Multiple adaptive mechanisms affect asparagine synthetase substrate availability in asparaginase-resistant MOLT-4 human leukaemia cells

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Childhood acute lymphoblastic leukaemia is treated by combination chemotherapy with a number of drugs, almost always including the enzyme L-asparaginase (ASNase). Although the initial remission rate is quite high, relapse and associated drug resistance remain a problem. *In vitro* studies have demonstrated an adaptive increase in asparagine synthetase (AS) expression in ASNase-resistant cells, which is believed to permit ASNase-resistant human leukaemia cells to survive *in vivo*. The present results, obtained with ASNase-sensitive and -resistant human MOLT-4 leukaemia cell lines, illustrate that several other adaptive processes occur to provide sufficient amounts of the AS substrates, aspartate and glutamine, required to support this increased enzymic activity. In both cell populations, aspartate is derived almost exclusively from intracellular sources, whereas the necessary glutamine arises from both intracellular and extracellular sources. Transport of glutamine into ASNase-resistant cells is significantly enhanced compared with the parental cells, whereas amino acid efflux (e.g. asparagine) is reduced. Most of the adaptive change for the amino acid transporters, Systems A, ASC and L, is rapidly (12 h) reversed following ASNase removal. The enzymic activity of glutamine synthetase is also enhanced in ASNase-resistant cells by a post-transcriptional mechanism. The results demonstrate that there are several sites of metabolic adaptation in ASNase-treated leukaemia cells that serve to promote the replenishment of both glutamine and asparagine.

Key words: acute lymphoblastic leukaemia, glutamine, membrane transport.

INTRODUCTION

Combination chemotherapy for childhood acute lymphoblastic leukaemia (ALL) involves a number of drugs, almost always including the enzyme L-asparaginase (ASNase). The proposed action of ASNase is the depletion of cellular asparagine and glutamine following efflux as a consequence of enzymic hydrolysis of the circulating levels of these two amino acids [1,2]. The exact molecular events that cause cell death following ASNase treatment are unknown. Whereas suppression of protein synthesis is an obvious potential target, it has been documented that ASNase treatment can initiate apoptosis [3–5,5a]. ALL cells are thought to be particularly sensitive to ASNase treatment, because of a relatively weak ability to synthesize sufficient asparagine due to a low expression level of asparagine synthetase (AS). ASNase, when used alone, causes complete remission in 40–60 % of pediatric ALL cases [6–8]. Although the remission rate for childhood ALL is quite high, relapse and associated drug resistance remain a problem with regard to disease-free survival rates [9]. Pieters and co-workers [10–12], as well as others [13], have documented that decreased drug sensitivity *in vitro*, including that to ASNase, is correlated with drug resistance and poor prognosis *in vivo*. The need to obtain a better understanding of the mechanism of ASNase action and ASNase resistance is underscored by studies that have concluded that ASNase treatment may also be helpful in some adult ALL patients [14,15], in certain subgroups of childhood acute myelogenous leukaemia [16,17], and in refractory AIDS-related non-Hodgkin's lymphoma [18].

Using the MOLT-4 human leukaemia cell line, Hutson et al. [19] isolated ASNase-resistant clones by maintenance in the presence of increasing concentrations of ASNase and documented elevated expression of AS mRNA, protein and enzymic activity [19]. Based on similar *in vitro* observations in other cell types [19–23], it is generally believed that this elevated AS activity permits ASNase-resistant human leukaemia cells to survive *in vivo*. The ability of the drug-resistant cells to provide sufficient amounts of the AS substrates, aspartate and glutamine, required to support the increased enzymic activity of AS, becomes critical. These substrates could come from an intracellular pool or may be acquired from the extracellular milieu by active transport across the plasma membrane. The anionic amino acid aspartate is transported into mammalian cells by both Na^+-independent System X_C^-, and Na^+-dependent System $X_{A,G}^-$ mechanisms [24]. For active accumulation of aspartate, the ion-driven activity would be the likely mediator and is encoded by a family of closely related genes that encode the excitatory amino acid transporters (EAATs) [24,25]. In contrast, active glutamine uptake can be catalysed by several different Na^+-dependent zwitterionic transporters, but in cells of lymphatic origin the two most likely are Systems A and ASC [24]. These two activities can be distinguished from each other by the selective inhibition of System A by N-monomethylated amino acids, such as the non-metabolizable alanine analogue 2-(methylamino)isobutryic acid (MeAIB) [26].

The present study was designed to investigate the relative contribution of extracellular and intracellular sources of aspartate and glutamine, by evaluating ASNase-dependent changes in the

Abbreviations used: ALL, acute lymphoblastic leukaemia; AS, asparagine synthetase; ASCT1, System ASC neutral amino acid transporter; ASNase, L-asparaginase; ATA2, amino acid transporter A2; EAATs, excitatory amino acid transporters; GS, glutamine synthetase; KRP, Krebs–Ringer phosphate buffer; MeAIB, 2-(methylamino)isobutyric acid; MSO, methionine sulphoximine; SAT2, System A transporter 2; SSC, standard sodium citrate; WST-1, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate.

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expression of glutamine synthetase (GS) and plasma membrane transporters in parental and drug-resistant cells. The resulting data document that in MOLT-4 leukaemia cells aspartate is derived primarily from intracellular sources, whereas the glutamine necessary for asparagine biosynthesis can arise from both intracellular and extracellular sources. Consistent with the concept that ASNase-treated cells adapt to increase asparagine synthesis and its retention within the cells, transport of glutamine into ASNase-resistant cells is significantly enhanced, whereas zwitterionic amino acid (i.e. asparagine) efflux is reduced. The results also indicate that ASNase-resistant cells are less dependent on glutamine for growth than parental MOLT-4 cells. Furthermore, the data demonstrate that ASNase-induced cellular adaptation of the zwitterionic amino acid transporters, Systems A, ASC and L, as well as GS activity are rapidly reversible, whereas the drug-resistant phenotype is not.

MATERIALS AND METHODS

Cell culture

The human ALL cell line MOLT-4 (A.T.C.C., CRL 1582) was propagated in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 10 ml/litre antibiotic/antimycotic solution (100 units/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B; Gibco BRL, Gaithersburg, MD, U.S.A.). All suspension cultures were maintained at 37 °C in a 5% CO_2 incubator. Cells were routinely collected by centrifugation for 5 min at 228 ***g***, and were resuspended at a density of approximately 5×10^5 cells/ml in fresh medium 24 h before all experiments. To establish a model of ASNase resistance, a MOLT-4 subline was selected by incubation of parental MOLT-4 cells in increasing concentrations of ASNase (Merck, West Point, PA, U.S.A.) from 1×10^{-5} to 1 unit/ml [19]. Cells from this selection process were subcloned by limiting dilution and were maintained in RPMI 1640 medium supplemented with 1 unit/ml ASNase. To examine the reversibility of ASNase effects, resistant MOLT-4 cells were transferred and maintained in RPMI 1640 medium lacking the drug for a period of at least 6 weeks. For acute ASNase treatment, MOLT-4 cells were collected by centrifugation, washed twice with PBS, and then resuspended to 4×10^5 cells/ml in RPMI 1640 medium with or without ASNase (1 unit/ml) for 12 h at 37 °C.

Analysis of cell growth and apoptosis

The growth rate of MOLT-4 cells following ASNase treatment was determined by a 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate (WST-1) cell proliferation assay (Boehringer Mannheim, Indianapolis, IN, U.S.A.). MOLT-4 cells were washed twice with PBS and then resuspended at a concentration of 4×10^5 cells/ml in RPMI 1640 medium. The cells were treated as described in the text and then seeded in 96-well plates (100 μl/well). After incubation for 48 h with 1 unit/ml ASNase at 37 °C and 5% CO_2, 10 μl of WST-1 reagent was added per well, and the cells were incubated for an additional 2 h. The absorbance of the supernatant, which is linearly related to viable cell number, was determined at 450 nm with a reference wavelength of 690 nm, as described by the manufacturer. The data are expressed as the percentage of the control [(mean absorbance of the treated wells/mean absorbance of the untreated wells) $\times$ 100]. The results are presented as means $\pm$ S.D. of assays performed in quadruplicate.

Apoptosis in MOLT-4 cells was determined by flow cytometry using an annexin V-FITC detection kit (R&D Systems, Minneapolis, MN, U.S.A). MOLT-4 cells were washed twice with PBS and then resuspended at a concentration of 1×10^6 cells/100 μl in binding buffer [10 mM Hepes (pH 7.4), 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1.8 mM $CaCl_2$]. To each 100 μl sample of cells, 10 μl of 50 μg/ml propidium iodide and 1 μl of 25 μg/ml FITC-conjugated annexin V was added, and the cells were incubated in the dark for 20 min. The samples were subjected to flow cytometry analysis on a Becton Dickinson FACScan instrument in the University of Florida Flow Cytometry Laboratory.

RNA analysis

Total cellular RNA was isolated with a QIAGEN RNeasy kit (Qiagen, Valencia, CA, U.S.A.). For Northern-blot analysis, 15 μg of total RNA/lane was size-fractionated in a 1% (w/v) agarose/6.6% (v/v) formaldehyde gel and then blotted by downward capillary transfer on to a Hybond-N nylon membrane (Amersham, Arlington Heights, IL, U.S.A.) in 10$\times$ standard sodium citrate (SSC; 1.5 M sodium chloride, 0.15 M sodium citrate, pH 7.0). The membranes were cross-linked by ultraviolet exposure in a Gene Linker UV chamber (Bio-Rad Laboratories, Hercules, CA, U.S.A.) and hybridized with the appropriate ^{32}P-radiolabelled cDNA probes. All radiolabelled probes were generated with a Strip-EZ DNA random primed StripAble DNA probe synthesis kit (Ambion, Austin, TX, U.S.A.). Unincorporated nucleotides were removed by centrifugation through a ProbeQuant G-50 micro-spin column (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). Hybridization was performed at 61 °C in a buffer solution [0.5 M sodium phosphate (pH 7.2), 7% (w/v) SDS, 1 mM EDTA (pH 8.0), 1% (w/v) BSA; pH values correspond to the pH values of stock solutions] with the exception of the System ASC neutral amino acid transporter (ASCT1) probe, which was hybridized at 42 °C in ULTRAhyb buffer (Ambion) in order to more easily visualize the signal. Following overnight incubation, all membranes were washed four times for 10 min with a high stringency wash solution [0.04 M sodium phosphate (pH 7.2), 1 mM EDTA (pH 8.0), 1% (w/w) SDS; pH values correspond to the pH values of stock solutions] at 65 °C, with the exception of the ASCT1 membrane, which was washed twice for 5 min with 2$\times$SSC containing 0.1% (w/v) SDS and twice for 15 min with 0.1$\times$SSC containing 0.1% (w/v) SDS at 50 °C, according to the manufacturer's directions (Ambion).

The membranes were exposed to BioMax MR film (Kodak, Rochester, NY, U.S.A.) and quantified by densitometry using an UN-SCAN-IT software program (Silk Scientific, Orem, UT, U.S.A.). Each experiment was repeated at least once using different batches of cells to ensure reproducibility. The GS cDNA probe (A.T.T.C.) corresponds to exon 1 of mouse kidney GS (GenBank® accession number J03820). The ASCT1 probe is a 2102 bp cDNA sequence [27], corresponding to the human System ASC transport activity. The System A probe corresponds to the 3′ end of the coding region of the rat SAT2/ATA2 gene (where SAT2 corresponds to System A transporter 2, and ATA2 corresponds to amino acid transporter A2) [28]. Of the three cDNAs that encode System A activity, ATA1 is brain specific and ATA3 is liver specific, so SAT2/ATA2 is the gene expressed in lymphoblast cells, such as MOLT-4 cells [28].

Amino acid transporter expression

Cells grown for 12 h under the conditions indicated in each Figure legend, were collected by centrifugation, washed twice in Na^+-free Krebs–Ringer phosphate buffer [choline KRP; 119 mM choline chloride, 5.9 mM KCl, 1.2 mM magnesium sulphate,

1.2 mM potassium bicarbonate, 5.6 mM glucose, 0.5 mM $CaCl_2$, 25 mM choline bicarbonate and 0.15% (w/v) BSA] and incubated for 30 min at 37 °C in choline KRP to partially deplete intracellular stores of amino acids. Following incubation, the cells were collected by centrifugation, resuspended at 5×10^7 cells/ml in choline KRP, and distributed in 96-well plates at 20 μl/well. To initiate transport, 180 μl of KRP containing either 5.5 μM glutamine (3.6 μCi [^{3}H]glutamine), 5.5 μM asparagine (0.18 μCi [^{14}C]asparagine) or 5.5 μM leucine (3.6 μCi [^{3}H]leucine) was added to the cells, either in Na^+-containing KRP [119 mM NaCl, 5.9 mM KCl, 1.2 mM magnesium sulphate, 1.2 mM potassium bicarbonate, 5.6 mM glucose, 0.5 mM $CaCl_2$, 25 mM choline bicarbonate and 0.15% (w/v) BSA] or Na^+-free buffer (i.e. choline KRP). After incubation at 37 °C for 1 min, the cells were harvested by aspiration (2.00–2.26 kPa) with a cell harvester (Brandel, Gaithersburg, MD, U.S.A.), and deposited on Whatman GF/B filters that had been pre-soaked with an ice-cold solution of the transported amino acid (5 mM) to reduce non-specific binding. Following aspiration, the filtered cells were washed four times with ice-cold choline KRP. The filters were dried overnight, placed in 3.5 ml of Scintisafe scintillation fluid (Fisher Scientific, Pittsburgh, PA, U.S.A.), and the radioactivity was determined using a Beckman LS 3801 liquid-scintillation counter (Beckman Coulter, Fullerton, CA, U.S.A.). To determine the protein content of the cell suspensions, 80 μl of 2% (w/v) SDS/0.2 M NaOH was added to six 20 μl aliquots of each cell suspension, and to a cell-free choline KRP buffer sample to subtract for the included BSA. In an independent series of experiments, it was determined that there was no significant difference in the total protein content per cell of parental cells, ASNase-resistant cells and resistant cells cultured without ASNase.

GS enzymic analysis

Parental and resistant MOLT-4 cells, incubated for 12 h in the absence or presence of 1 unit/ml ASNase, were collected by centrifugation at 4 °C, washed twice with ice-cold PBS, and resuspended in 1 ml of a solution containing 0.25 M sucrose, 0.2 mM EDTA and 2 mM 2-mercaptoethanol. The cell suspensions were sonicated on ice by eight 5 s bursts (setting 2 on a Model 60 Sonic Dismembrator; Fisher Scientific), with a cooling interval of 1 min after four bursts. The cell lysate was centrifuged at 30000 ***g*** for 1 h, and the supernatant was removed and stored at −80 °C. It was determined that there was no loss of GS activity following one cycle of freezing and thawing the cell extracts. To determine the GS activity of MOLT-4 cells, a modification of the assay described by Smith et al. [29] was used. To initiate the assay, 50 μl of cell extract (200 μg of protein) was added to 200 μl of 1.25× buffer, resulting in an assay of the following composition: 10 mM imidizole/HCl (pH 7.0), 2.5 mM glutamic acid, 0.25 μCi of L-[U-^{14}C]glutamic acid, 30 mM $MgCl_2$, 25 mM ammonium chloride, 7.5 mM ATP, 10 mM creatine phosphate, and 1.5 units of creatine phosphokinase. Background values, obtained from cell extracts incubated in buffer lacking ATP, creatine phosphate, creatine phosphokinase and $MgCl_2$, were subtracted from samples containing complete buffer in order to report the data as ATP-dependent GS activity. The extracts were incubated for 60 min at 37 °C, and the assay was stopped by the addition of ice-cold distilled water. A 1 ml sample of the resulting suspension was immediately placed on to a 2 ml bed volume AG 1-X8 anion exchange column (Bio-Rad; 200–400 mesh, 8% crosslinked, chloride form, equilibrated in distilled water) and rinsed with 6 ml of distilled water to collect the [^{14}C]glutamine. The entire effluent was collected in a 20 ml scintillation vial, to which 10.5 ml of ScintiSafe scintillation fluid (Fisher Scientific) was added, and the radioactivity determined. Two independent cell lysates were assayed in triplicate.

RESULTS

Selection of the ASNase-resistant MOLT-4 human leukaemia cells used in the present study and the concurrent increase in expression of AS mRNA, protein and activity have been described previously [19]. Furthermore, we have documented that ASNase treatment of MOLT4 cells causes depletion of both extracellular and cytoplasmic asparagine and glutamine (A. M. Aslanian and M. S. Kilberg, unpublished work). The latter effect

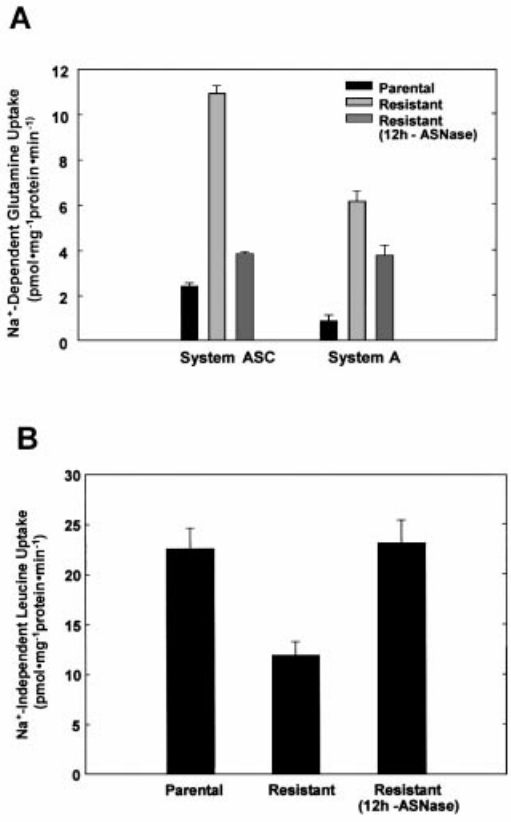

Figure 1 Amino acid transport in parental and ASNase-resistant MOLT-4 leukaemia cells

(**A**) The Na^+-dependent transport rates for the uptake of 50 μM [^{3}H]glutamine. To identify the individual transport systems contributing to glutamine uptake, a saturating concentration (5 mM) of unlabelled MeAIB was included to competitively inhibit transport via System A, and the additional inclusion of a saturating amount (5 mM) of unlabelled serine inhibited transport via System ASC. To examine the reversibility of ASNase effects on glutamine transport, resistant MOLT-4 cells were incubated in RPMI 1640 medium lacking ASNase for 12 h (12 h-ASNase) prior to transport. The Na^+-independent uptake rates for 50 μM [^{3}H]glutamine, that were subtracted to obtain the Na^+-dependent rates, were 1.5 ± 0.2 and 2.5 ± 0.2 pmol · mg of protein^{-1} · min^{-1} for parental and resistant cells respectively. (**B**) The activity of System L, an Na^+-independent amino acid transport system, was assayed in parental and drug-resistant cells by measuring the uptake of 50 μM [^{3}H]leucine in the absence of Na^+ and in the presence of a saturating concentration (5 mM) of the specific System L inhibitor, 2-amino-2-norbornanecarboxcylic acid. The reversibility of the change in System L was monitored by incubation of resistant cells in medium lacking ASNase for 12 h prior to transport. The data presented are the means ± S.D. for assays performed in quadruplicate and are representative of three independent experiments.

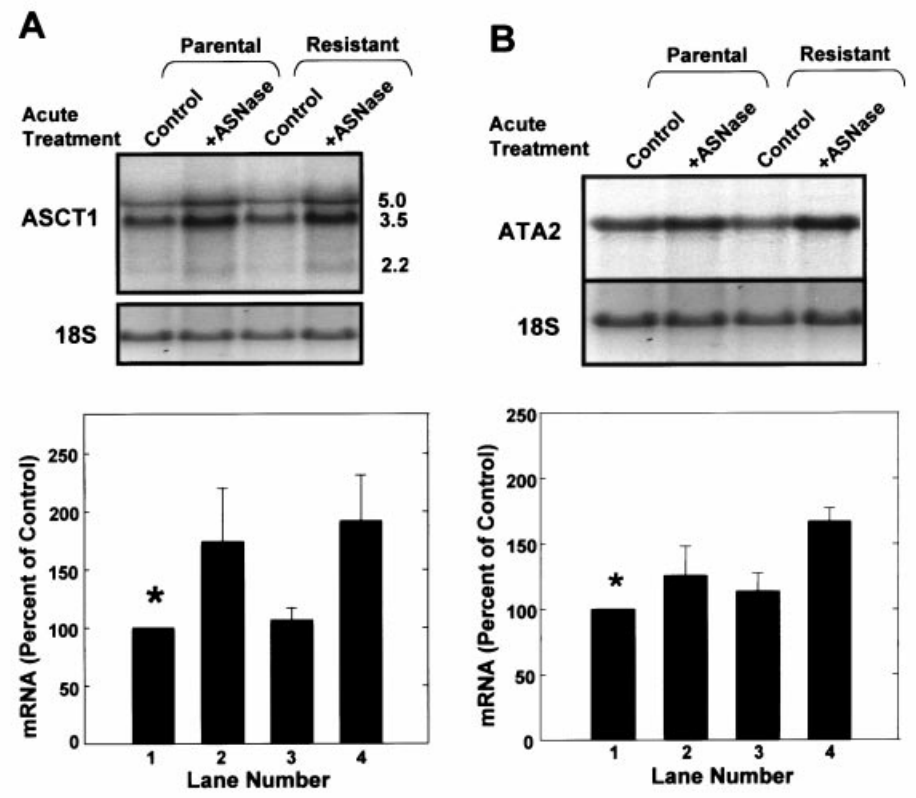

Figure 2 Transporter mRNA expression in MOLT-4 leukaemia cells

Parental and ASNase-resistant MOLT-4 cells (1×10^4 cells/ml) were incubated in the absence (Control) or presence (+ASNase) of 1 unit/ml ASNase for 12 h and then subjected to Northern-blot analysis by probing the same blots sequentially for ASCT1 (System ASC; **A**) and SAT2/ATA2 (System A; **B**) mRNA. The reversibility of the transporter mRNA induction was determined by incubating the drug-resistant cells in the absence of ASNase for 12 h before analysis. The total RNA blot (15 μg/lane) was probed with ^{32}P-radiolabelled cDNA probes, as described in the text, and the ethidium bromide stain of the 18 S ribosomal RNA served as a measure of lane loading. The quantified data were normalized to parental control cells (shown by the asterisk) and plotted as bar graphs. The quantified results are the means $\pm$ S.D. for three independent experiments. In (**A**) the sizes of the mRNA species are shown (in kb).

is due to the glutaminase activity inherently associated with the AS enzyme [2]. However, other metabolic changes that occur in human leukaemia cells in response to ASNase exposure have not been described, and neither has the source of the aspartate and glutamine to support the increased AS enzymic activity been identified.

Aspartate and glutamine transport following ASNase treatment

To determine the relative contribution of extracellular aspartate and glutamine delivery to MOLT-4 cells, plasma membrane transport was measured for parental cells, ASNase-resistant cells and resistant cells cultured in the absence of ASNase for 12 h. In several independent experiments, MOLT-4 cells exhibited no detectable System $X^-_{A,G}$ activity, as judged by the lack of inhibition by 5 mM D-aspartate of 5 μM L-[^{3}H]aspartate uptake (4.0 ± 1.0 and 5.3 ± 1.3 pmol·mg of protein^{-1}·min^{-1} in the absence and presence of D-aspartate respectively). Consistent with these data showing no mediated transport, Northern-blot analysis revealed no detectable mRNA content for any of the isoforms (EAAT1–4) likely to encode System $X^-_{A,G}$ activity in lymphocytes. These data document that ASNase treatment and the development of resistance in MOLT-4 leukaemia cells does not require the active uptake of extracellular aspartate to provide increased substrate for the AS reaction, and ASNase treatment does not induce aspartate transporter expression or activity.

MOLT-4 cells exhibited a readily measurable rate of Na^+-dependent glutamine transport activity that was significantly enhanced when ASNase-resistant cells were compared with the parental MOLT-4 cell line (Figure 1A). [^{3}H]Glutamine transport was measured at a substrate concentration of 50 μM. In the presence of a saturating concentration (5 mM) of the System A-specific inhibitor MeAIB, flux via this Na^+-dependent zwitterionic transport system was enhanced by more than 4-fold in the drug-resistant cells. Maintenance of the resistant cells in the absence of ASNase for 12 h resulted in a 33 % decrease in this enhanced System A activity (Figure 1A). Transport measurements in the presence of a saturating concentration (5 mM) of both serine and MeAIB revealed that the balance of the Na^+-dependent 50 μM [^{3}H]glutamine uptake in MOLT-4 cells was primarily mediated by System ASC (Figure 1A). The contribution of System ASC to the total Na^+-dependent glutamine uptake in the parental cells was more than twice that for System A (Figure 1A). Like System A, the System ASC activity was substantially increased (more than 4-fold) in the ASNase-resistant cells. When the resistant cells were incubated for just 12 h in the absence of ASNase (12 h-ASNase), the System ASC activity nearly returned to the rate observed in parental cells, illustrating an even more rapid reversibility than System A.

Given that ASNase functions in the extracellular space, but acts by depleting intracellular asparagine, efflux across the plasma membrane may play an important role in ASNase action. An

Na^+-independent bi-directional transport activity that would represent at least one contributor to asparagine efflux from human leukaemia cells is termed System L [30]. Asparagine breakdown precludes loading cells with radioactive amino acid, so the System L activity was monitored by measuring the Na^+-independent flux of leucine inhibited by a saturating concentration of 2-aminobicyclo-[2,2,1]-heptane-2-carboxylic acid, a non-metabolizable System L-specific amino acid analogue [31]. The data illustrate that ASNase-resistant cells have a 50% reduction in System L activity relative to the parental cells (Figure 1B). Culture of the resistant cells in the absence of ASNase for 12 h resulted in the complete normalization of System L, indicating that the drug-induced reduction is a transient adaptation that requires the continuous presence of ASNase, similar to the reversibility observed for System ASC. Trans-effects (i.e. trans-inhibition or trans-stimulation) due to changes in the cytoplasmic amino acid pool were minimized as a factor in all of these transport experiments by incubating the cells in amino acid-free medium for 30 min prior to the transport assays. Collectively, the transport data indicate that uptake of glutamine is enhanced in ASNase-resistant MOLT-4 cells, whereas System L, a potential mechanism for asparagine efflux, is reduced.

System ASC and System A mRNA content in ASNase-resistant cells

Two genes, ASCT1 and ASCT2, have been identified that encode ASC transport activity [24]. Although Northern-blot analysis of polyadenylated mRNA detected little or no ASCT2 (results not shown), ASCT1 mRNA was present as three species of 2.2, 3.5 and 5.0 kb (Figure 2A), in agreement with other cell types [27]. Consistent with the System ASC activity measurements, the ASCT1 mRNA content was increased approx. 2-fold in response to ASNase. This result was true for both short-term (12 h) treatment of parental cells (Figure 2A, compare lanes 1 and 2) and long-term maintenance of drug-resistant cells in the presence of ASNase (Figure 2A, compare lanes 1 and 4). Consistent with the System ASC transport activity measurements, the reversal of this change in ASCT1 mRNA content was rapid and nearly complete; 12 h after removal of ASNase from resistant cells, the amount of ASCT1 mRNA returned to the basal level (Figure 2A, compare lanes 3 and 4). Therefore the data illustrate that both ASC activity and ASCT1 mRNA are increased in response to ASNase treatment of MOLT-4 cells by a rapidly reversible mechanism.

The analysis of SAT2/ATA2 (System A) mRNA content revealed a 50% increase in parental cells treated with ASNase for 12 h (Figure 2B, compare lanes 1 and 2) or ASNase-resistant cells maintained continuously in the presence of the drug (Figure 2B, compare lanes 1 and 4). The reasons for a difference in the magnitude of increase between the transport activity (4-fold, see Figure 1) and the mRNA is unclear. Protein stability, mRNA translation rates or recruitment of pre-existing transporters are all possibilities. Given that the cDNAs for System A have just recently been cloned [28], the molecular mechanisms for activation have not been characterized. With regard to the reversibility of the System A mRNA, incubation of ASNase-resistant cells for only 12 h resulted in nearly a complete return to the parental cell level (Figure 2B, compare lanes 1 and 3).

Effect of System A transport on ASNase sensitivity

Increased System A amino acid transport activity occurs within each cell cycle and parallels increased cell division and cell growth [32,33]. Transformed cells express much higher levels of System A, presumably to maintain a higher rate of growth. To further investigate the role of elevated System A activity in the maintenance of ASNase resistance, both parental and ASNase-resistant MOLT-4 cells were incubated for 48 h in medium containing MeAIB concentrations ranging from 0 to 10 mM and then cell growth rates were estimated by measuring the number of viable cells using a WST-1 assay (Figure 3A). Inclusion of MeAIB, to block amino acid uptake by System A, had only a slight effect on cell growth of the parental MOLT-4 cells. In contrast, incubation of ASNase-resistant cells in MeAIB (0–5 mM) resulted in a concentration-dependent decline in cell growth relative to drug-resistant cells incubated in the absence of the amino acid analogue (Figure 3A). The data indicate that amino acid transport mediated by System A is an important contributor to cell growth of ASNase-resistant MOLT-4 cells. The effect of 5 mM MeAIB was less severe on induction of

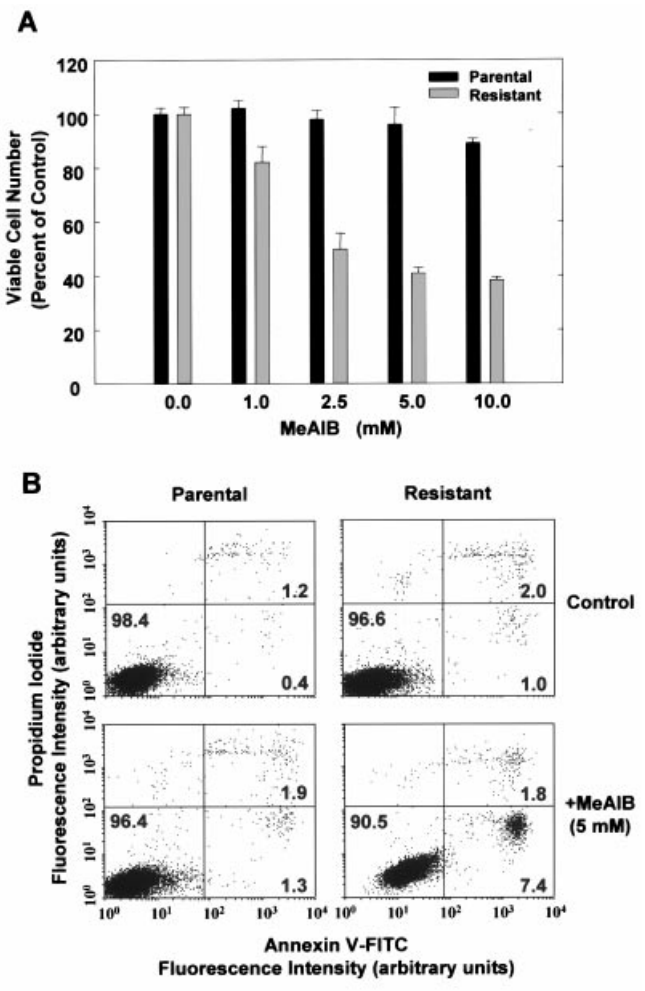

Figure 3 Effect of MeAIB on MOLT-4 leukaemia cell growth and viability

Parental and resistant MOLT-4 cells (1×10^4 cells/ml) were incubated for 48 h in medium with (resistant) or without (parental) 1 unit/ml ASNase. To block transport via System A, MeAIB was included in the medium at the indicated concentrations. The cells were then subjected to a WST-1 cell proliferation assay (**A**) or an annexin V-FITC apoptosis assay (**B**). For the WST-1 cell growth assay, the absorbance of the treated cells is plotted as a percentage ($\pm$S.D., $n = 4$) of the untreated control for each cell population. For the apoptosis assay, the numbers in each quadrant indicate the percentage of cells from a total of 10000 counted. The data presented are representative of three independent experiments.

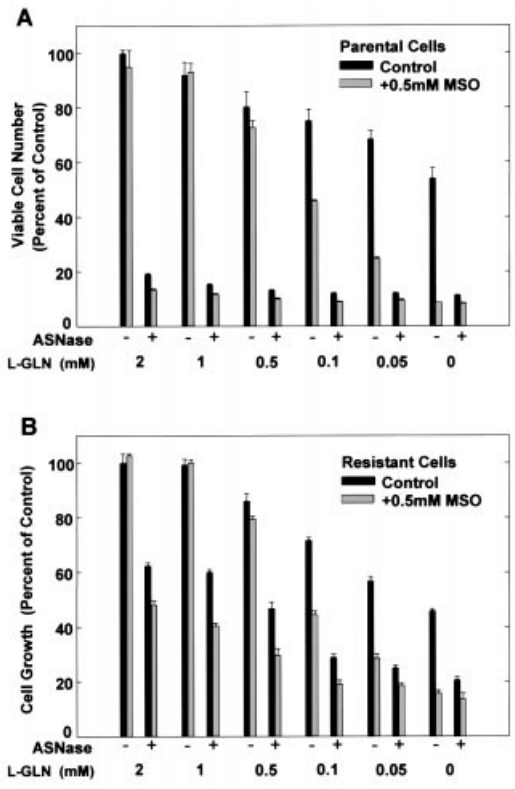

Figure 4 Effect of GS inhibition on cell growth in MOLT-4 leukaemia cells

The activity of GS was inhibited by incubating parental (**A**) or ASNase-resistant (**B**) MOLT-4 cells (1×10^4 cells/ml) with 0.5 mM MSO for 48 h. The cells were then analysed for cell growth using the WST-1 cell proliferation assay. In addition, the impact of decreasing the extracellular glutamine (L-GLN) concentration stepwise from 2 to 0 mM was investigated. The absorbance of the treated cells is plotted as a percentage of the untreated control for each cell population. The data are presented as the means ± S.D. for assays performed in triplicate and are representative of three independent experiments.

apoptosis. A 48 h incubation of parental cells in the presence of MeAIB caused no significant change in the percentage of cell viability (96 % compared with 98 %) or the percentage of cells undergoing apoptosis (1.3 % compared with 0.4 %) (Figure 3B). In contrast, the same MeAIB treatment of resistant cells caused a slight decrease in the number of viable cells (91 % compared with 97 %) and an increase in apoptotic cells (7.4 % compared with 1.0 %). Collectively, the results suggest that inhibition of amino acid uptake mediated by System A caused a cytostatic effect on ASNase-resistant cells, but altered the amount of apoptosis to a lesser extent.

Effect of glutamine availability on ASNase resistance

To assess the relative contribution of extracellular uptake and intracellular synthesis of glutamine to MOLT-4 cell growth and viability, cells were cultured for 48 h in decreasing concentrations of glutamine in the absence or presence of the GS inhibitor methionine sulphoximine (MSO). Culture of the parental cells in medium with decreasing glutamine concentrations resulted in a concentration-dependent reduction in cell proliferation (Figure 4A). Inhibition of glutamine synthesis by MSO had little effect at glutamine levels above the physiological concentration of 0.5 mM, but MSO reduced cell growth as the glutamine concentration in the medium was decreased from 0.5 to 0 mM. For example, the number of viable cells in glutamine-free medium was approximately 55 % of control in the absence of MSO after the 48 h incubation, but less than 10 % of control (2 mM glutamine) in the presence of the GS inhibitor (Figure 4A). Given that plasma glutamine content is approximately 0.5–0.7 mM, these data indicate that extracellular glutamine is the primary source of this amino acid at physiological concentrations, and that glutamine synthesis impacts parental MOLT-4 cell growth only when extracellular glutamine becomes limiting, as might be the case following ASNase treatment, because of its inherent glutaminase activity [2]. However, the decline in cell proliferation in the absence of MSO and at extracellular glutamine concentrations below 0.5 mM indicate that the basal cellular glutamine synthesis rate is not sufficient to maintain growth of the parental cells. When ASNase-resistant MOLT-4 cells were incubated in the absence of ASNase and in decreasing concentrations of glutamine, cell proliferation was reduced and further reduction occurred in the presence of MSO (Figure 4B). In the presence of ASNase for the 48 h culture period, the addition of MSO produced only a modest growth-inhibitory effect on the resistant cells, at all extracellular glutamine concen-

Table 1 Effect of GS inhibition on apoptosis in parental and ASNase-resistant MOLT-4 cells

The impact of GS inhibition by 0.5 mM MSO and 1 unit/ml ASNase on apoptosis/necrosis in parental and resistant MOLT-4 cells (1×10^4 cells/ml) was analysed. The cells were incubated for 48 h in 0, 0.1 or 2 mM glutamine with or without MSO and/or ASNase, and then subjected to an annexin V-FITC apoptosis assay, as described in the text. The values indicate the percentage of cells from a total of 10000 counted, and are presented as the means ± S.D. for three independent experiments.

	Percentage of cells											
	Control			+ MSO			+ ASNase			+ ASNase + MSO		
Glutamine	Viable	Apoptotic	Necrotic	Viable	Apoptotic	Necrotic	Viable	Apoptotic	Necrotic	Viable	Apoptotic	Necrotic
Parental Cells												
2 mM	91.4 ± 0.9	4.1 ± 0.9	3.8 ± 1.8	87.1 ± 4.1	6.6 ± 1.8	5.6 ± 4.3	35.4 ± 1.7	18.2 ± 7.8	44.1 ± 6.0	34.0 ± 14.6	29.8 ± 7.5	34.4 ± 14.7
0.1 mM	81.5 ± 2.6	7.9 ± 2.0	10.1 ± 4.2	48.6 ± 6.9	18.7 ± 6.9	31.5 ± 7.2	38.5 ± 3.3	16.8 ± 7.4	43.7 ± 6.2	27.2 ± 7.8	28.4 ± 9.9	42.5 ± 11.3
0	80.6 ± 1.0	8.2 ± 2.8	10.5 ± 3.4	27.8 ± 6.8	27.0 ± 9.1	43.0 ± 12.5	40.9 ± 1.4	16.0 ± 6.8	42.1 ± 6.9	28.5 ± 7.4	25.3 ± 8.4	43.3 ± 9.2
Resistant Cells												
2 mM	96.3 ± 1.9	1.1 ± 1.0	1.7 ± 1.0	95.9 ± 1.1	1.7 ± 0.4	1.8 ± 0.6	91.1 ± 3.4	4.0 ± 1.7	4.4 ± 1.6	92.6 ± 2.7	3.9 ± 1.1	2.7 ± 1.7
0.1 mM	92.9 ± 1.9	2.6 ± 0.9	3.5 ± 1.2	82.3 ± 5.6	8.4 ± 5.7	7.3 ± 2.4	78.6 ± 4.4	10.1 ± 1.7	10.3 ± 6.4	86.5 ± 2.7	7.1 ± 1.7	5.0 ± 1.8
0	92.3 ± 2.1	3.8 ± 1.4	3.2 ± 1.0	83.5 ± 3.0	9.7 ± 1.4	5.8 ± 2.2	79.1 ± 7.2	7.3 ± 2.3	12.0 ± 6.4	83.4 ± 2.5	8.7 ± 1.7	6.5 ± 2.4

trations. These data indicate that ASNase-resistant cells rely heavily on extracellular glutamine for optimal growth, and that in the presence of ASNase the rate of intracellular glutamine synthesis is not sufficient to protect the cells.

When parental MOLT-4 cells were incubated for 48 h in 0, 0.1 or 2 mM glutamine, annexin V binding assays indicated that cell viability declined by approximately 10 % at the two sub-physiological concentrations with a concurrent increase in the number of cells undergoing apoptosis and/or necrosis (Table 1). The presence of 0.5 mM MSO had little effect on cell viability in the presence of 2 mM extracellular glutamine, but significantly decreased cell viability when extracellular glutamine became limiting. Therefore the data in Figure 4(A) indicate that inhibition of glutamine synthesis in parental cells is cytostatic as extracellular glutamine becomes limiting, and the data of Table 1 demonstrate that glutamine deprivation is also cytotoxic. When the parental cells were treated with both ASNase and MSO, a reduction in cell viability beyond that in the presence of ASNase alone was observed.

GS expression in response to ASNase

The data presented in Figure 4 and Table 1 indicate that the rate of glutamine synthesis in parental MOLT-4 cells is not sufficient to maintain cell growth or viability in the absence of extracellular glutamine. Furthermore, it is known that ASNase has a glutaminase activity [1] that reduces circulating glutamine levels during ASNase therapy [2]. To establish whether or not ASNase treatment causes activation of GS expression, both the GS mRNA content and activity were monitored in parental and ASNase-resistant MOLT-4 cells. An acute 12 h ASNase treatment of parental MOLT-4 cells caused little or no change in GS mRNA content (Figure 5A, compare lanes 1 and 2), and ASNase-selected resistant cells exhibited only a small elevation (20 %) of GS mRNA relative to the parental cells (Figure 5A, compare lanes 1 and 4). Although modest in amount, this drug-induced increase did not decline when the ASNase-resistant cells were incubated for 12 h without the drug (Figure 5A, compare lanes 3 and 4) or cultured in the absence of drug for 6 weeks (Figure 5A, compare lanes 1 and 5).

It is known that GS enzymic activity is subject to regulation by translational and/or post-translational mechanisms [34,35]. Therefore enzyme activity was monitored in parental and drug-resistant MOLT-4 cells incubated with or without ASNase for 12 h (Figure 5B). When the GS enzymic activity was compared between control parental cells and ASNase-resistant cells maintained with the drug (Figure 5), there was a large ASNase-induced increase (425 ± 8 compared with 23 ± 4 pmol·mg of protein^{-1}·min^{-1}). Incubation of parental cells in the presence of ASNase for as little as 12 h caused a significant increase in GS enzymic activity (99 ± 12 compared with 23 ± 4 pmol·mg of protein^{-1}·min^{-1}). The data of Figure 5(B) also illustrate that even short-term (12 h) removal of the drug from the resistant cells resulted in a large decline in GS activity (113 ± 8 compared with 425 ± 8 pmol·mg of protein^{-1}·min^{-1}). Collectively, the data are consistent with previous reports documenting post-translational control mechanisms for regulation of GS activity and indicate that, although GS mRNA content is only slightly increased by ASNase treatment, the enzymic activity is highly responsive to asparagine/glutamine availability. Although the enhancement of enzyme activity was partially reversible when ASNase was removed from the medium of resistant cells for 12 h, the cells still retained higher GS enzymic rates than those observed in the parental cells (113 ± 8 compared with 23 ± 4 pmol·mg of protein^{-1}·min^{-1}), consistent with the small, but poorly reversible, adaptation of the GS mRNA content (Figure 5A).

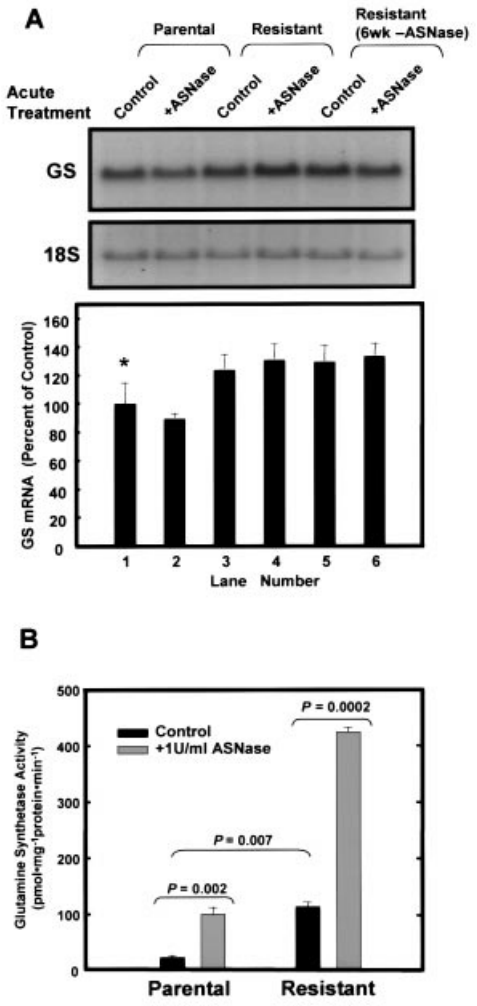

Figure 5 Expression of GS mRNA and enzymic activity in MOLT-4 leukaemia cells

MOLT-4 cells (1×10^4 cells/ml) were incubated for 12 h either in the absence (Control) or in the presence of 1 unit/ml ASNase (+ASNase) and then subjected to Northern-blot analysis for GS mRNA (**A**). The total RNA blot (15 μg/lane) was probed with a ^{32}P-radiolabelled cDNA corresponding to the coding region of human GS, with the ethidium bromide stain of the 18 S ribosomal RNA serving as a lane loading control. The bands were quantified, normalized to the 18 S absorbance, and then plotted as a percentage ($\pm$S.D.) of the parental control value (shown by an asterisk). The data used for quantification were taken from three independent experiments. (**B**) The GS enzymic activity in parental and resistant cells (1×10^4 cells/ml) following incubation for 12 h in the absence (control) or in the presence of 1 unit/ml ASNase. The data, representative of two independent experiments, are presented as the means $\pm$ S.D of enzyme assays carried out in triplicate. A paired t-test was performed to obtain the indicated P values for the differences between parental cell $\pm$ ASNase samples, between parental and resistant control samples, and between resistant cell $\pm$ ASNase samples. U, unit.

DISCUSSION

The results presented document the metabolic adaptations in asparagine and glutamine metabolism that occur in human leukaemia cells exposed to the drug ASNase. Collectively, the data reveal a global shift in cellular transport and metabolism to provide metabolic precursors to support an elevated asparagine

synthetic rate and to minimize loss of cellular asparagine by efflux at the plasma membrane. Given the induction of AS expression in the ASNase-resistant cells [19], the availability of the substrates, aspartate and glutamine, to provide sufficient flux through the enzyme becomes a critical factor. Transport analysis in MOLT-4 leukaemia cells revealed little or no transport activity for aspartate, an observation supported by the lack of detectable mRNA for any of the EAAT family of anionic amino acid transport proteins. Transport of glutamine into the ASNase-resistant cells was significantly elevated relative to the parental control cells and this increase was mediated by two different Na^+-dependent active transport processes, System A and System ASC. Bussolati et al. [36] also observed an increase in System A activity following ASNase treatment of mouse fibroblasts. In sharp contrast with the poor reversibility of the AS expression [21], removal of ASNase from the culture medium of the drug-resistant cells resulted in a reversal of System A and System ASC transport activities and mRNA content. Likewise, after only 12 h the decreased System L activity returned to the higher levels observed in parental cells. Taken as a whole, the results show that transport mechanisms for net influx or efflux are counter-regulated in ASNase-treated cells by adaptive mechanisms that are rapidly reversible and, therefore, quickly responsive to the presence or absence of the drug.

When the uptake of zwitterionic amino acids by System A transport activity in ASNase-resistant cells was blocked by MeAIB, a non-metabolizable substrate for which transport is restricted to System A [37], there was a significant inhibition of cell growth and a slight increase in the number of resistant cells undergoing apoptosis. There are other examples that illustrate the importance of elevated System A in maintaining increased cell proliferation. For example, blockade of System A activity results in suppression of DNA synthesis and delay in liver regeneration in a partial hepatectomy model [38]. These MOLT-4 transport data underscore the importance of the extracellular amino acid pool to continued growth of ASNase-resistant cells, and are consistent with the cell growth data documenting that at physiological concentrations extracellular glutamine was the principal source of this critical amino acid.

It is known that both the *Escherichia coli* ASNase and *Erwinia* ASNase, used in therapeutic protocols, have glutaminase activities that represent 1–3 % of the ASNase activity [2]. That this glutaminase activity plays an important role in the therapeutic effectiveness of ASNase has been suspected for some time. U937 lymphoma cells, selected for ASNase resistance, contained a 50 % increase in GS activity and were less sensitive to growth inhibition by reduction of extracellular glutamine [21]. These observations suggested that ASNase-induced asparagine/glutamine depletion caused an adaptive regulation of GS, which was important for continued growth in ASNase. Miller et al. [39] measured serum amino acid levels over a 10 day period in ALL patients treated with ASNase only and observed that both asparagine and glutamine levels fell to near zero during the initial 5 days of treatment. However, consistent with the up-regulation of GS for ASNase-resistant U937 cells [21] and MOLT-4 cells (Figure 5), even in the presence of a maintenance level of ASNase (50 units/kg/day), a full recovery of serum glutamine levels occurred [39]. Interestingly, despite a presumed adaptive increase in AS, asparagine content remained near zero throughout, even after treatment had been discontinued and ASNase activity was no longer detectable in the serum. The authors concluded that the primary anti-tumuor effect of ASNase was due to asparagine depletion. Conversely, Bussolati et al. [36] noted that their mouse fibroblasts are routinely cultured in medium lacking asparagine, so even in the absence of ASNase the sole source of cellular asparagine is intracellular synthesis. Therefore when they observed ASNase cytotoxicity they proposed that the effect was mediated via a reduction in extracellular glutamine. However, ASNase-dependent depletion of intracellular asparagine, following efflux, must also be considered as a factor.

The drug-resistant MOLT-4 cells were similar to the parental cells in their response to limitation of extracellular glutamine or blockade of intracellular synthesis when the WST-1 assay was used to monitor the cytostatic effects of ASNase. However, when apoptosis/necrosis was monitored, significant differences were revealed between the parental and ASNase-resistant cell populations. Limiting the extracellular glutamine concentration in the presence of the GS inhibitor MSO resulted in only a modest decline in ASNase-resistant cell viability, whereas greater than 70 % of the parental cells underwent apoptosis/necrosis. These data provide a striking contrast between parental and ASNase-resistant MOLT-4 cells and indicate that the drug-resistant cells are much less dependent on glutamine for cell survival.

Most mammalian cells produce glutamine by the enzyme GS, which uses glutamate and ammonia as substrates, but it is interesting to note that the allosteric control of liver GS activity is different than that for brain [40]. Given that the two protein sequences are identical, post-translational modification is thought to account for the regulatory differences observed between these tissue-specific enzymes. Acute treatment of either parental or ASNase-resistant MOLT-4 cells for 12 h did not result in any increase in GS mRNA, and, furthermore, GS mRNA was only slightly increased in ASNase-resistant cells when compared with parental control cells. These results argue that neither transcriptional regulation nor mRNA stability are primary regulatory mechanisms for GS expression. However, in both parental and resistant cells an acute treatment with ASNase (12 h) resulted in a substantial increase in GS enzymic activity, and when drug-resistant cells were incubated in the absence of ASNase for only 12 h, the extremely high levels of GS activity declined significantly. Therefore ASNase treatment results in activation of glutamine synthesis by an adaptive response through modulation of GS enzymic activity at a post-translational mechanism, consistent with the observation that GS protein stability is inversely proportional to the availability of glutamine [35]. The importance of increasing the glutamine synthesis rate for protein synthesis, as a possible carbon source, and for asparagine biosynthesis is clear from the cytotoxicity data. Therefore viewing all of the results together, the data indicate that ASNase resistance is associated with an adaptive enhancement of transport and the synthetic capacity for both asparagine and glutamine, the latter amino acid being a necessary precursor for the former.

The present data document that both short-term and long-term ASNase treatment of human leukaemia cells causes a wide range of metabolic changes to occur. Despite these adaptive mechanisms, ASNase treatment of parental leukaemia cells caused both a cytostatic and cytotoxic effect; 65 % underwent apoptosis/necrosis within 48 h of exposure to the drug. An explanation may be provided by Broome [41] who showed that ASNase-sensitive lymphoma cells preferentially use extracellular asparagine for protein synthesis, rather than intracellularly generated asparagine. This preference was not as strong in ASNase-resistant cells, an observation that led Broome to hypothesize that this difference was why the sensitive cells were killed, despite a cytoplasmic asparagine concentration equal to that in the drug-resistant cells. From a global perspective, ASNase alters the transport and metabolism associated with aspartate, glutamate, glutamine and asparagine in ways that support increased biosynthesis and cellular retention of asparagine. These

processes may well become targets for the prevention of drug resistance.

This research was supported by a grant (to M.S.K.) from the Institute of Diabetes, Digestive and Kidney Diseases, the National Institutes of Health (DK-52064). We wish to thank Dawn Beachy for secretarial support, and other members of the laboratory for helpful discussions.

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Received 6 February 2001/30 April 2001; accepted 21 May 2001