# Asparaginase Antibody and Asparaginase Activity in Children With Higher-Risk Acute Lymphoblastic Leukemia

## *Children's Cancer Group Study CCG-1961*

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**Abstract:** We investigated the anti-asparaginase antibody (Ab) and asparaginase enzymatic activity in the sera of 1,001 patients (CCG-1961) with high-risk acute lymphoblastic leukemia (HR-ALL). Patients received nine doses of native *Escherichia coli* asparaginase during induction. Half of rapid early responders (RER) were randomly assigned to standard intensity arms and continued to receive native asparaginase. The other RER patients and all slow early responders received 6 or 10 doses of PEG-asparaginase. Serum samples (n = 3,193) were assayed for determination of asparaginase Ab titers and enzymatic activity. Three hundred ninety of 1,001 patients (39%) had no elevation of Ab among multiple evaluations—that is, were Ab-negative (<1.1 over negative control)—and 611 patients (61%) had an elevated Ab titer (>1.1). Among these 611 patients, 447 had no measurable asparaginase activity during therapy. Patients who were Ab-positive but had no clinical allergies continued to receive *E. coli* asparaginase, the activity of which declined precipitately. No detectable asparaginase activity was found in 81 of 88 Ab-positive patients shortly after asparaginase injections (94% neutralizing Ab). The Ab-positive patients with clinical allergies subsequently were given Erwinase and achieved substantial activity (0.1–0.4 IU/ml). An interim analysis of 280 patients who were followed for 30 months from induction demonstrated that the Ab-positive titers during interim maintenance-1 and in delayed intensification-1 were associated with an increased rate of events. The CCG-1961 treatment schedule was very immunogenic, plausibly due to initially administrated native asparaginase. Anti-asparaginase Ab was associated with undetectable asparaginase activity and may be correlated with adverse outcomes in HR ALL.

**Key Words:** asparaginase, antibody, high-risk acute lymphoblastic leukemia

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Asparaginase (ASNase) is an important drug for the treatment of childhood acute lymphoblastic leukemia (ALL).[1–6] Its ability to catalyze asparagine (Asn) and glutamine (Gln) deamination in the central circulation makes it specifically useful in ALL because the lymphoblasts, which cannot effectively synthesize Asn,[7–10] undergo *p53*-dependent apoptosis due to inhibition of protein biosynthesis.[5,10–12] Available ASNase preparations used in the United States include native *Escherichia coli* ASNase as a first-choice formulation, and its pegylated analog (PEG-ASNase).[1,3–5] An alternative ASNase, Erwinase, isolated from *Erwinia chrysanthemi*, is used as a substitute when patients have clinical allergies to native *E. coli* ASNase.[3,13,14]

A series of studies found improved results with more intense or longer ASNase regimens in pediatric leukemia.[1,4,5,15–17] These studies demonstrated that constant moderate to high levels of ASNase enzymatic activity have to be achieved in serum for sustained Asn deamination, which is postulated to be the cornerstone of the antileukemic mechanism of the ASNase.[3,5,7–10] The randomized comparison of *E. coli* ASNase and PEG-ASNase for childhood standard-risk ALL (CCG-1962) showed rapid clearance of lymphoblasts from days 7 and 14 bone marrow aspirates in patients on the PEG-ASNase arm, which was supported by the fact that ASNase activity was prolonged in patients treated with PEG-ASNase compared with the native enzyme.[5]

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Unfortunately, the bacterial origin of ASNase causes the undesired antigenicity of the drug, which triggers host immune response in the form of anti-ASNase antibody (Ab) production (immunoglobulin [IgG], IgG4 bivalent Ab),[5,18] followed by subclinical or clinical hypersensitivity.[19–24] In patients treated with different ASNase preparations and regimens, circulating Ab were found in an average of 58% (range 28–96%).[18,20,21,25–27] In the above-mentioned studies, severe clinical allergic reactions were seen in only 24% of children and 29% of adults after their first exposures to the ASNase,[18,20,21,25–27] versus 58% Ab-positive presentation. Thus, approximately half of the patients historically must have had silent hypersensitivity, as described recently.[3,5] The precise role of Ab in altering ASNase pharmacokinetics is not known, and the direct relationship between the Ab and outcome has not been established.[21,23] However, some studies showed faster clearance of the ASNase, lower ASNase $t_{1/2,el}$, and decreased enzymatic activity in patients with hypersensitivity or high anti-ASNase Ab titers.[3,24] Severe clinical hypersensitivity requires that the drug be withdrawn from the therapeutic regimens. In a Pediatric Oncology Group (POG) study on B-lineage ALL, a high frequency of allergic reactions prevented 25% of patients from receiving their entire planned course of ASNase therapy. According to this study, allergic reactions could be the primary reason for the inability to show an advantage of the regimen offering intensive ASNase.[28] In contrast, another POG study demonstrated that intensive high-dose ASNase consolidation improved survival for children with T-cell ALL and advanced-stage lymphoblastic lymphoma.[29]

In CCG-1961, it was hypothesized that the prevalence of Ab formation in the high-risk (HR) ALL patients is a predictor of poor treatment outcome. Evaluation of the whole cohort with known Ab levels and longer follow-up in CCG-1961 will permit a definitive evaluation of this hypothesis. However, the clinical allergy and outcome data currently remain under the control of an external data monitoring committee (DMC), and examination of the role of Ab levels in treatment outcome of the entire cohort is planned as part of the future analyses.

We determined the correlations between anti-ASNase Ab and ASNase enzymatic activity in children with HR ALL, particularly in connection with the type of ASNase used.[30,31] We report here the overall summary of clinical ASNase pharmacology observations, the results from a large database of 1,001 patients monitored for anti-ASNase Ab and ASNase enzymatic activity in serial serum samples from CCG-1961, and the results from an interim outcome analysis that showed that anti-ASNase Ab may adversely affect treatment outcome.

## METHODS

### Patient Eligibility and Treatment Protocol

From November 1996 until May 2002, 2,077 patients with newly diagnosed ALL and Rome/NCI higher-risk features were entered in CCG-1961, a randomized prospective study, and assigned to one of the protocol arms (Fig. 1, Table 1). Patients met the following criteria for eligibility: ages 1 to 9 years old with an initial white blood cell count (WBC) of 50,000/μM or higher, or from 10 to 21 years with any WBC. Patients who had central nervous system leukemia or overt testicular leukemia at diagnosis were eligible, and patients with FAB L3 leukemia were ineligible. We received specimens from 1,202 patients, 1,048 of whom had more than one sample withdrawn, but only 1,001 of the 1,048 were fully analyzed according to the eligibility criteria, since remaining considered indecipherable. Thus, the patients with single specimens (n = 154) were excluded from these analyses.

Multiple specimens (n = 3,193) from 1,001 patients were obtained at diagnosis and after induction treatment of ASNase pharmacology studies. Patients were treated according to the CCG-1961 study protocol (see Table 1 and Fig. 1). The backbone of induction therapy comprised prednisone, vincristine, daunomycin, and asparaginase plus intrathecal methotrexate and cytosine arabinoside. The next phases (eg, consolidation, one or two interim maintenance and delayed intensification cycles and two [for girls] or three [for boys] years of maintenance therapy) also included cyclophosphamide, cytarabine, vincristine, dexamethasone, methotrexate, mercaptopurine, thioguanine, and doxorubicin or idarubicin (see Table 1). Three different ASNase preparations were used: native *E. coli* ASNase, 6,000 IU/m$^2$, given intramuscularly (IM); PEG-ASNase, 2,500 IU/m$^2$, IM; and *Erwinia*-ASNase, 6,000 IU/m$^2$, IM. ASNase injection frequencies, selection of preparation, and treatment modifications were performed according to protocol and the randomization arm and depending on toxicities.

Treatment was stratified between rapid and slow early responders (RER and SER) based on the day 7 bone marrow status (<25% blasts [RER] or >25% blasts [SER]). All patients received nine doses of native *E. coli* ASNase during induction, on a Monday, Wednesday, and Friday schedule (three doses per week). RERs assigned randomly to standard-intensity arms (eg, arms A and B) received 6 or 12 additional doses of native ASNase during intensifications 1 and 2, while RERs assigned randomly to stronger-intensity arms (eg, arms C and D) received 6 or 10 doses of PEG-ASNase during consolidation, interim maintenance, and intensifications 1 and 2. All SERs subsequently received 10 doses of PEG-ASNase after induction. The *Erwinia* ASNase was used only if the patient developed clinical signs of allergy to the *E. coli* or PEG preparation. A Monday, Wednesday, and Friday schedule was used in case of *Erwinia* ASNase, substituting dose for dose of native *E. coli* ASNase. Six doses of *Erwinia* ASNase were given for each missed PEG-ASNase dose in patients with clinical allergies to PEG-ASNase. Figure 1 and Table 1 summarize the CCG-1961 protocol.


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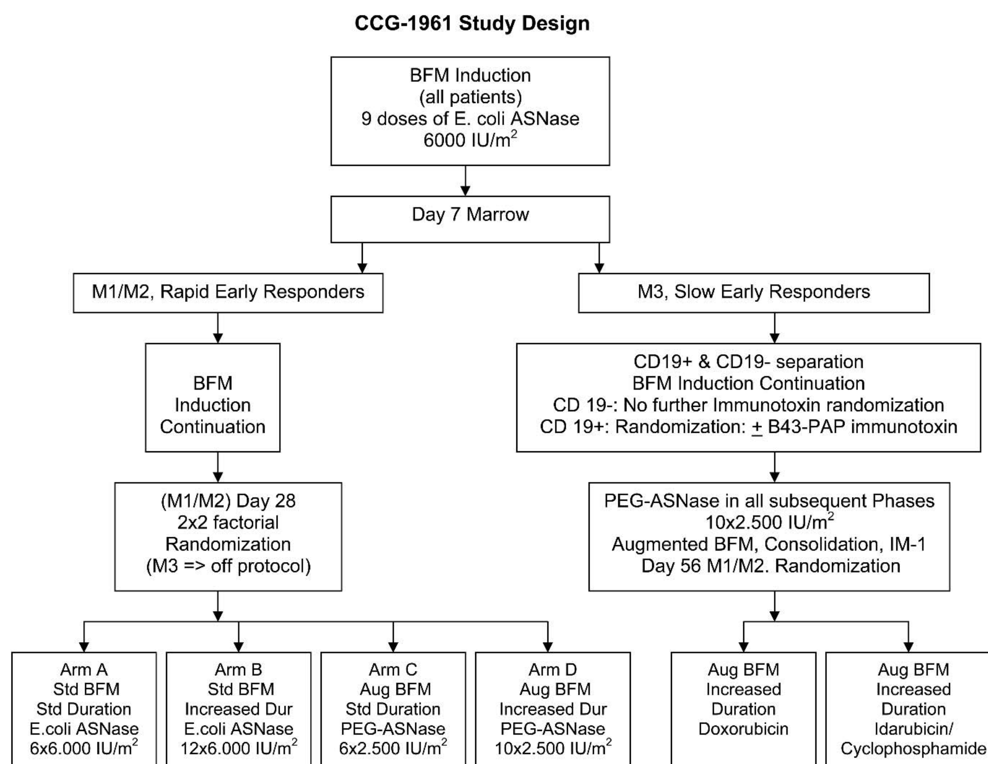


**Summary of ASNase doses in CCG-1961**

| Regimen | *E. Coli* ASNase | *PEG-* ASNase | Total |
|---|---|---|---|
| Arm A | 90.000 IU/m$^2$ | -- | 90.000 IU/m$^2$ |
| Arm B | 126.000 IU/m$^2$ | -- | 126.000 IU/m$^2$ |
| Arm C | 54.000 IU/m$^2$ | 15.000 IU/m$^2$ | 69.000 IU/m$^2$ |
| Arm D | 54.000 IU/m$^2$ | 25.000 IU/m$^2$ | 79.000 IU/m$^2$ |
| SER | 54.000 IU/m$^2$ | 25.000 IU/m$^2$ | 79.000 IU/m$^2$ |

**FIGURE 1.** CCG-1961 study design and the summary of asparaginase doses in different arms of the regimen.

## Serum Specimens

The pre-ASNase (control) and multiple post-ASNase serum specimens from 1,001 patents (3,193 specimens) were collected during different phases of therapy. Samples were shipped on dry ice and stored at –80°C to prevent Ab and ASNase denaturation and ex vivo amino acid deamination.[5] The specimens from these patients were analyzed for anti-ASNase Ab and ASNase enzymatic activity.

## Determination of ASNase Enzymatic Activity

Serum ASNase activity was measured using an enzymatic reaction that converts L-Asn to L-aspartate and ammonia in the presence of ASNase. Reacting solutions were placed in an enzyme-linked immunosorbent assay (ELISA) plate. Then ammonia nesslerization was monitored at optical density $OD_{405}$ nm. Asparaginase enzymatic activity was calculated from an ASNase standard curve in the range of 0.0125 to 0.6 IU/mL. The standard curves from each assay were compared with previous curves to ascertain quality control and calculate standard deviations.[5]

## Determination of Anti-ASNase Ab

Anti-ASNase antibody titers were measured using an antibody-capture ELISA.[3,5] Commercially available native *E. coli* ASNase and PEG-ASNase were the antigens used. A standard curve was generated from the reference standard pool (dilutions ranging from 1:1,000 to 1:64,000) in each ELISA run. Reference standard Ab dilutions were prepared from the serum

**TABLE 1.** Chemotherapy Protocol for CCG-1961

| Phase, Drug, Route | Dosage, Days | Dosage, Days |
|---|---|---|
| Induction therapy | Days 0–7 (All patients) | |
| Vincristine IV | 1.5 mg/m$^2$ days 0, 7 | |
| Prednisone PO | 60 mg/m$^2$ days 0–7 | |
| *E. Coli ASNase IM* | *6000 IU/m$^2$ days 3, 5, 7* | |
| Daunomycin IV | 25 mg/m$^2$ days 0, 7 | |
| Cytarabine IT | 30, 50 or 70 mg day 0 | |
| Induction continuation, 4 weeks (All patients) | | |
| Vincristine IV | 1.5 mg/m$^2$ days 14, 21 | |
| Prednisone PO | 60 mg/m$^2$ days 7–27 then 10 day taper | |
| *E. Coli ASNase IM* | *6000 IU/m$^2$ days 9, 11, 13, 15, 17, 19* | |
| Daunomycin IV | 25 mg/m$^2$ days 14, 21 | |
| Methotrexate IT | 8, 10 or 12 mg days 7, 28[a] | |
| Consolidation Phase, (RER patients) | | |
| | Arms A & B | Arms C & D |
| Prednisone PO | Taper continuation | Taper continuation |
| Cyclophosphamide IV | 1000 mg/m$^2$, days 0 and 14 | 1000 mg/m$^2$, days 0 and 28 |
| Cytosine | 75 mg/m$^2$/day, days 1–4, 8–11, 15–18, 22–25 | 75 mg/m$^2$/day, days 1–4, 8–11, 29–32, 36–39 |
| Arabinoside IV | | |
| 6-Mercaptopurine PO | 60 mg/m$^2$/day days 0–27 | 60 mg/m$^2$/day days 0–13, 28–41 |
| Methotrexate IT | 8, 10 or 12 mg days 1, 8, 15, 22 | 8, 10 or 12 mg days 1, 8, 15[b], 22[b] |
| Vincristine IV | | 1.5 mg/m$^2$ days 14, 21, 42, 49 |
| *PEG ASNase IM* | | *2500 IU/m$^2$ days 14, 42* |
| Interim maintenance #1 & 2 (RER patients) | | |
| | Arm A (IM #1 only) | Arm C (IM #1 only) (Capizzi I) |
| | Arm B same as A + IM #2 | Arm D same as C + IM #2 |
| 6-Mercaptopurine PO | 60 mg/m$^2$/day day 0–41 | |
| Methotrexate IT | 8, 10 or 12 mg, days 0, 28 | 8, 10 or 12 mg, days 0, 30[b] |
| Methotrexate PO | 15 mg/m$^2$/day days 7, 14, 21, 35 | |
| Methotrexate IV | | 100 mg/m$^2$ days 0, 10, 20, 30, 40 |
| Vincristine IV | | 1.5 mg/m$^2$ days, 0, 10, 20, 30, 40 |
| *PEG ASNase IM* | | *2500 IU/m$^2$ days 1, 21* |
| Delayed Intensification #1 & 2 | 7 weeks- A, B | 8 weeks- C, D |
| Reinduction + Reconsolidation | 4 + 3 weeks- A, B | 4 + 4 weeks- C, D |
| | Arm A (DI #1 only) | Arm c (DI #1 only) |
| | Arm B same as A + DI #2 | Arm D same as C + DI #2 |
| Vincristine IV | 1.5 mg/m$^2$ days, 0, 7, 14 | 1.5 mg/m$^2$ days, 0, 7, 14 |
| Doxorubicin IV | 25 mg/m$^2$ days 0, 7, 14 | 25 mg/m$^2$ days 0, 7, 14 |
| Dexamethasone PO | 10 mg/m$^2$/day, days 0–20 (Arm-B, Days 0–6, 14–20, no taper) | 10 mg/m$^2$/day days 0–6, 14–20, (Arm-D Days 0–20, no taper) |
| Methotrexate IT | 8, 10 or 12 mg day 0 | 8, 10 or 12 mg day 0 |
| *E. Coli ASNase IM* | *6000 IU/m$^2$ days 3, 5, 7, 10, 12, 14* | |
| *PEG ASNase IM* | | *2500 IU/m$^2$ day 3* |
| Cyclophosphamide IV | 1000 mg/m$^2$, day 28 | 1000 mg/m$^2$, day 28 |
| 6-Thioguanine PO | 60 mg/m$^2$/day, days 28–41 | 60 mg/m$^2$/day, days 28–41 |
| Cytosine Arabinoside IV | 75 mg/m$^2$/day, days 29–32 and 36–39 | 75 mg/m$^2$/day, days 29–32 and 36–39 |
| Methotrexate IT | 8, 10 or 12 mg days 28, 35 | 8, 10 or 12 mg days 28, 35[b] |
| Vincristine IV | | 1.5 mg/m$^2$ days 42, 49 |
| *PEG ASNase IM* | | *2500 IU/m$^2$ days 42* |


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**TABLE 1.** *Continued*

| Phase, Drug, Route | Dosage, Days | Dosage, Days |
|---|---|---|
| Maintenance Phase, 12 weeks per course (All patients) | | |
| Methotrexate IT | 8, 10 or 12 mg day 0 of each course (arms A & C—additional MTX on day 28 of courses 1–4) | |
| Vincristine IV | 1.5 mg/m$^2$, days 0, 28, 56 | |
| Prednisone PO | 40 mg/m$^2$, days 0–4, 28–32, 56–60 | |
| 6-Mercaptopurine PO | 75 mg/m$^2$/day, days 0–83 | |
| Methotrexate PO | 20 mg/m$^2$/day, days 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77 | |

SER patients: similar to RER Arm D, with randomizations on ±/B43-PAP in continuation of induction, and Daunorubicin vs. Idarubicin arms on delayed intensification.

[a]Patients with CNS disease at diagnosis IT Methotrexate (MTX) was given on induction days 14 & 21.

[b]Quit IT MTX for the RER patients on Arm D and SER patients, who had the CNS leukemic involvement at diagnosis.

Abbreviations: ASNase-asparaginase, CNS- central nervous system, IM- intramuscular, IT- intrathecal, IV- intravenous, PO- *per os,* RER- rapid early responders, SER- slow early responders.

of anti-ASNase Ab-positive patients with clinical allergy from the CCG-1962 study (approximate titer $1 \times 10^3$). A dilution of 1:500 was used as the positive control. Positive and negative controls (normal volunteer's serum) were used with each ELISA plate along with the "unknown" assayed samples. Plates were read at $OD_{490}$ nm. The Ab strength per unit volume of serum (1 mL) (titer) for patients' serum was calculated from the standard curve generated from each ELISA plate and expressed as the number over the negative control from the same healthy volunteer.[5] The titer 1.1 (>110% of negative control) was considered Ab-positive.

## Ex Vivo Neutralization Assay

Ex vivo neutralization experiments were conducted using the patients' serum specimens as a source of the anti-ASNase Ab. After 1 hour of interaction of the patient's serum at 1:1 volume with 1.0 IU/mL and 0.1 IU/mL native and PEG-ASNase antigen solutions, the remaining ASNase enzymatic activity was measured as described previously.[5]

## Statistical Methods

Nonpaired *t* tests were used to evaluate the possible differences in averaged values of some characteristics between the patients in the Ab-positive versus the Ab-negative groups.

# RESULTS

## Patient Characteristics

Table 2 shows the patient characteristics. Cytogenetic analyses for t(9:22), t(4:11), and t(1:19) translocations were positive in 1.6%, 1.1%, and 2.2% of total patients, respectively. Institutional immunophenotypes for CD2, CD7, CALLA, and CD19 were 30% or more in 23.7%, 25.9%, 74.1%, and 77.0% of cases, respectively. Central review on ploidy classification yielded these results: normal, 27.8%; hypoploidy, 9.2%; pseudoploidy, 37.8%, and hyperploidy, 25.2%. Lymphadenopathy was observed in 54.2% of cases, with normal nodes in 45.8%, moderately enlarged nodes in 45.0%, and markedly enlarged nodes in 9.2% of patients at diagnosis. There was moderate splenomegaly in 47.2% of patients and marked splenomegaly in 11.2%. Massive hepatomegaly was present in 8.0% of patients and moderate hepatomegaly in 44.0%. A large mediastinal mass was observed in 15.1% of patients.

Patient characteristics were compared in the Ab-positive versus Ab-negative groups as well (see Table 2). No significant differences were obtained for most characteristics at diagnosis. The only potentially important difference was observed when Ab-positive presentation was compared in boys versus girls. The ratio of Ab-positive to Ab-negative patients among boys was 1.77 (386/217) and among girls it was 1.3 (225/173), indicating a slight dominance of anti-ASNase Ab formation in boys.

## Anti-ASNase Ab Levels

Among 1,001 patients, 611 (61%) tested positive for at least one elevated anti-ASNase Ab titer (titer >1.1, Ab-positive) at some time during treatment (10–12 months for different randomized arms); the remaining 390 of 1,001 (39%) were anti-ASNase Ab-negative. Table 3 presents the Ab-positive ratio values over negative control per phase of treatment. The control Ab ratio over negative control averaged $0.99 \pm 0.18$. Once high-titer Ab-positivity appeared, generally it persisted in all subsequent samples (Fig. 2). When patients received *E. coli* native ASNase formulations, the Ab titers rose. When patients received alternative ASNase formulations, the Ab titers fell.

## ASNase Activity

The first half of the patients entered in the 6-year study (n = 507) were examined for Ab and ASNase activity in each

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**TABLE 2.** Patient Characteristics in General and According to the Anti-ASNase Ab Status, CCG-1961

| Characteristics at Diagnosis | Total (n = 1,001) | Ab(+) (n = 611) | Ab(−) (n = 390) |
|---|---|---|---|
| Sex | | | |
| Male | 603 (60.2) | 386/603 (64%) | 217/603 (36%) |
| Female | 398 (39.8) | 225/398 (56.5%) | 173/398 (43.5%) |
| Race | n = 989 | n = 604 | n = 385 |
| White | 696 (70.4%) | 430 (71.2%) | 266 (69.1%) |
| Hispanic | 192 (19.4) | 109 (18.05%) | 83 (21.6%) |
| Black | 56 (5.7%) | 38 (6.3%) | 18 (4.7%) |
| Asian | 15 (1.5%) | 10 (1.66%) | 5 (1.3%) |
| Other | 30 (3.0%) | 17 (2.8%) | 13 (3.38%) |
| Age distribution (range in yrs) | 1 (4.9%)–16+ (11.3%) | 1 (4.9%)–16+ (12.8%) | 1 (4.9%)–16+ (8.9%) |
| Majority of patients' age | 10–15 years (50.5%) | 10–15 years (50.1%) | 10–15 years (51.3%) |
| Mean patients' age* | 10.1 ± 5 years | 10.3 ± 5.1 years | 9.93 ± 4.96 years |
| Median patients' age | 11.25 years | 11.33 years | 11.08 years |
| Mean WBC at diagnosis (n = 999 pts)* | 103 ± 342 (×1,000/µL) | 92 ± 140 (×1,000/µL) | 120 ± 520 (×1,000/µL) |
| Range WBC | <5–9999 (×1,000/µL) | <5–1,226 (×1,000/µL) | <5–9999 (×1,000/µL) |
| Median WBC | 54.7 (×1,000/µL) | 54.2 (×1,000/µL) | 55.485 (×1,000/µL) |
| Percent of patients with >50K WBC | 53. 35% | 53.52% | 53.1% |
| Mean % bone marrow blasts (n = 987 pts)* | 88.9% ± 13.0% | 88.99% ± 13.01% | 88.8% ± 13.1% |
| Median % bone marrow blasts | 93.0% | 93.0% | 92.5% |
| Mean % peripheral blood blasts (n = 992 pts)* | 56.4% ± 35.2% | 56.53% ± 34.95% | 56.2% ± 35.6% |
| Median % peripheral blood blasts | 69.0% | 68.0% | 70.0% |
| Mean hemoglobin at diagnosis (n = 978 pts)* | 8.5 ± 3.41 g/dL | 8.45 ± 3.2 g/dL | 8.56 ± 3.7 g/dL |
| Median hemoglobin | 8.2 g/dL | 8.2 g/dL | 8.2 g/dL |
| Mean platelet count (n = 995 pts)* | 120.3 (×1,000/µL) | 99.66 (×1,000/µL) | 153.04 (×1,000/µL) |
| Median platelet | 47.0 (×1,000/µL) | 47.0 (×1,000/µL) | 46.0 (×1,000/µL) |
| Testicular involvement (n = 580 pts) (enlarged) | 9 (1.55%) | 6/375 (1.6%) | 3/205 (1.46%) |
| CNS disease at diagnosis (n = 975 pts) | 37 (3.8%) (Tr. Taps 16; 1.6%) | 20/599 (3.3%) | 17/376 (4.5%) |
| CNS-2 at diagnosis | 91 (9.4%) | 58/599 (9.7%) | 33/376 (8.8%) |
| Down syndrome (n = 999 pts) | 17 (1.7%) | 13/611 (2.1%) | 4/388 (1.03%) |
| T-cell lineage (n = 743 pts) | 206 (27.7%) | 80/289 (27.7%) | 126/454 (27.75%) |
| B-cell lineage | 537 (72.3%) | 209/289 (72.3%) | 328/454 (72.25%) |

*No statistical differences were obtained between the averages of these parameters in the Ab(+) vs. Ab(−) groups of patients, according to nonpaired *t*-test evaluations P = 0.1 to P = 0.9.

phase of treatment: consolidation, interim maintenance 1 (IM1), delayed intensification 1 (DI1), interim maintenance 2 (IM2), and delayed intensification 2 (DI2). Table 4 shows the distribution of the averaged Ab titers and ASNase activity values per treatment phase of anti-ASNase Ab-negative patients with positive ASNase activity. Of 141 Ab-positive patients (among 372) at consolidation, 88 had Ab-positive titers and ASNase activity measurements done on the same specimens, withdrawn shortly after PEG-ASNase, known to be administrated 7 to 14 days before sampling. No anticipated ASNase

**TABLE 3.** Average Ab Titers Over Negative Control per Treatment Phase for Ab(+) Patients

| Pre-Tx | Consolidation | IM1 | DI1 | IM2 | DI2 |
|---|---|---|---|---|---|
| 0.99 ± 0.18 | 8.48 ± 87.4 | 36.62 ± 108.7 | 33.51 ± 123.3 | 17.5 ± 55.18 | 20.85 ± 78.2 |

Data are given as mean ± SDEV.

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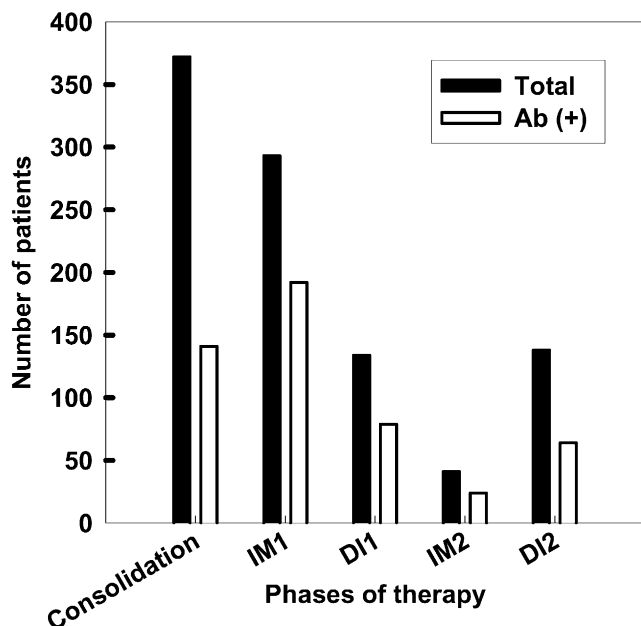


**FIGURE 2.** Numbers of Anti-ASNase antibody (Ab)-positive patients and total patients tested for Ab presentation per each treatment phase after induction in CCG-1961.

activity was detected in 81 of 88 Ab-positive patients (thus, 94% neutralizing activity). The neutralizing capacity of the Ab was confirmed also by our ex vivo verifications. The remaining seven patients either had partial neutralization or their ASNase activity was due to alternative ASNase formulation. There was also one each nonneutralizing Ab specimen during interim maintenance 1 and 2 and two during delayed intensification 1 among 263 separate data sets (98.48% neutralizing Ab-positive).

Among 1,001 patients, 611 were Ab-positive, 447 (73.2%) of whom had no ASNase activity at any time during treatment. These results, taken in conjunction with our ex vivo neutralization assay verifications, strongly suggest the neutralizing character of the anti-ASNase Ab. One hundred sixty four of 611 Ab-positive patients (26.8%) had ASNase activity at some time during treatment. These patients were examined for timing of Ab appearance in relation to the time of positive ASNase activity detection and divided into three groups. In group 1, 67 of 164 patients (40.9%) had ASNase activity 16.1 ± 10 weeks before the first appearance of Ab-positivity. Thus, these patients had ASNase activity before the Ab appeared. However, in a short time the ASNase activity declined precipitately in all. In group 2, 21 of 164 patients (12.8%) had ASNase activity 21.7 ± 19.3 weeks after the first appearance of Ab-positivity. In group 3, 76 of 164 patients (46.3%) had ASNase activity 14.7 ± 14.6 weeks after treatment started, with coexisting Ab. The last two groups had ASNase activity due to alternative ASNase formulation given many weeks after Ab-positivity appeared or concurrently with Ab positivity. That was because of randomization or clinical allergy presentation, which allowed administration of non–cross-reacting *Erwinia* ASNase. All of them had substantial activity, ranging from 0.1 to 0.4 IU/mL.

Many patients (39%) had no elevated Ab ratios during treatment. One hundred thirty of 390 Ab-negative patients (33.3%) had measurable positive ASNase activity (ASNase-positive) at some time during treatment; the remaining patients had no detectable ASNase activity, mostly due to inopportune timing of the sample collection (ie, before the ASNase dose was given).

### Interim Analysis of Ab Correlations With Poor Clinical Outcome

We report here the results of the interim analysis of 280 patients who were evaluated for at least 30 or more months after induction, since the results from the complete cohort are unavailable at this point of time due to DMC restrictions. Fifty-seven of 280 patients (20%) had no clinical allergies and were Ab-negative throughout their treatment (Table 5). An additional 27 patients (10%) had mild allergy symptoms but were Ab-negative. These reactions can be attributed to the other drugs they received or EMLA, the topical anesthetic gel used to reduce the pain of the IM injection.[32] In contrast, the largest subset of patients (115/280 [41%]) developed anti-ASNase Ab-positivity and had obvious clinical allergy symptoms. These patients were switched to *Erwinia* ASNase, a formulation that does not cross-react with the anti-*E. coli* ASNase Ab. This subset had beneficial outcomes by receiving effective ASNase therapy as the subset A, indicated by a hazard ratio of 0.6 versus 1.0. *Erwinia* ASNase doses varied in these patients; thus, the ASNase treatment may not have been pharmacodynamically equivalent to that of PEG-ASNase. The data suggest that some effective ASNase dosing is better than none for treating ALL Ab-positive patients.

In contrast, the fourth subset of patients (81/280 [29%]) had no clinical allergies but had anti-ASNase Ab-positivity in sera. Anti-ASNase Ab is negating the ASNase enzymatic activity, thus allowing the serum Asn to rebound, which counteracts the therapeutic aims. Ab-positivity has an adverse effect on treatment outcome that was statistically significant (Table 5). This subset of patients received no effective ASNase therapy after induction. Therefore, patients with anti-ASNase Ab-positivity and no clinical allergy symptoms had poorer outcomes than any other subset.

## DISCUSSION

Native and pegylated ASNases have major differences in pharmacokinetic and pharmacodynamic parameters and immunogenicity.[3,5] Recently, a randomized comparison between

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**TABLE 4.** ASNase Activity and Ab Titer in Ab(−) Patients

| Tx Phase | Pre-Tx | Consolidation | IM1 | DI1 | IM2 | DI2 |
|---|---|---|---|---|---|---|
| ASNase (IU/mL) | 0.0 ± 0.0 | 0.124 ± 0.19 | 0.135 ± 0.35 | 0.11 ± 0.27 | 0.073 ± 0.27 | 0.07 ± 0.23 |
| Ab titer over negative control | 0.99 ± 0.18 | 1.02 ± 0.12 | 0.99 ± 0.14 | 0.94 ± 0.11 | 0.98 ± 0.07 | 0.98 ± 0.07 |
| Number of patients* | 507 | 231 | 101 | 55 | 17 | 74 |

Data are given as mean ± SDEV.

*Patient numbers for antibody titer averages only. The same number of patients (507) was averaged for asparaginase activity and Ab titer before treatment. Patients were averaged for asparaginase activity in subsequent treatment phases unless their asparaginase value was zero.

native and PEG-ASNase and their pharmacokinetic evaluations after IM administration in children newly diagnosed with standard-risk (SR) ALL was reported.[5] The development of anti-ASNase antibody can cause mild, severe, or life-threatening clinical reactions that may prevent further use of this important class of antileukemic drugs.[14,18–24,28] In CCG-1962, only 2 of 59 SR ALL patients exhibited clinical allergic reactions after second exposures to PEG-ASNase.[5] This incidence appeared low compared with reported frequencies of hypersensitivity reactions from other studies.[14,19–21,23,27,28] Before this report, studies of ASNase pharmacology have been carried out in a relatively limited number of pediatric HR ALL patients.[4,20,21,33]

In the CCG-1961 study, where all patients started from nine doses of *E. coli* ASNase at induction, the overall treatment schedule appeared to be very immunogenic. In this study, anti-ASNase Ab-positivity was detected in 61% (611/1.001) of HR ALL patients, which is similarly high as the average percentage from reported series.[18,20,21,24–27] In greater than 90% of cases, Ab titers higher than 1.1 were neutralizing the ASNase enzymatic activity in patients, which was verified by in vitro neutralization assays. Other investigators also have reported up to 47% in vitro inhibition of the ASNase enzymatic activity by the patients' serum with high titers of anti-ASNase Ab.[24] Most of the Ab-positive cases were detected for the first time during consolidation and interim maintenance 1 phases, with the highest titers during interim maintenance 1 and delayed intensification 1 (see Table 3 and Fig. 2). We conclude that such a high incidence of Ab-positivity and a pattern of Ab distribution could be dependent on the treatment protocol—that is, all patients received nine doses of more immunogenic *E. coli* native ASNase at induction. The native ASNase treatment during induction most likely produced the highest titers at the interim maintenance 1 and delayed intensification 1 phases. After the induction phase, only subgroups (protocol arms A and B; see Table 1 and Fig. 1) of the patients were exposed again to the *E. coli* ASNase, six doses in each phase, delayed intensification 1 and 2, at which the Ab titers are also high (see Table 3). That supported our secondary hypothesis that anti-ASNase Ab would increase over time after subsequent exposures to native ASNase as a result of anamnestic immune response. The relative decrease of Ab titers in the interim maintenance 2 phase might be explained by the absence of patients in this phase from arm A and B who received *E. coli* ASNase. Subgroups of patients in arms D and all SER patients received PEG-ASNase in the interim maintenance 2 phase. PEG-ASNase was confirmed to be less immunogenic in CCG-1962.[5]

There was no standardized uniform time specification as to when the samples had to be drawn, but for the limited num-

**TABLE 5.** Interim Analysis of Anti-ASNase Ab and Outcome in Patients With High-Risk ALL—CCG-1961

| Groups | Clinical Allergy | Ab(+) | Number (%) of Patients | Events (30 mo) | Hazard Ratio | |
|---|---|---|---|---|---|---|
| | | | | | Observed | Expected |
| A | No | No | 57 (20%) | 3/57 | 1.0 | 0.66 |
| B* | Yes | No | 27 (10%) | 2/27 | 1.3 | 0.86 |
| C* | Yes | Yes | 115 (41%) | 3/115 | 0.6 | 0.38 |
| D** | No | Yes | 81 (29%) | 13/81 | 3.2*** | 2.11 |
| Total | | | 280 (100%) | 21/280 | | |

*Patients were treated with *Erwinia* ASNase after the clinical allergy symptoms appeared.

**Silent hypersensitivity patients. These patients had the highest hazard ratio, which was statistically significant over the other groups of patients.

***Log rank $P = 0.01$.


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ber of Ab-positive patients, sample withdrawal was known to be done shortly (7–14 days) after PEG-ASNase administration. As described in the results section, in 81 of these 88 Ab-positive patients, no anticipated ASNase enzymatic activity was found in the serum. The randomness of many samples is obvious due to the fact that ASNase activity was detected after the treatment had began in many serum specimens. However, this limitation, with all of its negative consequences due to timing, should not affect the main thrust of this manuscript, which is to demonstrate the high incidence rate of neutralizing anti-ASNase Ab-positivity to native *E. coli* ASNase, which was the drug all patients received during the induction phase.

Among the 611 patients who had elevated Ab ratios over control, a significant number had no ASNase activity at any time. The other patients who had Ab-positivity and ASNase activity were divided into two groups: (A) many of these patients (40.9%) had activity before Ab-positivity appeared (group 1 in the Results section) and (B) the remaining (59.1%) were due to the administration of an alternative ASNase formulation (groups 2 and 3). In effect, ASNase activity was present before the Ab appeared or it mainly was due to Erwinase.

Patients who had been treated with native or PEG-ASNase and who had obvious clinical reactions were treated with Erwinase, which had therapeutic ASNase activity in anti-*E. coli* native ASNase Ab-positive patients, while PEG-ASNase rarely did.[31] This confirmed that the anti-*E. coli* ASNase Ab cross-reacted and neutralized PEG-ASNase, which is also an *E. coli*-derived enzyme, but did not cross-react with Erwinase, as documented in vitro. The most plausible explanation is that the epitopes in *E. coli* ASNase and PEG-ASNase proteins are the same, whereas Erwinase most likely has a different antigenic epitope. A recent study verified these findings on the cross-reactivity between patient Ab raised against native *E. coli* and PEG-ASNase, but not *Erwinia* ASNase.[34] Assessment of the amino acid sequence and crystal structure of *E. chrysanthemi* ASNase and comparison with other bacterial L-ASNases shows certain differences.[35] Future Erwinase exposures might eventually bring maturation of the IgG Ab toward this drug, but patients to some extent may benefit from switching to this ASNase formulation.

The significance of the subclinical hypersensitivity phenomenon and substitution of *E. coli* ASNase by Erwinase will be determined in the final outcome analyses of CCG-1961. In contrast, Ab-positive patients without clinical manifestations of allergy (eg, silent hypersensitivity patients) may have poorer outcomes because of no antileukemic contribution from an important component (ASNase) in multidrug chemotherapy. This idea was supported by the results of the interim analysis conducted in approximately one third of the patients who by December 1999 had 30 or more months of follow-up evaluation. Patients with Ab-positivity and clinical allergy symptoms during treatment with native or PEG-ASNase were treated with *Erwinia* ASNase. These patients had a hazard ratio as good or better than the patients with Ab-negative and sufficient ASNase activity (see Table 5). Assuming that the *Erwinia* ASNase is not bioequivalent in pharmacodynamic terms to native or PEG-ASNase, these results suggest that some effective ASNase treatment is better than none. In contrast, the patients with Ab-positivity and no clinical allergy symptoms (silent hypersensitivity patients) had a significant hazard ratio. Kurtzberg et al., suggested in 1993 that "silent hypersensitivity" due to anti-ASNase Ab formation may result in suboptimal Asn depletion and diminished efficacy of ASNase therapy.[24] Our analyses have shown that the anti-ASNase Ab has a predictive clinical value and that switching patients with Ab-positive from native ASNase to Erwinase may benefit their outcome, as indicated by the reversal of the hazard ratio from 3.22 to 0.6.

Since the silent hypersensitivity subset was large (29% of evaluable patients), representing approximately the 25% of all HR ALL patients who relapse in 5 years from diagnosis, the anti-ASNase Ab could be a surrogate marker responsible for the poor outcomes in this disease. Therefore, we propose that the final analyses will allow us to verify the interim results in the total population of patients with HR ALL over a longer time of 5 to 7 years of follow-up. Verification of these results should strongly recommend the standard monitoring of serum anti-ASNase Ab in clinical settings.

The potential differences of outcome between boys and girls have not been evaluated yet in CCG-1961 study, and the differences may have been insignificant due to the adjusted and more intensive (maintenance) therapy for boys than girls, as reported in previous CCG studies.[36,37] However, the poor prognostic significance of the males persisting since 1960 was still apparent in recent large studies by others[38–41] and was explained partially by different distributions of ALL immunophenotype and DNA index.[38] Unsuccessful attempts were made to demonstrate the potential gender differences in the pharmacology of other commonly used antileukemic agents (eg, mercaptopurine, methotrexate, cytarabine and teniposide).[38]

Our findings indicate a higher prevalence of average anti-ASNase Ab formation in boys, as evidenced by a 1.4 times higher ratio of Ab-positive to Ab-negative patients in boys compared with girls (1.77 vs. 1.3, respectively). This finding, in concert with the provisional acceptance of the adverse effect of the anti-ASNase Ab-positivity on clinical outcome, may be an explanation for the worse outcome of the ALL-affected boys observed historically.[42] Future outcome analyses will focus on this issue when the individual randomized arms are evaluated.

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