Abstract. This is an ambitious effort attempting to present as many aspects as possible in a review article on asparaginases (ASNase), and their use against acute lymphoblastic leukemia (ALL) and T-cell lymphomas. In the process, the modes of drug resistance are described both of the host and in the leukemia cells themselves. These modes of drug resistance, developed by the ALL cells, are an attempt to overcome the toxic insult this class of anti-leukemic drugs causes to them. It is expected that by reading this article one would obtain a better understanding of the initial events in the leukemia development, its microenvironment, and the many issues that a leukemia specialist has to deal with, especially in the treatment of refractory and relapsed patient populations. The specific issues addressed in this review deal with the importance of nutrients in tumor growth and progression of malignancies; the cytogenetics of ALL, as well as its chemotherapy, are also briefly presented. The emphasis will turn to ASNase, their mechanisms of action, the immune responses they cause in a significant percentage of the ALL patients, the significance of the up-regulation of glutamine synthetase and asparagine synthetase and the complexity of the elucidation of the mechanisms of action of ASNase. Additional details on the ASNase epitope mapping of anti-ASNase antibodies, the degradation of the protein, and the unmet needs in producing an optimal ASNase protein, will be also presented. Finally, a brief description of the toxicity, as well as the correlative factor of ALL treatment with ASNase is given.

Nutrients, Gene Expression, and Progression of Malignancies

Malignancies have their etiology in malformations of DNA, such as point mutations, chromosomal deletions, inversions, copy number variants (CNV), loss of heterozygocity (LOH), and other chromosomal abnormalities. However, protein biosynthesis is fundamental in all living cells because it is the mechanism that drives the cellular growth and development of all normal (healthy) and cancerous cells. For the most part, malignancies express variants of protein biosynthesis due to mutations leading to abnormal up-regulation of protein biosynthesis. Furthermore, many determinant causative proteins act in concert with hormones [growth hormone (GH), androgens, estrogens, etc.], about 20 families of growth factors and their ligands, which along with ample nutrients [glucose, amino acids (AA), lipids, minerals, vitamins, etc.] play an important role in the control of gene expression leading to cellular growth and proliferation and/or differentiation. AAs, along with other nutrients, serve as the building blocks of protein biosynthesis, as well as signal transduction messengers transmitting the nutritional status of the entire organism to individual cells. Given the critical roles AAs exert in cell survival, growth, metabolism, and signaling, it is not surprising that mammalian tumors have evolved...
mechanisms to adapt to protein malnutrition and related AA deprivation for short time intervals, i.e., during chemotherapy. The steady state of the cellular pool for each of the nonessential AAs is the result of a balance between de novo biosynthesis plus nutrient input and utilization and removal (degradation). Diet and proteolysis provide the essential and the majority of the nonessential AA pool. Certain key nonessential AAs, such as L-glutamine (Gln) and L-asparagine (Asn), which are necessary for neuronal function and survival of malignant cells, are in great demand for protein biosynthesis and as sources of carbon and nitrogen in highly proliferative conditions. Therefore, the molecular basis of gene regulation by AAs is an important field of research for the study of regulation of global protein inhibition under physiological and pharmacological conditions. However, we must leave the broad spectrum of cell survival aside, and focus on the aspects of lymphoproliferative malignancies, which is an exciting field in oncology.

**Acute Lymphoblastic Leukemia (ALL)**

Leukemias are a cancer of the blood cells, characterized by uncontrolled growth and accumulation of immature white blood cells of the myeloid or lymphoid lineage. Normally, hematopoiesis is a balanced biological process of pluripotent hematopoietic stem cell (HSC) proliferation and differentiation that mainly occurs in the bone marrow (BM) and thymus microenvironments. In the case of leukemia, there is an uncontrolled expansion of immature malignant cells with the immortality of a leukemia stem-like cell, which results in anemia (red and white blood cell deficiencies), leading to increased risk of infections. Further proliferation of the leukemia cells will drive them from the BM and circulation to invade most tissues in the body. Leukemic transformation is caused by multistep pathogenesis involving at least two or numerous genetic abnormalities (DNA), the altered proteins of which drive these cells into uncontrolled cell growth and clonal expansion. Surveillance, Epidemiology and End Results (SEER) on the US cancer incidence estimated that ~44600 men~55% and women~45% will be diagnosed with and ~21780 men and women died of ALL, the majority of whom where adults, in 2011. Approximately 10.8% of those diagnosed were under the age 20 (Table I) (1).

Without effective treatment, leukemias have always been lethal. Lymphoid leukemia is dichotomized into T- and B-lineage leukemia, with further subdivision into acute or chronic leukemia. ALL is an aggressive malignancy and occurs predominantly in young children and young adults, with peak incidence between 3-5 years old, decreasing in incidence with increasing age, before increasing again at around 50 years old (Table I).

<table>
<thead>
<tr>
<th>Age at diagnosis</th>
<th>Percentage of incidence per (10^5) population</th>
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<tbody>
<tr>
<td>0-20 years</td>
<td>10.8%</td>
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<tr>
<td>21-34 years</td>
<td>4.8%</td>
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<td>35-44 years</td>
<td>5.3%</td>
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<td>45-54 years</td>
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<td>55-65 years</td>
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<tr>
<td>65-74 years</td>
<td>19.7%</td>
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<tr>
<td>75-84 years</td>
<td>22.5%</td>
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<tr>
<td>85 years+</td>
<td>10.8%</td>
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**Cytogenetics of ALL**

Conventional karyotyping, comparative genome hybridization (CGH), and related studies (e.g. fluorescence in situ hybridization, FISH) of chromosomes and their abnormalities have evolved into a specialized science with significant sub-classifications of the various types of leukemias, along with commensurate intensity of treatment protocols. Many genetic factors, such as chromosomal abnormalities and karyotypic changes, lead to polymorphisms of class I of major histocompatibility complex (MHC) with the human leukocyte antigens (HLA-A, HLA-B, HLA-C, etc.) and T-cell receptors playing important roles in immune responses to antigens. In addition, microenvironmental factors in the BM, CNS, and elsewhere influence the survival and growth of leukemia stem cells (LSC). Many other factors, such as optimal drug dosing, age, gender, obesity, growth factors in circulation, immune system maturity, supportive care, and response to foreign antigens of the host, may play important roles in the overall response to chemotherapy treatments.

Translocations and other chromosomal aberrations are important in ALL treatment outcome. Monosomies, trisomies, breakpoint cluster region- Abelson tyrosine-protein kinase (BCR-ABL), Fms-related tyrosine kinase 3 (FLT-3), the mixed lineage leukemia t(4;11)(q21;q23) involving the genes MLL and transcription factor (chromosome) 4, AF4 (MLL-AF4 fusion protein), the cryptic t(12;21)(TEL/AML1), which is a gene fusion resulting from a t(12;21)(p13;q22) chromosomal translocation, and many other oncogene expressions play important roles in the outcome of leukemia (2-4). Thus, all these mutant proteins must be inhibited by a global protein inhibitor if we wish to eliminate all leukemia cells. Yet, as the surviving minimum residual disease (MRD), leukemia cells are enriched by these drug-refractory blast cells, the majority of those patients who responded initially to
targeted therapy [e.g. imatinib mesylate in (Ph+)] chromosome leukemia] are likely to be subject to disease progression due to imatinib-resistant Ph(+) ALL blasts (5, 6). Furthermore, patients whose leukemia blasts exhibit significant differences in the metabolism and interactions of purine and pyrimidine nucleoside anabolite drugs, such as fludarabine, cladribine, and cytosine arabinoside (ara-C), may have differences in the initial and subsequent dose responses (7, 8). All these possible negative factors, along with the notion that tumor mutations continue to occur with cytotoxic pressure from the chemotherapy drugs, influence the long-term ALL outcomes. In addition, progression of the malignancy lead to the acquisition of additional mutations, which make neoplasms difficult to efficaciously treat with cytotoxic drugs. At that point, immune-based treatment modalities or more intensified drug treatments, followed by BM transplantation are recommended. Therefore, reasons for ALL relapse are many and complex. This brief synopsis highlights that ALL is a very heterogeneous disease requiring multiple and specific drugs in order to be treated effectively. One might ask, in such a complex disease how can one expect that one of the many oncology drugs can make a difference in treatment outcomes. It is hoped that by the end of this article the reader may have been persuaded that this is the case for asparaginases.

About 4000 cases of pediatric ALL are diagnosed every year in the US and many more throughout the world. The majority of these cases are in children and young adults, making ALL the most common form of malignancy in these age groups. The treatment protocols of ALL are complex and use 6-12 drugs (2-4). Approximately 48% of patients are afflicted with the higher risk ALL features and just above this percentage (58%) for patients with standard features. Despite this success in long term ALL outcomes, approximately 15% of patients experience disease relapse and most of them die from progression of ALL, making leukemic relapse the most common cause of treatment failure in pediatric oncology (2).

Chemotherapy of ALL

The era of cancer chemotherapy was initiated in the past 50 or more years. Cytotoxic chemotherapy drugs, along with improved surgical techniques and radiation therapy, are the principal means of effectively controlling malignancies. Many classes of antineoplastic drugs were investigated and developed in the 1960s and 1970s, including the bacterial L-asparaginases (ASNases), which are still in use today. The first discovery of the tumor-inhibitory properties of ASNase occurred 50 years ago, with the observation that lymphoma-bearing mice (particularly 6C3HED) treated with guinea-pig serum underwent rapid and often complete regression (9). In the 1960s, and continuing research in this field, Broome reported that ASNase activity in guinea-pig serum was responsible for the anti-lymphoma effects (10, 11). Escherichia coli bacterium was shown to possess two such enzymes, one expressed constitutively (EC1, Km=5 mM) and another induced by anaerobiosis (EC2, Km 12.5 μM); only the latter was tumor inhibiting. Asparaginases are tetrameric bacterial enzymes which deaminate Asn and Gln, inducing apoptosis (12-15). PK–PD analyses show that PEG-ASNase provides a better day 7/14 bone marrow response (12). The optimal ASNase administration which in the range of enzymatic activity of 0.4-0.7 IU/mL, at trough levels, provides greater than 90% depletion in vivo (with hepatic asparagine biosynthesis -CCG-1961 and CCG-1962), of Asn and Gln deamination. Such a level of depletion is associated or correlated with improved long term EFS (12-14, 17). Maintaining optimal ASNase enzymatic activity at trough times of 0.4-0.7 IU/mL, independent of the ASNase formulation, yields optimal depletion of Asn and Gln in patients with ALL (12-14). Naturally, major improvements have been made in the treatment outcomes of many malignancies, especially in leukemias and lymphomas, with the use of classical and novel anticancer drugs. The major classes of cytotoxic drugs targeting leukemias are summarized below: i. Alkylating agents: cyclophosphamide, etc.; ii. Corticosteroids: prednisone, dexamethasone; iii. Antimetabolites: cytosine arabinoside (ara-C), methotrexate (MTX), 5-fluorouracil (5-FU), gemcitabine, etc.; iv. Anthracyclines and topoisomerase inhibitors: adriamycin, daunorubicin, doxorubicin, Vp-16, and related congeners; all these classes of oncology drugs target and damage DNA. v. Mitotic inhibitors: vincristine, taxanes -target mitosis and stop cell division; vi. Protein inhibitor drugs: asparaginases.

Recently, the development of specific target-oriented molecules (imatinib mesylate, rapamycin and their related congeners, inhibiting specific pathways which target mostly mutant proteins and/or their antibodies, has provided further success in specific malignancies. As a result, significant steps have been made in prolonging patients’ lives and curing many cancer patients, especially pediatric ALL patients (2-4). Only in one of these classes of drugs is the bacterial-origin global protein inhibitor L-ASNase used in its native or pegylated form. ASNases inhibit global protein biosynthesis of many cell cycle and antiapoptotic proteins in cells undergoing chemoradiation; hence, they have become the drug of choice in many induction and re-induction combination regimens against leukemia and lymphomas in both pediatric and adult patient populations (12-14). The average IC50 concentration of PEG-ASNase is 0.4 IU/mL (12, 15). To this end, assays for the determination of the pharmacodynamic effectiveness of these bacterial proteins and their antibodies have been developed and applied in leukemia evaluations.
Useful Bacteria in Oncology

E. coli is gram negative, facultative anaerobic, and nonsporulating. Cells are typically rod shaped and are about 2-μm long and 0.5 μm in diameter, with a cell volume of 0.6-0.7 μm^3. E. coli is commonly found in the lower intestine of warm-blooded organisms (endotherms). Most E. coli strains are harmless (saprophytes), but some, such as serotype O157:H7, can cause serious food poisoning in humans and are occasionally responsible for food product recalls. Erwinia chrysanthemi (Dickeya chrysanthemi) is a gram-negative bacillus that belongs to the family Enterobacteriaceae. Erwinia is a genus of Enterobacteriaceae bacteria containing mostly plant pathogenic species. It is a close relative of E. coli and other animal pathogens that include Salmonella, Shigella, Klebsiella, Proteus, and Yersinia (Y. pestis). Members of this family are facultative anaerobes, able to ferment sugars to lactic acid, contain amidase and nitrate reductase but lack oxidases. Even though many clinical pathogens are part of the Enterobacteriaceae family, most members of this family are plant pathogens.

Both the E. coli and Erwinia chrysanthemi bacteria produce an amidase (ASNase with glutaminase activity), which for the past 50 years has been successfully used against lymphomatous malignancies. ALL cells are auxotrophs for Asn, thus they require Asn from external sources. As their name implies, ASNases deaminate Asn and, to a lesser extent, Gln, releasing aspartate and glutamic acid, respectively, plus ammonium ion. Depletion of Asn leads to a lesser extent, Gln, releasing aspartate and glutamic acid, and inhibition of protein biosynthesis. Hence, once the first few malignant cells have been generated, in order to grow, they need to synthesize proteins in a continuous and more rapid manner than healthy cells. Therefore, the use of the few protein inhibitor drugs, which are bacterial proteins themselves, like ASNases, has been shown to be very effective in combination therapies.

Classical immunology teaches us that protein therapeutics may elicit an immune response upon a single or multiple administrations (16, 17). When cross-reactive polyclonal antibodies are developed, they may cause unexpected adverse effects, including the discontinuation of biological drug treatments. For example, antibodies to ASNases in ALL patients will cause discontinuation of ASNase therapy, which most likely will have deleterious effects on long-term event-free survival (EFS) (17). Therefore, it is essential that immunological assays of therapeutic proteins be developed and applied during clinical trials for better safety and efficacy treatment assessments (16, 17).

ASNase, Asparagine Synthetase (ASNS), Glutamine Synthetase (GS) and Protein Synthesis

Protein synthesis is fundamental in all living cells, dividing and resting, both healthy and malignant. Translational control in eukaryotic cells is critical for gene regulation during nutrient deprivation stress, development and differentiation, neuronal function, disease progression, and aging. Therefore, translation of the genetic code to proteins and its regulation matter to healthy and malignant cells.

The plasma concentrations of AAs are relatively stable but they are affected by nutritional, pathological and pharmacological conditions. In patients, the plasma concentration of essential and non-essential AAs is affected by many external and internal factors. For example, an alteration in the AA profile has been reported when there is a deficiency of any one or more of the essential AAs, a dietary imbalance of AAs, or an insufficient intake of protein or after the deamination of certain AA is desired as part of anti-leukemia drug regimen. Specifically, deprivation of glutamine, arginine, cystine and all essential AAs leads to induction of insulin-like growth factor-binding protein-1 (IGFBP-1) mRNA and protein expression in a dose-dependent manner.

AA availability regulates the expression of several genes involved in the regulation of growth, cellular function or AA metabolism. Specifically, enzymatic activities or mRNA levels that are regulated by AA availability have been described. Amino acid limitation (Asn, Gln, etc.), especially induces the expression of asparagine synthetase (ASNS), glutamine synthetase (GS), insulin-like growth factor (IGF-I and -II), IGFBP-1, C/EBPβ homologous protein (CHOP), ornithine decarboxylase, the oncogenes c-JUN and c-FOS and the ribosomal proteins L17 and L25 (18, 19). Moreover, upon up-regulation of GS and ASNS proteins, the anti-apoptotic oncoproteins Myc and BCL-2 and its congeners are also stabilized and up-regulated (20-22). The BCL-2 family of oncoproteins induces many anti-mitochondrial lysing pathways, thus generating clones of leukemia cells which are refractory to many other classes of drugs, and to gamma radiation (23-25). The inverse experiment, demonstrated that
Gln deprivation initiated an intrinsic apoptotic pathway in Sp2/0-Ag14 hybridoma cells. In this study, Gln deprivation triggers intracellular events, which rapidly target the mitochondria, activating an intrinsic pathway of apoptosis, leading to a commitment to the cell death program less than 2 h after Gln withdrawal (26). It is noteworthy that a significant induction of GS and ASNS activities in ASNase-resistant cells, requires a long exposure to alternative Erwinia ASNase in order to render a degree of ASNase sensitivity in cell populations, which present unequivocal signs of cellular apoptosis. Gln itself activated mTOR separately from the Phosphatidylinositol 3-kinase (PI3k) → protein kinase B/Akt (Akt) → mammalian Target of Rapamycin (mTOR) pathway in a number of haematological diseases, including ALL, chronic myeloid leukemia (CML), mantle cell lymphoma, anaplastic large cell lymphoma, and lymphoproliferative disorders (27). Most importantly, a recent analysis performed in B-lineage ALL has indicated that the expression of at least 54 genes significantly discriminate between ASNase-resistant and sensitive cells (28, 29). Conversely, ALL cells find pharmacological sanctuaries in the BM or adipose tissues provided by mesenchymal or fat cells in the form of Asn, ASNS, Gln and GS, respectively (30, 31). In support of these reports, a recent study demonstrated the significance of Gln depletion in solid tumor carcinomas, whereby in the presence of genes responsible for AA homeostasis, such as ASNS and GS; for example, deficiency of dietary protein or an imbalance of essential AAs activates the AARE signal transduction pathway of genes responsible for AA homeostasis, such as Gln and Asn. Moreover, following AA deprivation, malignant cells increase transcription of ASNS from its mRNA via stabilization, thus avoiding Gln starvation and certain cell death (Gln-less death) (38-41).

**An Elucidation of the Mechanism of Action of Asparaginases**

Assuming that most asparaginases have both the ability to deaminate Asn but also have a glutaminase moiety which deaminates Gln, it is interesting to evaluate the effect that depletion of these AAs may have on other AAs [Figure 1 (12-14)]. As has been shown previously (41), a number of other AAs were depleted in patients post ASNase treatments. Therefore, it could be that the true mechanism of pro-apoptotic action of these protein inhibitors is related to the depletion of Gln, Asn and the essential AAs, in response to which the ASNS and GS proteins are up-regulated. An alternative hypothesis could be that ASNS protein and all other oncogene co-expressed proteins make these malignant cells refractory to chemotherapy by up-regulating MYC, BCL-2 with possible mutations in p53 proteins (23).

**Clinical Use of ASNases in Lymphoid Malignancies and Adverse Effects**

In the US, three ASNase formulations are available: native *E. coli* ASNase (ELSPAR), the pegylated form ONCASPAR (monomethoxypolyethylene glycol–L-asparaginase, PEGASNase), and *Erwinia* ASNase from *E.
Chrysanthemi. Intermittent or continuous use of ASNase has been shown to be active against ALL through the depletion of serum Asn and to a lesser extent, of Gln. However, after multiple ASNase administrations, patients develop antibodies to this bacterial protein (12-14, 17).

ASNases are administered either intramuscularly or, currently, intravenously to patients (2-4, 12-14, 17). Although ASNases do not diffuse into the extracellular space, the population of helper T-cells in the circulation, along with the dendritic cells, is exposed to this bacterial antigen protein, thus initiating a host response. The pharmacokinetic handling of *E. coli*-pegylated ASNase in children and in young adults (2-19 years old) with ALL is identical (12-14,17). Pharmacodynamic evaluations in both pediatric and adult patients with ALL post-ASNase treatment have demonstrated the common biochemical condition which must be met at trough times of 90% inhibition of both Asn and Gln, using each patients’ pre-therapy control. In antibody-negative patients, Asn should be depleted from ~50 μM to 3 μM or lower and Gln from ~600 μM to less than 80 μM concentrations, respectively (12, 13, 41-43).

The four active sites of ASNase tetramer protein are each made up from the N-terminal domain of a given subunit and the C-terminal domain of the other member of the respective close pair. This is illustrated in Figure 2, in which the A/C pair is depicted in shades of blue and the B/D pair depicted in green and yellow; the active site of subunits, recognizable by the 4 bound aspartate (Asp) in the active pockets (Figure 2). However, once the immune response is activated, most often the antibody neutralizes the enzymatic activity of ASNase, thus the formulation has to be either discontinued or a non-cross-reacting bacterial ASNase (erwinase) has to be administered, when available (13, 17, 42, 43). As many as 70% of high-risk ALL patients treated with the native *E. coli* ASNase may develop antibodies to ASNase after intramuscular administrations, many of whom become antibody-positive without demonstrating any clinical evidence of antibody formation (silent hypersensitivity), while others have higher antibody levels after the presentation of clinical allergic reaction (12, 17, 44-48). In patients who develop hypersensitivity post-ASNase treatment, there appears to be an inverse relationship between high antibody levels and low or undetectable enzymatic activity, due to neutralization of the antigen-ASNase (17, 47, 48). Moreover, high titers of antibodies to ASNase are associated with a lower risk of osteonecrosis and adverse pharmacodynamic effects of therapy (47). In addition, patients with high titers of antibody to ASNase were shown to have inversely lower exposure to the enzymatic activity; the rebounding of Asn and Gln are associated with increased systemic clearance of dexamethasone, thus reducing this drug’s exposure (AUC). Both of these events are associated with higher risk for ALL relapse (48).
Patients who experience disease relapse, mostly after the development of neutralizing antibodies against this antigen, develop drug resistance to these and many other classes of oncology drugs. This second form of ASNase resistance may be exacerbated and possibly correlated with the up-regulation of the Gln-dependent ASNS (17, 23, 49-51). To ameliorate this insidious condition, a non-cross reactive to the \textit{E. coli} ASNase antibody, \textit{E. Chrysanthemi} ASNase is a preferred alternate formulation for depletion of serum Asn and Gln during re-induction therapies (17). This ASNase has more than 10-fold better glutaminase activity in comparison to \textit{E. coli} ASNase with favorable \textit{Km} and faster \textit{kcat} (52, 53); hence, it is a better drug to deaminate Gln, the co-substrate of ASNS. This becomes more important since, as stated above, Gln deprivation (<2 h) triggers intracellular events affecting the mitochondria, leading to an irreversible commitment to apoptosis (25). Thus, many investigators have developed the hypothesis that patients in long-term remission had optimal treatment with ASNases, at least in part, due to limited presence or lack of antibodies and ASNS expression.

Numerous pharmacokinetic (PK) and pharmacodynamic (PD) studies have been reported from European and US centers (12, 13, 17, 42, 54-57). Universal population PK and PD models describing the handling of these ASNase formulations in young children, teens and young adults with ALL in remission induction and in remission re-induction have been published (12, 14, 42). The significance of the similarity of these reports is that a limited sampling approach may be used in the determination of the pharmacokinetic and pharmacodynamic evaluations in the many thousands of newly diagnosed patients with ALL. This is the goal of clinical pharmacology.

\textbf{Treatment-related Adverse Effects: Modes of Resistance to ASNases}

\textit{Up-regulation of ASNS in leukemia cells.} ASNS up-regulation makes ALL cells refractory to ASNase (15, 23, 24). AAs serve as signal transduction messengers to transmit the nutritional status of the entire organism to individual cells. One of the
mechanisms by which AAs mediate this signaling is altered transcription for specific genes via a signal transduction process referred to as the AA response pathway. Under these conditions, increased expression occurs for genes containing AA response elements i.e. ASNS, GS, c-MYC, thiopurine methyltransferase, BCL-2, dihydrofolate reductase, and many others], as several enzymes favor glutathione (GSH) biosynthesis (58). For a considerable number of years the up-regulation of ASNS was considered the most important factor in ASNase drug resistance. Human T-cell leukemia cells developed resistance to ASNase after a 24-h treatment with various concentrations, washing, and plating in soft agar (23, 24). Of greatest interest is that out of the many dozens of monoclonal lines there was variable resistance to ASNase and cross-resistance to many other antimetabolite drugs and gamma radiation. These clones were found to have up-regulation of ASNS and BCL-2, and mutated P53 (23, 24). In effect, among these clones, resistance was determined to many commonly used salvage regimens, such as Capizzi I and II. However, even though the up-regulation of ASNS mechanism of drug resistance is ASNase-induced in leukemia cells, as it has been demonstrated in vitro (23, 24, 59) and in vivo (60-62), no serious clinical effort has been made to date to address this problem (43, 43). Yet up-regulation of ASNS may not be predictive of treatment outcomes in clinical trials (11, 61, 62).

In confirmatory clinical evaluations, it was shown that ASNase treatment of pediatric patients with ALL induced expression of mRNA for ASNS. In addition, another study from the same group of investigators had reached a similar conclusion in t(12;21) translocation (TEL-AML1 ALL) leukemic blasts post-ASNase treatment. Specifically, they reported that the sensitivity to ASNase was not dependent on ASNS. They concluded that their results disagreed with the prevailing notion that ASNS expression would alter ASNase sensitivity in the ALL cells (60, 62). Moreover, the same group of investigators recently reported that about 70 genes were expressed in discordant patterns in the various subtypes of ALL-post-ASNase exposure. However, one common gene protein associated with pan-resistance of the ALL cells was that of BCL-2 overexpression. Further statistical analyses demonstrated that BCL-2 overexpression was an independent (p=0.01) prognostic factor (28, 29).

Epitope mapping of antibodies to asparaginase. The host immunological effects of formation of antibodies to ASNase induced by ASNase protein, and the augmented Asn input from the de novo biosynthesis of Asn by the liver, a reaction which is catalyzed by ASNS and/or the contributions from nutrient intake, make the further use of E. coli ASNase drugs pharmacodynamically useless in the continuing treatment of ALL patients (Figure 1) (11-15, 17, 42, 63, 64). Many phase II and phase III clinical studies have reported a wide range of the presence of human antibacterial antibodies to ASNase. In addition, the rates of anti-ASNase antibody formation vary significantly (12-13, 17, 44-48). However, these were assayed by an antibody-capture enzyme-linked immunosorbent (ELISA) assay, which did not always specify the immunoglobulin subtype (12-13, 17, 44-48, 65, 66). Recently, BIACORE with surface plasmon resonance (SPR) protein chips has been shown to produce accurate, sensitive, precise and rapid antibody results (66). Clinically, despite the compromise of the immune system upon initiation of induction treatment of ALL, allergic or anaphylactoid reactions can occur after administration of many drugs, including native E. coli ASNase or PEG-ASNase. The most frequent and severe allergic reactions have been observed in patients after multiple doses of ASNase and, less frequently, PEG-ASNase. These obvious clinical reactions can occur after the second or third doses, or even after many months from post-induction therapy (12-13, 17, 44-48). Clinical manifestations of ASNase hypersensitivity reactions are: anaphylaxis (rare); allergic reactions; edema; serum sickness; bronchospasms; urticaria and rash; itching and swelling of extremities; erythema – local or generalized; other clinically related reactions. Other toxicities to ASNases include: organ toxicities, pancreatitis and related hyperglycemia, glycosuria, ketoaderosis, liver dysfunction cerebral dysfunction, decreased protein synthesis, hypoproteinemia, hyperfibrinogenemia, hypercoagulable state – coagulopathies, alteration in clotting factors (antithrombin III – ATIII) (Figure 2) (67).

In an attempt to minimize these dose-limiting side-effects of ASNases, numerous efforts have been made to identify the immunogenic epitope(s) in both the Erwinia and the E. coli ASNases and compare them (17, 47, 64, 65). Linear peptides of ASNase proteins that are recognized by antibodies, frequently correspond to flexible loops on the protein surface. The sequence of 252SVFDTLA258, is of the epitope residues 252 to 258 of the E. coli ASNase. Furthermore, about 12% to 20% will be the antibody was dependent on Pro 285, and Pro 286, since Pro 285 and Pro 286 make the further use of E. coli ASNase, whereas the sequence 254SVSVRGI260 represents the residues 254 to 260 from Erwinia ASNase. Therefore, there is no mismatch between these results and the data from Moola et al. (68), because they named the residues 255 to 260 of the Erwinia ASNase (68). In addition, the sequence 258NYVYKSVF254, represents the residues 248 to 254 of E. coli ASNase, whereas the sequence 254SVSVRGI260 represents the residues 254 to 260 from Erwinia ASNase. It has to be taken into account, however, that Moola et al., used in patients with clinical allergy to E. coli ASNase, after repetitive use of Erwinia ASNase, about 12% to 20% will develop antibodies to Erwinia ASNase (47, 70, 71). The region 253VPDPE258 near the C-terminus was an immunodominant epitope for Erwinia ASNase. Furthermore, the antibody was dependent on Pro285, and Pro286, since their replacement via a site-directed mutagenesis by almost any other AA resulted in reduced binding.
epitopes at the N-terminus (37DTLI40, 84DDVDGV89), and two at the C-terminus (208VFDVRG213 and 255VSVRGI260) were identified as the immunodominant sites, recognized by antibodies raised against the E. coli ASNase (68, 69, 72). Two of these epitopes, residues 37-40 and 208-213, were also recognized by a polyclonal antibody raised against the Erwinia chrysanthemi ASNase. This cross-reactivity may be due to structural similarities between the two enzymes, since both have similar AA sequences. Apparently, the similarity in AA sequence is not immediately neutralizing the Erwinia ASNase enzyme’s catalytic activity (72, 73). Moreover, the dominant E. coli epitope identified in a recent study did not react with antibodies raised against Erwinia ASNase. This is not surprising, as the respective regions in the two enzymes show only low sequence similarity i.e. 254NLYKSVF260 vs. 254SVSVRGI260 for E. coli and Erwinia ASNase, respectively (68, 69). Many of the E. coli ASNase epitopes surround the four enzymatic active sites, thus when the antibody binds to the antigen it neutralizes the ASNase catalytic activity [(Figure 3) taken from (69)]. Moreover this figure shows the precise catalytic pocket of ASNase (69).

In the era of recombinant DNA gene product expression in biological oncology therapeutic products, a number of recombinant ASNases have been made and their crystalline structures are being resolved. Of greater interest is the identification of the active sites and the epitopes responsible for antibody production. Furthermore, serious efforts have been made in an attempt to modulate immunogenic epitopes via bioengineering of these proteins without reducing their enzymatic activities. The AA 25NIGS28 in the E. coli ASNase sequence is one of the four major indentified antigenic peptides (68). Recently, Patel et al. demonstrated the presence of the lysosomal cysteine endopeptidases cathepsin B (CTSB) and asparaginyl endopeptidase (AEP) in lymphoblasts that can degrade ASNase (74). The Asn24 residue on the flexible active loop was identified as the primary AEP recognition site. AEP first cleaves native ASNase at Asn24, thus exposing the 25NIGS28 epitope (68, 69). The result of this hydrolysis at this exact point in the protein sequence generates a highly antigenic fragment of E. coli ASNase and, hence modulates the therapeutic response to ASNases. Therefore, leukemia cells contain within their cells endopeptidases which can precisely generate the antigen for immune response, and thus ensure survival of the remaining ALL cells (69, 74).

However, it is certain that if it has not been already bioengineered, with site-directed mutagenesis, a possible optimal AA substitution in this position could be made, leading to enhanced resistance to AEP degradation, while reducing its immunogenic epitope at the adjacent sites, improving its thermal stability and hence, maintaining its enzymatic activity better than the existing E. coli-pegylated products. We can readily conclude from the ASNase structural comparisons that the main immunogenic epitopes that have been identified in the E. coli and Erwinia ASNases and their AA differences explain the non-reactivity of the antibody-antigen.

Clinical implications of anti-asparaginase antibodies and glutaminase activities. In recent years, new native ASNases and recombinant ASNase proteins, encoded by their respective CDNASs have been purified and characterized. At least 16 ASNase formulations have been reported, either from fermentations or from recombinant DNA methods in their native or pegylated formulations. Among them there are ASNases with undetected glutaminase activity, others with low to moderate activity, and a few others with augmented glutaminase activities (75). In the US, only three ASNases are licensed by the FDA, all of which are fermentation products. Two are the E. coli ASNases with relatively low glutaminase activity. As a result, weekly or q2w, administrations of PEG-ASNase, during re-induction, are required to trigger the refractory to native ASNase patients with ALL (13, 17, 53, 56, 76, 77).

Erwinia ASNase has a higher glutaminase moiety (~10-fold greater than the E. coli ASNase) and hence, more favorable Km and Vmax for Gln deamination. Erwinia ASNase has PK deficiencies vs. the E. coli proteins, however, it has been proven beneficial to ALL patients who had either developed an antibody to the E. coli formulations or had relapsed ALL disease (13, 17, 42, 43, 47). These clinical studies clearly demonstrated that by using Erwinia ASNase as a second line treatment, an ASNase with improved glutaminase activity is clinically beneficial to patients with refractory ALL. This is, most likely, the result of depleted Glu levels in serum below a minimum effective Gln concentration (14, 17, 42, 43). Thus, under these conditions, ASNs cannot exert its de novo biosynthesis of Asn. A similar effect of depleted Asn and Gln can be achieved by shortening the dosing intervals of Peg-asparaginase. Under these higher serum enzymatic activities this ASNase formulation can effectively deplete Asn and Gln in patients with high risk or relapsed ALL (56, 57, 76).

A new approach for overcoming ASNS up-regulation is by depleting Gln to a greater degree. To this end, recombinant proteins have been produced and their crystalline structure has been resolved (53, 77). Recently, and in following this line of thought, recombinant DNA techniques have allowed the modulation of the glutaminase enzymatic activity in the E. coli ASNase protein (78, 79). Such recombinant bioengineered E. coli protein products along with Erwinia ASNase may provide a novel approach in overcoming the ASNS mode of ALL resistance. Alternatively, other investigators seek to reduce the glutaminase moieties of ASNase to reduce toxicity. Such efforts are shown in recent publications (75, 77-81).

Degradation of ASNases. Patel et al. (74) found CTSB and AEP in lymphoblasts that can degrade ASNase and therefore
modulate therapeutic response to ASNases. The elimination of ASNases from the systemic circulation is subject to the lysis of leukemia cell enzymes in circulation. This infers that patients with higher leukemic burden at diagnosis, who respond favorably to the other anti-leukemic drugs before ASNase is administered, may release more of these peptidases in serum, which would thus reduce the stability of ASNase. Perhaps this mechanism may explain the wide variability of ASNase enzymatic PK profiles (13-14, 54-57, 52,79, 82-86).

Unmet need for determination of antibodies to ASNase. ALL is a very complex malignancy of multiple etiologies. Hence, the mechanisms of treatment failure in ALL are poorly understood (2-4, 74, 85). Recently, attention has been focused on the antibody development to ASNase; hence, an intrinsic form of drug antigen-induced resistance to ASNases is utilized by the leukemia blasts, these drugs then become ineffective in improving efficacy and long-term outcomes in ALL. However, there are many unmet needs in deciphering why some patients develop antibodies to ASNase (12-14, 16, 17, 43-48). Are the variable rates due to altered immunoglobulin profiles in patients? Are the same idiosyncratic immunoglobulin profiles which provide differences in the severity of infectious diseases or the severity of transplanted BM or blood stem cell rejection post BM transplantation? Are there similarly variable host responses in developing antibodies to ASNases taking place in the ALL patients? Do the T-cell and/or dendritic cells process the antigen (ASNase) differently in some patients? Or do these cells have a different expression of their pattern recognition receptors and thus a specific, but variable recognition of the offending antigen?

A search for a global bioassay for the determination of pharmacodynamics post-ASNase therapy was undertaken. Indeed, despite the availability of ELISA antibody assay, no coordinated efforts have been made in the last decade for a centralized application of the antibody determination in sera from the many thousands of ALL patients diagnosed every year in the United States. Clearly the immune response to ASNase in ALL patients is very heterogeneous; thus, methods must be able to detect different antibody isoforms, affinities, and neutralizing specificities. In previous decades, serum samples have been used for screening of antibodies to ASNase using an inverse sandwich antibody-antigen bioassay (ELISA) in both pediatric (12-13, 17, 43-48) and

Figure 3. Protein crystallography depicts in greater detail the mobile loop (in its closed position) highlighted in blue. Three dominant epitopes surround the active site, i.e., residues 53–58 (light red) of subunit D amino acids correspond to residues 253-257 of subunit B (dark red), and residues 283-289, also from subunit B (pink). Adapted with permission from Dr. Med. Hans-Joachim Muller (69).
adult patients with ALL (14). IgG immunoglobulin is the most abundant isotype, whereas IgE is the least abundant class of immunoglobulins circulating in human serum (86). Only a limited number of publications reporting ELISA quantifiable results have been published, and even fewer have been reported comparing ELISA with other assays for antibodies in patients who receive biological oncology drugs for their immunogenicity determination (66, 87).

Replacement of the multiple injections of native E. coli ASNase with the long half-life PEG-ASNase have improved the complete remission rates (2-4, 12, 17). However, replacement of native E. coli ASNase with an equal dose and schedule of the shorter half-life Erwinia ASNase eroded outcomes in the context of multiantigen therapy (13, 17, 64-66). Recently, in patients with either relapsed ALL or antibody-positive, much higher doses of Erwinia ASNase were administered intravenously, with equal or perhaps improved efficacy without apparent evidence of increased toxicity of this drug (42, 43). Could a pegylated Erwinia ASNase formulation provide a better solution for the antibody-positive patient treatment?

Since the existing ASNase formulations are good, but imperfect (75) new efforts are needed to use recombinant techniques and make ultra-pure asparaginase proteins in order to: minimize allergic reactions, maintain Asn and Gln depletion, amugment glutaminase activity, improve PK parameters, and improve thermodynamic stability by inhibiting the action of lysosomal endopeptidases (74). Such efforts have been initiated (77-81).

ELISA and SPR (Biacore) Assays

When antibodies to ASNase are detected by an ELISA assay, then a biochemical enzymatic assay determining the percentage neutralization of ex vivo – added antigen (1 IU ASNase) over a short time frame is performed at great expense of time and effort to validate the neutralization of enzymatic activity by the antibody (12, 17, 43-48, 66). Therefore, a simpler, accurate, and rapid immunological method is needed to determine the antibody type and its antigen neutralization. SPR-Biacore has emerged as a biosensor assay that allows for the sensitive detection of both low- and high-affinity antibodies, their isoforms, and their binding characteristics to the antigen (ASNase (66).

Toxicity of ASNases

The toxic effects of ASNase are related primarily to immune reactions to this bacterial protein and to the effects of Asn depletion, and subsequent inhibition of protein synthesis in major glands such as the liver and pancreas. The allergic reactions are the most prominent toxicities, and although grade 3 and 4 are lower than grade 1 and 2, they are primarily due to antibodies to ASNase in circulation. In the high-risk ALL study (CCG-1961), clinical allergy and high titer antibody were very common with the augmented regimen on CCG-1961 (12-13, 17, 56, 57, 67).

Panosyan et al. (17) and Avramis and Tiwari (67) provided some additional and important clues to the preponderance of antibody-positivity and its adverse effects, exerted in long-term EFS in patients with high-risk ALL. Several laboratories have shown that treated patients can develop a silent allergy, where circulating antibodies neutralize ASNase activity, resulting in an ineffective contribution by the drug to the anti-leukemic PD effect, without causing obvious clinical allergic symptoms. When this occurs the duration of effective ASNase treatment is shorter for these patients (17, 47, 48). This suggests that monitoring for silent antibodies to ASNase or loss of ASNase activity could provide a better understanding of this class of anti-leukemic drugs and thereby improve outcome.

Correlative Factors in ALL Treatment Outcomes

A statistical interim analysis was released four years after the opening of the CCG-1961 study. According to this study’s protocol, specimens were tested for antibodies in 1200 pediatric patients with high-risk ALL, all of whom received nine doses of native E. coli ASNase during induction and then two doses of PEG-ASNase during consolidation. Four or more specimens were obtained from the vast majority of patients per study protocol CCG-B951. Patients were selected randomly for the ASNase antibody assays. Among these patients, 410 had four or more sera specimens assayed for antibody titer (13, 17). The results were expressed as a ratio over the negative control per ELISA assay. The data were transmitted to the statistician’s office at the CCG Statistical Center, who performed an interim analysis with treatment outcome. Among the 410 patients, 280 had all the clinical data available for evaluation of efficacy, clinical allergy, and outcome for 30 or more months (17). The remaining patients lacked clinical data or they had not completed 30(+) months from treatment induction. According to the interim analysis, the antibody and clinical event data (EFS) showed that there were four sub-sets of patients, the three proposed ones and a fourth consisting of patients with clinical allergy symptoms and antibody-negative sera (false-negative reaction or allergic reaction to other agents) (17). The historical data from ALL studies in CCG show an 80% EFS in 5 years, with very few relapses after this time.

The results of this interim analysis demonstrated that the largest subset of patients (41%) developed antibodies to ASNase and had clinical allergy symptoms. We have shown in other studies that these antibodies inhibit ASNase enzymatic activity, thus allowing the serum Asn and Gln to rebound. The antibody has an adverse effect on treatment outcomes, which was statistically significant in the survival of these patients (17). This subset (29%) of silent hypersensitivity patients
received no effective ASNase therapy post-induction. Therefore, patients with antibodies to ASNase have a worse outcome than any other subset. Since the silent hypersensitivity subset is large (29% of total evaluable patients), approximately representing 25% of all high-risk ALL patients who relapse in 5 years from diagnosis, the anti-ASNase antibody in these ALL patients could be a surrogate marker of poor outcome in this disease. A recent study reported a higher percentage (~34%) of silent hypersensitivity subset of ALL patients (47). The interim analyses have shown that the ASNase antibody has a predictive clinical value and that switching antibody-positive patients from native *E. coli* ASNase to *Erwinia* ASNase may benefit their outcome, as indicated by the reversal of the hazard ratio from 3.22 to 0.6 (17, 48). In addition, the presence of antibody-positivity in sera of ALL patients also resulted in faster clearance of dexamethasone from the circulation (48). Therefore, an unpleasant pattern develops where antibodies to ASNase, combined with reduced dexamethasone exposure yield poorer PD effects of both drugs, thus adversely affecting treatment outcomes in ALL. These two poor PD effects are, most likely, the principal causes responsible for the majority of relapses in ALL. Should the final outcome analyses be as important as the interim ones, the antibody to ASNase model can then strongly recommend the use of alternative ASNase formulations for improved treatment outcomes in ALL. Therefore, antibody-positivity should be the primary surrogate marker for treatment outcome in future clinical trials.

**Conclusion**

ASNase is a tetramer protein that deaminates Asn and Gln. ASNase inhibits protein synthesis in T-cells. Gln deamination is necessary for optimal Asn deamination and, therefore, leukemia blast kill. The most important mode of resistance to ASNase is the high titer antibody to ASNase, which is detected in patients with no overt clinical allergy (silent hypersensitivity). IgG isoform of antibody neutralizes ASNase activity; When this pharmacodynamic deficiency remains undetected (silent hypersensitivity) then early ASNase activity; When this pharmacodynamic deficiency remains undetected (silent hypersensitivity). IgG isoform of antibody neutralizes ASNase, which yield poorer outcomes after combination treatments on ALL patients. Hence, appropriate monitoring of these parameters during therapy is of the utmost importance.

The up-regulation of ASNS is considered an important mode of resistance to ASNases. The up-regulation of ASNS may be partially responsible for the antigenic capability of bacterial ASNases. Other modes of ASNase resistance include the release by the leukemia cells the cytosolic lysosomal cysteine endopeptidases cathepsin B (CTSB) and asparaginyl endopeptidase (AEP). Hydrolysis by these endopeptidases exposes a highly antigenic oligo-peptide in *E. coli* ASNase. Novel, recombinant ASNases are available with variable enzymatic activities against Asn and Gln. A number of new ASNase proteins have been engineered with site-directed mutagenesis to be refractory to CTSB and AEP hydrolysis and perhaps to have lower immunogenicity. Future studies should examine additional ASNase formulations that would promote effective ASNase therapy, reduce antigenicity, and limit the role of asparagine synthetase & other gene expression profiles. Then, the era of truly individualized ASNase therapy may bring high percent of long term cures.

**References**


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