = 0.7$).

Asparagine

In accordance with Berlin–Frankfurt–Münster (BFM) criteria, we defined complete depletion as an asparagine level lower than $0.1 \mu\text{mol/L}$, and almost complete depletion as a level between 0.1 and $0.5 \mu\text{mol/L}$ [17]. Above $0.5 \mu\text{mol/L}$, depletion was considered insufficient. The pre-treatment mean of asparagine was $96.2 \pm 42.8 \mu\text{mol/L}$. We did not find any significant difference between the level of asparagine in the non-allergy, clinical allergy, and silent inactivation groups ($p = 0.635$), or within the different phases of each group ($p = 0.219$). More than 50% of allergic patients and 27% of non-allergic patients failed to obtain a sufficient depletion (Table III). For all of these patients, the schedule

of asparaginase injections was respected, and L-asparaginase activity was above 100 IU/L when L-asparagine was measured at a level greater than $0.5 \mu\text{mol/L}$. Patients with silent inactivation were always completely or almost completely depleted.

Outcome

With a median follow-up of 5 years, we did not detect any difference in terms of outcome between the different ‘immunization’ groups. Two patients died of relapse. One of these two patients did not show any sign of allergy but had poor cytogenetics. The other patient was in the clinical allergy group, and presented initially with standard risk leukemia.

Discussion

The primary endpoint of this study was to analyze the incidence of silent hypersensitivity to *Escherichia coli* asparaginase in children with ALL. We found that more than 18% of patients presented IgG anti-asparaginase antibodies without clinical hypersensitivity. This incidence varies in the literature from 5 to 46% according to time of measurement, associated chemotherapy, or L-asparaginase dose [4,5,8,9,11]. Most of the patients presenting a silent hypersensitivity were in the FRALLE B-T group. Conversely, patients with clinical allergy were treated in the FRALLE A group. The latter arm of the FRALLE 2000 protocol is considered to be the less intensive. The main differences with the B-T arm are the absence of concurrent prednisone therapy and the different L-asparaginase schedule in second intensification, and the less intensive chemotherapy induction, consolidation and second intensification. Indeed, previous reports demonstrated that the probability of an allergic reaction was significantly increased in patients who did not receive concurrent prednisone–vincristine therapy and when a hiatus between courses of asparaginase existed [18–21]. In a

Table III. Asparaginase activity and asparaginase depletion in the different immunization groups.

	Non-immunized group		Silent immunization group		Clinical allergy group	
	Samples, % (n)	Patients, % (n)	Samples, % (n)	Patients, % (n)	Samples, % (n)	Patients, % (n)
Asparaginase activity						
≥100 IU/L	100 (92)	100 (10)	98.4 (63)	83.3 (5)	98.6 (144)	85.7 (12)
<100 IU/L	0 (0)	0 (0)	1.6 (1)	16.7 (1)	1.4 (2)	14.3 (2)
Total	(92)	(10)	(64)	(6)	(146)	(14)
L-asparagine						
≤0.1 μM	86.2 (50)	54.5 (6)	94.3 (33)	83.3 (5)	81.5 (66)	46.2 (6)
>0.1–≤0.5 μM	3.4 (2)	18.2 (2)	5.7 (2)	16.7 (1)	3.7 (3)	7.7 (1)
>0.5 μM	19.4 (6)	27.3 (3)	0 (0)	0 (0)	14.8 (12)	53.8 (7)
Total	(58)	(11)	(35)	(6)	(81)	(13)

recent trial, systematic use of corticosteroids given before each course of asparaginase was associated with few clinical allergic reactions (two out of 118), whereas the incidence of silent antibodies increased between 26 and 42% during the first delayed intensification [5]. On the other hand, no formal association has been demonstrated between treatment schedule, dose, and allergy [1,20,22,23]. Moreover, the picture is likely more complicated since corticosteroid (especially dexamethasone) pharmacokinetics is highly variable and is related to the concurrent use of particular drugs, age, and treatment intensity. Thus, patients allergic to asparaginase suffer from diminished exposure to asparaginase while maintaining a high clearance of dexamethasone [24]. In the second intensification, the FRALLE A group presented a peak of antibody production. In contrast, FRALLE B and T patients, who were exposed to intensive corticosteroids and chemotherapy, maintained positive but low antibody titers and had few allergies during this phase. Therefore, it seems that the immunosuppression induced by concomitant corticotherapy and chemotherapy may have prevented the development of antibody or lower antibody synthesis. These low IgG titers defined the silent antibodies. It was impossible for us to determine the impact of each factor.

It has been demonstrated in several reports that asparaginase activity or half-life is lowered in immunized patients, suggesting the neutralizing power of anti-asparaginase antibodies [5,9,10,12]. In this report, we have not found any significant decrease of activity between groups or phases. Most of the patients even had high asparaginase activity, from 3- to 10-fold higher than the minimal recommended threshold. It is likely that such levels attenuated differences of activity between groups, explaining the absence of a decrease of asparaginase activity in the clinical allergy group. Also, we did not measure such activity in the second intensification, in which most of the allergic reactions occurred. High enzymatic activity has already been measured using the asparaginase Medac, identical to Kidrolase [10,17,25–27]. American studies using a different brand with potentially less potency may have detected the impact of antibodies where we could not with a more potent asparaginase. Only one report of the Children's Cancer Group (CCG) protocol 1962 studied the impact of silent antibodies on asparaginase activity. In the first delayed intensification, half of the patients with silent hypersensitivity presented an ineffective range [5]. This association was not found in other phases.

In 1981, Riccardi *et al.* demonstrated that an asparaginase activity greater than 100 IU/L was necessary to ensure a good cerebrospinal fluid

(CSF) and plasma asparagine depletion [28]. The results of the present study revealed an insufficient asparagine depletion in more than 50% of the patients from the clinical allergy group. Strikingly, despite sufficient levels of asparaginase, 27% of the patients in the non-allergy group were not sufficiently depleted in asparagine. Indeed, one must admit that serum asparagine concentration is usually difficult to measure because of its *ex vivo* destruction. Therefore, and despite the rigorous design of our study in terms of sample collection and transport, this may represent a limitation of our findings. Other reports investigating pegylated asparaginase or *Erwinia* showed such a lack of asparagine depletion in non-immunized patients, without any explanation related to the existence of alternative mechanisms of resistance to asparaginase [29–32]. In 2004, Hak *et al.* compared asparagine concentrations after asparaginase therapy by Elspar or Oncaspar in 53 patients included in the St Jude XIV protocol. In the first and second re-inductions, serum and CSF depletion were less important in immunized than in non-immunized patients [33]. In our study, silent immunized patients had an optimal depletion, but the small number of patients of this group did not allow us to draw firm conclusions.

In conclusion, the present study highlights the importance of immunosuppression induced by concomitant chemotherapy and corticotherapy in some phases, in order to maintain low antibody titers unable to initiate allergic reactions, defining silent antibodies. The pharmacokinetic and pharmacodynamic consequences of the silent IgG remain unclear.

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