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An investigation into the mechanism of L-asparaginase resistance in L5178Y murine leukemia cells

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Summary. Resistance of leukemia cells to L-asparaginase is presumed to be due to increased expression of asparagine synthetase activity by resistant cells, so they are no longer dependent on an exogenous source of L-asparagine for growth. The mechanism by which cells acquire the ability for increased enzyme expression, however, has not been clearly defined. Evidence presented here indicates that genomic alterations in the form of translocations, gene amplification, or increased P-glycoprotein expression, do not account for the phenotypic transformation from L-asparaginase sensitivity to L-asparaginase resistance. Instead, both sensitive and resistant L5178Y cells contain immunoreactive material detected by Western blotting with an antiserum prepared against bovine pancreatic asparagine synthetase. This suggests that the mechanism of resistance might involve modification of asparagine synthetase in L-asparaginase-resistant cells by an as-yet-unidentified mechanism or by inhibition of enzyme activity in the L-asparaginase-sensitive cells.

Keywords: Amino acids – Asparaginase – Leukemia – Resistance – Asparagine synthetase – Chemotherapy

Abbreviations: CHO: Chinese hamster ovary; DMEM: Dulbecco's modified Eagle medium; EtBr: ethidium bromide; I.U.: international unit; PBS: 0.14 M NaCl, 0.01 M KCl, 0.02 M phosphate, pH 7.4; SDS: sodium dodecyl sulfate.

Introduction

The development of resistance to chemotherapeutic agents is one of the major obstacles to the successful treatment of cancer. Fundamental processes operative

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in generating drug-resistant mutants cover a broad spectrum of mechanisms at the level of DNA modifications, membrane changes, and specific metabolic alterations. The seemingly inherent ability of cancer cells to adapt to chemotherapeutic agents can perhaps best be understood if examined within the context of a well-defined system in which acquisition of resistance occurs concomitantly with a detectable change in cell function.

In mammalian cells asparagine synthetase is responsible for the ATP-dependent synthesis of asparagine from glutamine and aspartic acid (Kartner et al., 1983; Mehlhaff et al., 1985). In contrast to the situation in most normal cells, asparagine synthetase activity in certain tumor cells is undetectable (Cooney and Handschumachar, 1970; Horowitz et al., 1968; Patterson and Orr, 1967; Pragar and Bachynsky, 1968a, 1968b), making them dependent on an exogenous source of asparagine for survival. The enzyme L-asparaginase, by catalyzing the hydrolysis of asparagine to aspartic acid and ammonia, deprives the malignant cells of the asparagine available from extracellular fluid, resulting in cell death (Boyse et al., 1967).

The purpose of this study was to investigate the basis of resistance of L5178Y murine leukemia cells to L-asparaginase, in light of the mechanisms previously described as mediating chemotherapeutic resistance in other systems. These included gene amplification, P-glycoprotein expression, chromosomal rearrangements that might alter regulation of asparagine synthetase expression, and production of an inactive gene product by L-asparaginase-sensitive cells. The results indicated that immunoreactive material can be detected in L-asparaginase-sensitive L5178Y cells by Western blotting with an antiserum prepared against bovine pancreatic asparagine synthetase. This suggests that the absence of enzymatic activity in the sensitive cells is most likely effected at the level of the translation product of the asparagine synthetase gene.

Materials and methods

Cell lines

L5178Y cells, originally derived from a methylcholanthrene-induced lymphoma in DBA/2 mice (Fischer, 1957), were obtained from the Division of Cancer Treatment, National Cancer Institute (DCT Tumor Bank), Frederick, MD. They are Thy-1.2-positive, surface immunoglobulin-negative, nonspecific esterase-positive, and L-asparaginase-sensitive (Yang et al., 1981). Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated, undialyzed fetal calf serum, 0.34 mM asparagine, 2mM L-glutamine, and gentamicin sulfate (50 μg/ml). L-asparaginase-resistant cells were maintained in the same medium without the asparagine supplement. Cells were grown at 37°C in a humidified 10% CO_2-90% air atmosphere.

Derivation of L-asparaginase-sensitive and -resistant L5178Y cells

The genealogy of the L5178Y sensitive and resistant cells was unknown, since they were both originally obtained from the DCT Tumor Bank. Therefore, it was possible that genetic anomalies unrelated to L-asparaginase sensitivity or resistance could have accumulated in the cell lines and could obscure any results obtained in subsequent analyses. To circumvent this problem, it was necessary to derive sensitive and resistant cells that had recently

diverged. To do this, the parental L-asparaginase-sensitive L5178Y cells from the DCT Tumor Bank were cloned twice by limiting dilution in 96-well microtiter plates at an average density of 1 cell per well. Three clones (D10, C1, and F8) were isolated from the second subcloning and were screened for L-asparaginase sensitivity in the cytotoxicity assay described below. The clone L5178Y D10 was used as the sensitive cell line in all subsequent investigations.

To derive L-asparaginase-resistant cells from the parental sensitive population, 4×10^6 L5178Y D10 cells were exposed to 1×10^{-4} I.U.'s of L-asparaginase, which was the highest drug concentration in which a substantial portion of the sensitive cells remained viable (Table 1). The cells were maintained in this concentration of L-asparaginase for 2 weeks, and the medium was changed as it became spent. After 2 weeks the L-asparaginase concentration was increased to 2.5×10^{-4} I.U.'s.

Table 1. Effect of L-asparaginase on the growth of L-asparaginase-sensitive L5178Y D10 cells and L-asparaginase-resistant L5178Y D10/R cells

L-asparaginase	Cell type			
	L5178Y D10		L5178Y D10/R	
Conc. (I.U./ml)	Cells/ml[a]	% Inhib.[b]	Cells/ml	% Inhib.
0	3.0×10^6	N.A.	3.35×10^6	N.A.
10	$<1.1 \times 10^4$	>99.2	$<1.1 \times 10^4$	>99.7
1	$<1.1 \times 10^4$	>99.2	2.4×10^5	92.8
10^{-1}	$<1.1 \times 10^4$	>99.2	2.5×10^6	19.4
10^{-2}	$<1.1 \times 10^4$	>99.2	2.9×10^6	6.5
10^{-3}	3.3×10^4	98.9	3.2×10^6	4.5
10^{-4}	1.9×10^6	36.7	3.3×10^6	1.5
10^{-5}	2.6×10^6	13.3	3.4×10^6	6.9
10^{-6}	2.9×10^6	3.0	3.3×10^6	1.5

L5178Y D10 or L5178Y D10/R cells were inoculated into 24-well plates at an initial concentration of 2×10^5/ml in 2 ml of asparagine-supplemented complete DMEM and the indicated concentration of L-asparaginase. After 48 hours of incubation at 37°C, the number of viable cells was determined by trypan blue exclusion, and the percent inhibition was calculated as shown below

[a] Viable cells per ml as determined by trypan blue exclusion

[b] Percent inhibition = {(viable cells in neg. control − viable cells in exp. sample)/viable cells in neg. control} × 100

After 48 hours the cells were centrifuged at 200 g, and the pellet was resuspended in 1 ml of complete DMEM. The suspension was layered gently onto 5 ml of fetal calf serum and centrifuged at 40 g for 4 minutes. The supernatant fluid, which contained most of the dead cells, was discarded, while the pellet, which was enriched for viable cells, was resuspended in complete medium containing 1×10^{-4} I.U.'s of L-asparaginase/ml and cultured in a 24-well plate. Seventy-two hours later the cells were again exposed to an L-asparaginase concentration of 2.5×10^{-4} I.U.'s/ml for 48 hours. After centrifugation through fetal calf serum, the cells were cultured in medium containing 5×10^{-4} I.U.'s of L-asparaginase per ml. Within 72 hours healthy clumps of cells could be seen. This was 24 days after the first exposure to L-asparaginase and 10 days after exposure to increased L-asparaginase concentrations. This cell line was designated L5178Y D10/R and was used for all subsequent experiments involving L-asparaginase-resistant cells.

L-Asparaginase cytotoxicity

Cells were grown at an initial concentration of 2×10^5/ml in 2.0 ml of asparaginase-supplemented DMEM and varying dilutions of L-asparaginase from 10 to 1×10^{-6} I.U./ml in duplicate wells of a 24-well plate. A control with no L-asparaginase was also included. After 48 hours incubation at 37°C, the number of viable cells was determined by trypan blue exclusion with a hemacytometer.

Chromosome spread preparations

Metaphase chromosome spreads were prepared according to a modification of the procedure of Sun et al. (1974). L5178Y D10 and L5178Y D10/R cells were suspended at a concentration of 2×10^6/ml in 10 ml of PBS, to which was added 0.1 ml of colchicine (Sigma Chemical Co.) from a 10 μg/ml stock solution. This preparation was incubated at 37°C for 45 minutes. The cells were centrifuged at 200 g for 6 minutes and then washed twice with PBS.

The cells were gently resuspended in 6 ml of 0.56% KCl prewarmed to 37°C and incubated for 12 minutes. The suspension was then centrifuged for 6 minutes at 200 g, and the cells were resuspended in 4 ml of freshly-prepared fixative (methanol : glacial acetic acid, 3 : 1, v/v). The cells were immediately centrifuged for 6 minutes at 200 g. The supernatant fluid was removed, and the fixative was added to the cells as before. The centrifugation and addition of fixative were repeated as above, and the third aliquot of fixative was allowed to stand at room temperature for 30 minutes. The cells were centrifuged at 200 g for 6 minutes and resuspended in a volume of fresh fixative determined empirically to give a sufficient number of chromosome preparations when dropped onto slides.

One drop of the cell suspension was dropped onto a slide from a height of 3–4 inches, and the fixative was ignited by bringing the slide momentarily into contact with an alcohol flame. Slides were allowed to air dry at room temperature for one week, then overnight at 60°C.

G-banding staining procedure

A 0.25% solution of Wright's stain in anhydrous methanol was prepared and mixed for 30 minutes. The solution was allowed to sit at room temperature for 1 month, then filtered through Whatman 1MM filter paper. Immediately before use, 1 part of Wright's stain was added to 3 parts of 0.06 M phosphate buffer, pH 6.8. Unstained slides were treated with 0.025% trypsin for 15–20 seconds, rinsed with PBS, and stained with Wright's stain for 3–5 minutes. The slides were rinsed with water and allowed to air dry.

Metaphase spreads were examined under a bright field at 1000X magnification with a green filter. Photographs were taken with Kodak Technipan film (ASA 25) in a Nikon photomicroscope. Photographs were printed with a polycontrast #2 filter (Kodak) on Polycontrast Rapid II RC paper (Kodak).

The nomenclature of Nesbitt and Francke was employed for karyotypic classification (Nesbit and Francke, 1973). Pictures of individual chromosomes were cut out and classified. The lengths of all chromosomes were added together to obtain the total haploid length. Each chromosome length was then divided by the total haploid length to obtain its relative length.

Southern blot analysis of L5178Y D10 and L5178Y D10/R DNA

L5178Y D10 and L5178Y D10/R cells were grown as described above. Approximately 5×10^7 cells were lysed by incubation in 0.5 M EDTA with 1% Sarcosyl and 100 μg/ml proteinase K for 2 hours at 50°C. DNA was extracted with phenol/chloroform and precipitated with ethanol/acetate. The precipitate was dried under vacuum and dissolved in a minimal amount of TE buffer.

The restriction enzymes BamHI, EcoRI, and HindIII, were used according to the manufacturer's directions. Each reaction mixture was electrophoresed in 0.8% agarose at 20 V for 16 hours, after which the gel was stained with ethidium bromide and photo-

graphed. DNA was transferred to a nylon membrane (Zetaprobe, Bio-Rad, Richmond, CA) according to the procedure of Reed (Reed and Mann, 1985).

An asparagine synthetase-specific cDNA derived from a human fibrosarcoma cell line with an amplified asparagine synthetase gene was obtained from Dr. Irene Andrulis (Hospital for Sick Children, University of Toronto). The probe was labeled with ^{32}P by random priming. The reaction mixture was then passed through a 1-ml Sephadex G-50 spin column equilibrated with TE buffer.

Filters were prehybridized for 5 minutes at 60°C in a solution of 0.5 M NaH_2PO_4, pH 7.2, containing 1% BSA, 1 mM EDTA and 7% SDS. The hybridization was performed in the same solution, with approximately 10 ml of solution per 200 μl of probe. After incubation for 16 hours at 60°C, the membrane was washed twice at 60°C for 30 minutes in 40 mM NaH_2PO_4, pH 7.2, containing 0.5% BSA, 1 mM EDTA, and 5% SDS. Additional washes were performed twice for 30 minutes each in 40 mM NaH_2PO_4, pH 7.2, containing 1 mM EDTA and 1% SDS. Membranes were exposed to Kodak XAR film with a Lightning Plus intensifying screen (Dupont, Wilmington, DE) at −70°C.

Multidrug resistance assays

Cells at an initial concentration of 2×10^5/ml were incubated in 24-well tissue culture plates (Costar, Cambridge, MA) with various concentrations of the drug being tested. All drugs were purchased from Sigma Chemical Co., St. Louis, MO. Test samples and control samples with no drug were plated in triplicate. After 48 hours incubation at 37°C, cell viability was determined by trypan blue exclusion with a hemacytometer.

The D_{10} value, which was the concentration of the drug which decreased the number of viable cells within 48 hours to approximately 10% of the control with no drug, was determined for each drug with each cell line. Relative resistance was obtained by dividing the D_{10} value of the L-asparaginase-resistant cells by that of the sensitive cells.

Western blot analysis

Cells were suspended at a concentration of 1×10^7/ml in PBS and sonicated 3 times for 15 seconds each time, with 15-second intervals between sonications. The lysate was centrifuged at 10,000 g for 10 minutes, and the supernatant fraction was used for analysis. Samples were analyzed by SDS-polyacrylamide gel electrophoresis on a 5–15% polyacrylamide gradient according to the procedure of Laemmli (1970). An aliquot of extract corresponding to $0.3–1.0 \times 10^6$ cells was loaded onto each lane. Transfer to a nitrocellulose filter was done at 30 V overnight in a Trans-Blot Cell (Bio-Rad, Richmond, CA). The filter was blocked by incubation in a solution of 5% nonfat dry milk in PBS for 90 minutes at room temperature. After being washed three times in Tris-saline buffer (10 mM Tris-Cl, 15 mM NaCl, pH 7.4) containing 0.1% Triton X-100 and once in Tris-saline buffer without Triton, the filter was further incubated at room temperature for 2 hours in the presence of a rabbit antiserum specific for bovine asparagine synthetase. The filter was then washed as above, followed by incubation in goat anti-rabbit IgG conjugated to horseradish peroxidase for 90 minutes at room temperature. After this, the filter was washed with at least five changes of Tris-saline buffer with 0.1% Triton X-100 over a 30-minute period, followed by two rinses with Tris-saline without detergent. Diaminobenzidine in the presence of cobalt chloride was added to allow for color development, after which the reaction was stopped by washing with water.

Enzyme assays

To obtain cell extracts for enzymatic assays, cells were placed in 4 ml of the appropriate enzyme assay buffer (see below) in 30-ml Corex tubes at a concentration of 1×10^8 cells per ml. They were then sonicated 3 times at full power for 1 minute each time in a water-bath sonicator (Laboratory Supplies Co., Hicksville, N.Y.). The solution was centrifuged at 12,000 g

for 20 minutes in an SS-34 rotor in an RC5-B centrifuge to remove cell debris. The supernatant fluid was then applied to a Sephadex G-25M column (Pharmacia Fine Chemicals, Piscataway, N.J.) to remove endogenous enzyme substrates, products, and effectors. The columns were equilibrated prior to use with 30 ml of assay buffer. A two-ml aliquot of the cell extract was added to the column, followed by 0.75 ml of sample buffer, and an additional 2-ml aliquot of buffer. The first 2 ml of effluent from the column was collected and used for enzyme assay.

Serine hydroxymethyl transferase, asparagine synthetase, and L-asparaginase were assayed by slight modifications of the HPLC assay described by Unnithan et al. (1984). Isocratic separation of the enzymatic products was accomplished with 14% methanol for the L-asparaginase assay and 18% methanol for the SHMT assay. SHMT substrate mixture was as described by Schirch and Gross (1968), and the L-asparaginase assay mixture was as described by Tower et al. (1963). In all cases the reaction was terminated by the addition of 20% (w/v) TCA to a final concentration of 5%. The samples were centrifuged at 12,000 g for 5 minutes in a microcentrifuge, and the supernatant fluids were saved for HPLC analysis.

To assay glyoxylate-asparagine aminotransferase activity, the incubation mixture contained 150 μl of cell extract, 150 μl of 2 mM glyoxylate (including 250,000 cpm [1-^{14}C]-glyoxylate) and 50 mM asparagine in EPPS/EDTA buffer (24 mM 4-[2-hydroxyethyl]-1-piperazine propane sulfonic acid and 8 mM EDTA, pH 8.5). The samples were incubated at 37°C for 2 hours, after which the reaction was stopped by addition of 20% TCA. The tubes were centrifuged as above, and glycine was separated from glyoxylate in the supernatant fluids by the use of 5-ml SCX silica-based cation exchanger columns and the Vac Elut vacuum apparatus (Analytichem). After the column had been conditioned by the addition of methanol followed by 0.1 M H_2SO_4, 150 μl of sample plus 1 ml of H_2O was added to the column. The column was then washed with four 2-ml aliquots of H_2O and eluted with four 2-ml aliquots of NH_4OH. The amount of radioactivity in the NH_4OH fractions was determined in a liquid scintillation counter.

Protein determination

Protein concentrations were determined by the Coomassie blue dye binding method (Bradford, 1976), with bovine serum albumin as the standard.

Results

Before the L5178Y D10 cells were used for derivation of a resistant cell line, experiments were performed to investigate the dependence of the L5178Y D10 cells upon an exogenous source of asparagine. The cells were grown in medium without asparagine and in medium containing both asparagine and 10^{-3} I.U.'s L-asparaginase per ml. The number of viable cells was determined at 12-hour intervals. As seen in Fig. 1, either treatment resulted in 99% inhibition of growth within 48 hours, indicating that this cell line exhibited the L-asparaginase-sensitive phenotype.

Cytotoxicity assays demonstrated that the L5178Y D10 cells showed almost complete inhibition of growth within 48 hours in DMEM with L-asparaginase at concentrations of 10^{-3} I.U.'s per ml or above (Table 1). At a concentration of 10^{-4} I.U.'s per ml, the percent inhibition of growth decreased to 36.7%. Therefore, in an attempt to isolate resistant variants, the sensitive cells were initially exposed to this concentration of asparaginase, with the concentration gradually increased to 5×10^{-4} I.U.'s per ml, as described in Materials and methods.

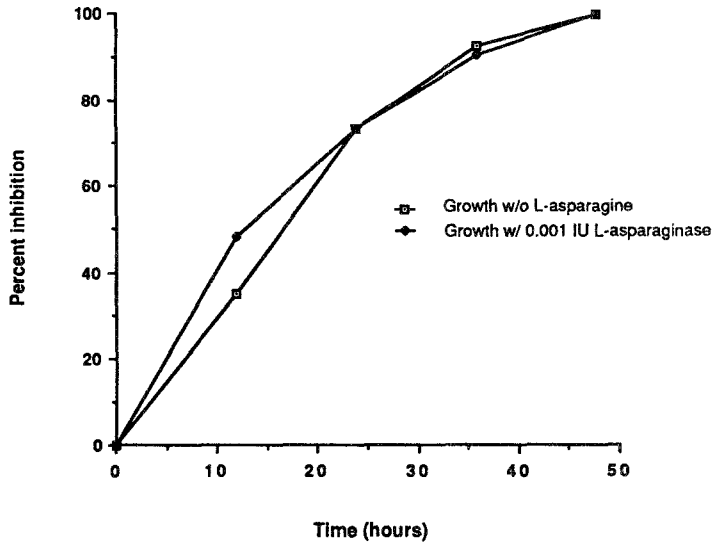

Fig. 1. Inhibition of growth of L5178Y D10 cells by asparagine deprivation and exposure to L-asparaginase. Cells were incubated in medium containing no supplemental asparagine or medium with asparagine and 10^{-3} I.U./ml of L-asparaginase. The number of viable cells was determined at twelve hour intervals by trypan blue exclusion, and the percent inhibition was determined as shown below by comparison with a control containing supplemental asparagine with no L-asparaginase. % inhibition = {(No. of cells in control − No. of cells in exp. sample)/No. of cells in control} × 100

Subsequent assays demonstrated that a majority of the L5178Y D10/R (L-asparaginase-resistant) cells, which were derived by exposure of the sensitive cells to L-asparaginase, were killed only when the L-asparaginase concentration reach 1.0 I.U. per ml, which is at least 1000-fold greater resistance than shown by the sensitive cells (Table 1). In other experiments in which resistant cells were derived by exposure to higher initial concentrations of asparaginase or by asparagine deprivation, the resistant cells obtained were also resistant to this same concentration of L-asparaginase (data not shown). Therefore, the method of selection did not seem to influence the degree of asparaginase resistance. In addition, resistant cells maintained in medium with asparagine for 11 months did not demonstrate increased sensitivity to the drug, indicating that, once acquired, L-asparaginase resistance was a stable phenotype.

An attempt was made to induce a higher degree of resistance by exposure to concentrations of L-asparaginase greater than 10^{-1} I.U.'s per ml. Incubation of resistant cells for up to 6 months in concentrations of L-asparaginase alternating between 10^{-1} and 5×10^{-1} I.U.'s per ml did not result in resistant cells capable of growth in increased concentrations of asparaginase (data not shown). Since exposure to high concentrations of drugs used for selection has been shown in other systems to induce gene amplification, this result suggested that amplification of the gene for asparagine synthetase was not responsible for L-asparaginase resistance.

To determine whether acquisition of the resistant phenotype was accompanied by detectable chromosomal alterations, the karyotypes of both L5178Y

D10 and L5178Y D10/R cells were analyzed. The modal chromosome number (2C) was determined by counting 100 mitoses in both cell lines. Both were aneuploid, with 58/100 L5178Y D10 cells and 61/100 L5178Y D10/R cells displaying the diploid mouse chromosome number of 40 (Fig. 2). Although both had a minimum modal number of 37, L5178Y D10 cells had a maximum chromosome number of 40, while the L5178Y D10/R had up to 44, with 28/100 cells having over 40 chromosomes.

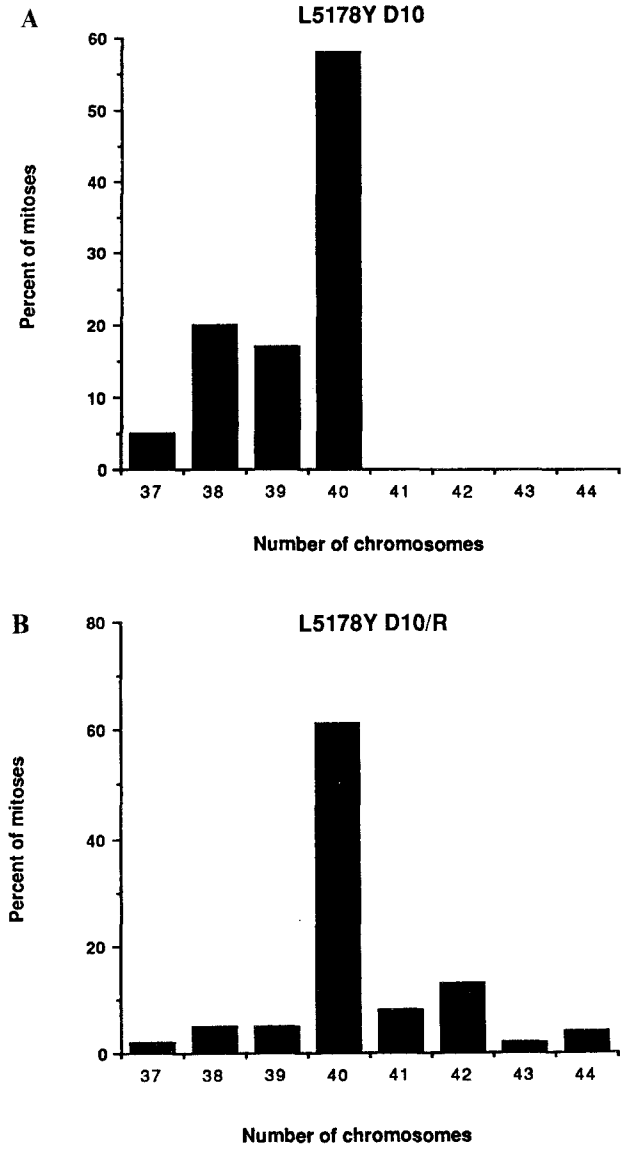

Fig. 2. Distribution of chromosome number in L5178Y D10 and L5178Y D10/R cells. Metaphase spreads were prepared as described in Materials and methods, and the number of chromosomes in 100 mitoses from each cell type was determined. Results are expressed as the percentage of mitoses containing the indicated number of chromosomes. **A** L5178Y D10 cells; **B** L5178Y D10/R cells

A

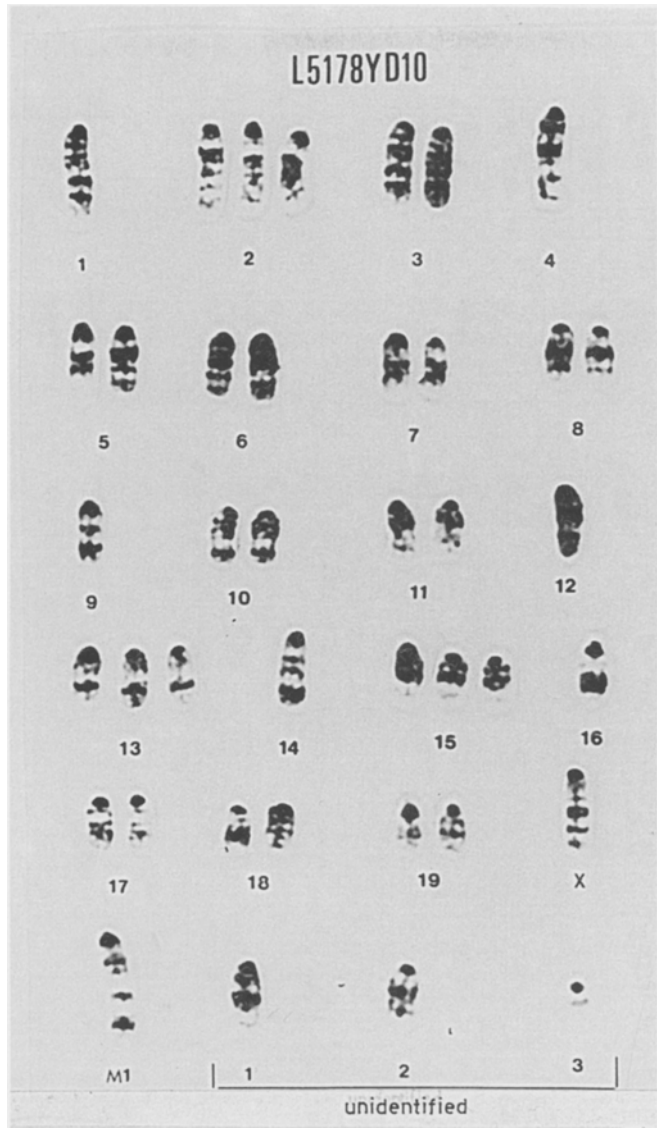

Fig. 3. Karyotype of G-banded L5178Y D10 and L5178Y D10/R cells. Metaphase chromosomes were prepared by incubation of the cells in the presence of colchicine, and the chromosomes were treated with trypsin and stained with Wright's stain, as described in the Materials and methods. Chromosomes were identified as proposed by the Committee on Standardized Genetic Nomenclature for Mice (Nesbit and Francke, 1973). **A** L5178Y D10 cells; **B** L5178Y D10/R cells (see p. 60)

G-banded metaphase chromosome spreads are shown in Figs. 3A and B for L5178Y D10 and L5178Y D10/R cells, respectively. These examples are from metaphase spreads which contain the maximum number of chromosomes seen in each cell line. Because of the aneuploidy present within both cell types, these examples do not reflect the distribution of chromosomes seen in all metaphase spreads. They do, however, illustrate the typical banding patterns that permitted the identification of each chromosome according to standard mouse chromosome nomenclature (Nesbit and Francke, 1973).

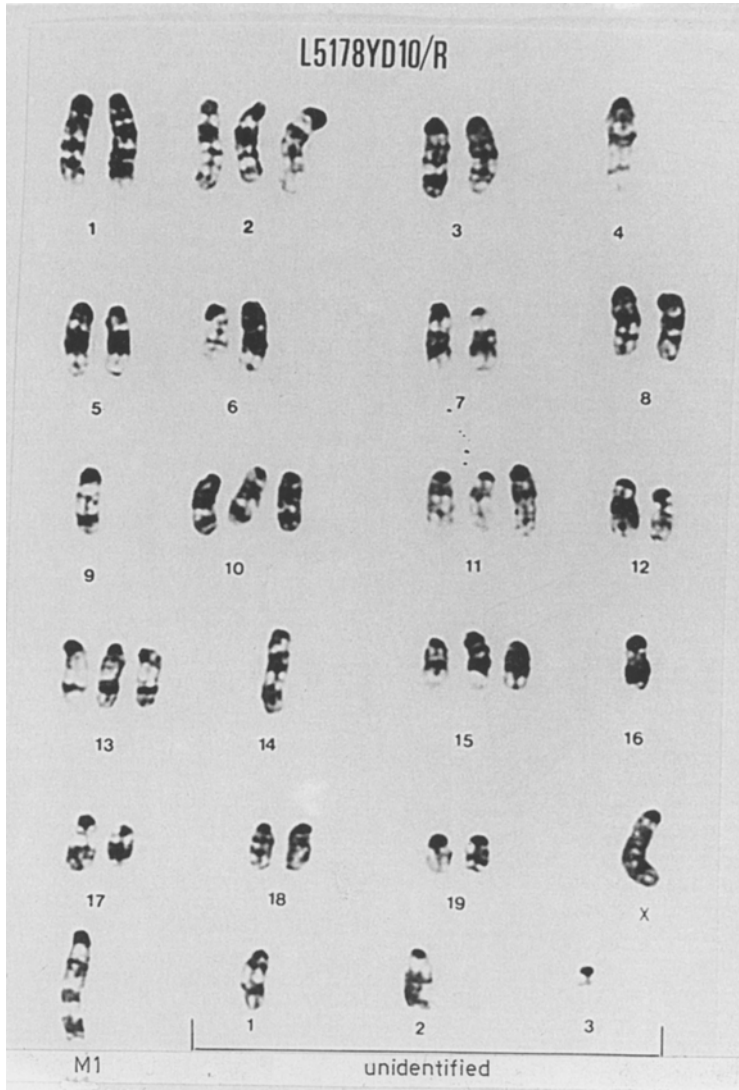

Fig. 3B

The chromosomes of the cell lines show some variability in morphology and banding patterns from normal DBA/2 chromosomes, perhaps due to translocations, deletions, or additions that have accumulated over the 35 years since these cells were first isolated. One marker chromosome (M1), which was possibly chromosome 4 with a terminal addition, was consistently seen in each of the 200 metaphase spreads analyzed. This chromosome is not thought to participate in L-asparaginase resistance, since it is present in both cell lines. None of the 100 metaphase spreads analyzed in each cell line revealed evidence of large homogeneously staining regions. There was also no evidence of double minute chromosomes or extrachromosomal fragments, which can be indicative of unstable gene amplification (Schimke et al., 1981). While there was some variability in the number of chromosomes per cell, this appeared to be random. Analysis of 3 L5178Y D10 and 5 L5178Y D10/R metaphase spreads indicated there was no

Table 2. Results of karyotype analysis of 8 metaphase spreads Chromosome number

Cell line	Chromosome number																					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	X	M1	Unidentified
L5178Y D10																						
1[a]	1[b]	1	2	1	3	2	2	2	1	2	2	1	3	1	3	1	2	1	3	1	1	2
2	1	3	2	1	2	2	2	2	1	2	2	1	3	1	3	1	2	2	2	1	1	3
3	2	2	1	1	2	2	1	2	1	2	2	2	2	1	3	1	2	2	2	1	1	3
L5178Y D10/R																						
1	2	3	2	1	2	2	2	2	1	3	3	2	3	1	3	1	2	2	2	1	1	3
2	1	3	2	1	3	1	2	2	2	1	2	2	2	2	3	1	3	3	2	1	1	3
3	2	3	2	2	2	2	2	1	2	2	2	1	2	1	2	2	2	1	3	1	1	1
4	2	2	2	1	2	2	2	2	2	2	2	1	2	1	2	1	2	2	2	1	1	4
5	2	2	2	1	2	2	2	2	3	2	2	1	3	1	2	1	2	1	2	1	1	2

[a] Number represents the chromosome preparation from the indicated cell line. Results from three preparations from L5178Y D10 cells and five from L517Y D10/R cells are included in this analysis

[b] Number represents the number of copies of the indicated chromosome found in that particular chromosome preparation

Table 3. Relative length of each chromosome as a percentage of the mitotic complement

Chromosome Number	Relative length	
	L5178Y D10	L5178Y D10/R
1	5.90 ± 0.34	5.95 ± 0.41
2	5.52 ± 0.32	5.60 ± 0.36
3	5.41 ± 0.30	5.35 ± 0.38
4	5.59 ± 0.44	5.65 ± 0.38
5	5.13 ± 0.32	5.02 ± 0.29
6	4.25 ± 0.25	4.18 ± 0.20
7	4.10 ± 0.30	4.08 ± 0.28
8	4.15 ± 0.21	4.20 ± 0.27
9	3.85 ± 0.35	3.78 ± 0.30
10	4.15 ± 0.22	4.22 ± 0.31
11	4.14 ± 0.25	4.25 ± 0.30
12	4.12 ± 0.28	4.22 ± 0.18
13	4.58 ± 0.22	4.49 ± 0.17
14	4.52 ± 0.30	4.60 ± 0.24
15	4.18 ± 0.25	4.24 ± 0.22
16	3.95 ± 0.16	3.81 ± 0.29
17	3.71 ± 0.28	3.88 ± 0.17
18	3.65 ± 0.11	3.55 ± 0.25
19	3.22 ± 0.34	3.17 ± 0.12
X	5.55 ± 0.25	5.45 ± 0.31
M1	6.80 ± 0.35	7.00 ± 0.34

chromosome found either exclusively or in increased numbers in the resistant cells (Table 2).

The relative length of each chromosome expressed as a percentage of the mitotic complement is shown in Table 3. While variability is seen, these data do not support a consistent increase in chromosomal length due to enlargement associated with homogeneously staining regions. Therefore, gene amplification, at least to the extent that would lead to HSR formation, is not involved in acquisition of L-asparaginase resistance in L5178Y cells.

To verify that gene amplification does not participate in L-asparaginase resistance, the relative hybridization intensities with an asparagine synthetase-specific cDNA probe was determined for genomic DNA from both cell lines. In addition to detection of gene amplification, Southern blot analysis was intended to discover variability in sizes of restriction fragments and methylation differences between the two cell lines. Figure 4 illustrates the results of restriction endonuclease digestion of genomic DNA. BamHI and EcoRI digestion is shown in Fig. 4A, while HindIII digests are represented in Fig. 4B.

A comparison of the DNA fragment lengths generated by digestion with each endonuclease showed no evidence of chromosomal rearrangements involving the asparagine synthetase gene. Digestion with BamHI enabled detection of distinct bands at 19, 6.6, 4.9 and 4.1 kb in both cell lines. There were 4 faint bands, 2.9, 2.6, 2.2, and 1.9 kb seen with only the L5178Y D10 DNA, which could

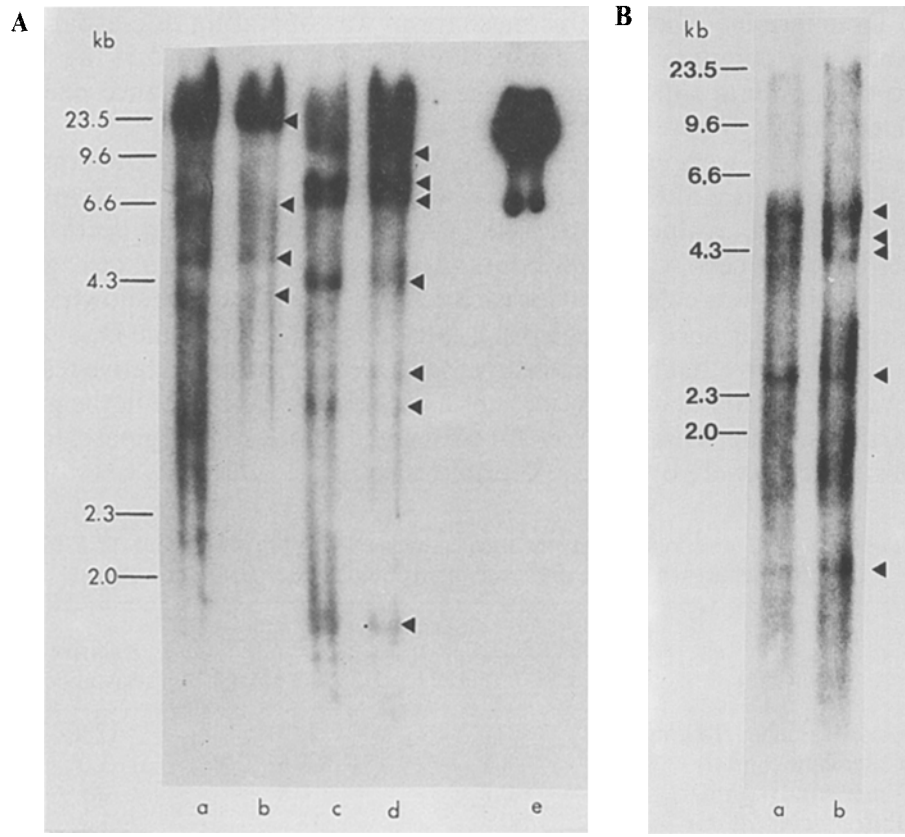

Fig. 4. Hybridization of cDNA specific for human asparagine synthetase with endonuclease-digested genomic DNA from murine L5178Y L-asparaginase-sensitive and -resistant cells. Genomic DNA was isolated, digested with the indicated endonucleases and transferred to nitrocellulose as described in Materials and methods. **A** Hybridization results with BamHI- and EcoRI-digested genomic DNA. *a* and *b*, respectively, represent genomic DNA from L5178Y D10 and L5178Y D10/R cells; *c* and *d*, respectively, are EcoRI-digested genomic DNA from L5178Y D10 and L5178Y D10/R cells; *e* is BamHI-digested genomic DNA from HT5 human tumor cells containing an amplified asparagine synthetase gene. Ten μg of DNA from the appropriate L5178Y cell line were added to each well, while 1 μg of HT5 DNA was added. Arrows indicate major bands common to both cell types. **B** Hybridization results with HindIII-digested genomic DNA. *a* and *b*, respectively represent genomic DNA from L5178Y D10 and L5178Y D10/R cells. Ten μg of DNA from the appropriate cell line were added per well. Arrows indicate major bands common to both cell types

be accounted for by these bands in the DNA from resistant cells being below the detection threshold. EcoRI digests generated 7 bands with lengths of 9.5, 7.4, 6.3, 4.2, 3.4, 3.1, and 1.7 kb, all of which were seen in both cell lines. A faint band of 1.3 kb is seen only in the L5178Y D10 DNA. HindIII digests revealed 5.2, 4.6, 2.6, and 1.2, kb fragments in both cell lines.

It has been observed that cancer cells can acquire resistance simultaneously to several chemotherapeutic drugs, some of which have different modes of action (Gerlach et al., 1986; Juliano and Ling, 1976; Kartner et al., 1983, 1985). In most cases the resistant phenotype is associated with increased expression of a high molecular weight glycoprotein, originally termed P-glycoprotein (Kartner et al.,

1983). To determine whether this mechanism was operating in L-asparaginase resistance, the comparative resistance of L5178Y D10 and L5178Y D10/R cells to chemotherapeutic agents implicated in the cross-resistance phenotype was determined.

Both cell types were grown for 48 hours in the presence of various concentrations of these drugs, after which cell viability was determined by trypan blue exclusion. The D_{10} value, which is the drug concentration that decreased the number of viable cells within 48 hours to 10% of that of control cells growing without the drug, was calculated for each cell line. The results are shown in Table 4. The relative resistance for each drug, calculated by dividing the D_{10} value for the resistant cells by that for the sensitive cells, is also shown. A relative resistance of 1.0 would indicate that both cell lines had the same D_{10} value in the presence of a particular drug. This was seen for all drugs except L-asparaginase, to which the resistant cells were over 1000X more resistant.

Table 4. D10 and relative resistance values of L5178Y D10 and L5178Y D10/R cells grown in the presence of various chemotherapeutic agents

Agent	D10 value[a]		Relative resistance[b]
	L5178Y D10	L5178Y D10/R	
L-asparaginase (I.U./ml)	7×10^{-4}	9×10^{-1}	1286
Colchicine (ng/ml)	8.7	8.6	1.0
Vincristine (ng/ml)	0.7	0.8	0.9
Puromycin (μg/ml)	50	58	1.2
Actinomycin D (ng/ml)	0.5	0.47	0.9

[a] The D10 value is the concentration of drug which decreases the number of viable cells to 10% of a control without the drug within 48 hours. Cell viability was determined by the trypan blue dye-exclusion test

[b] Relative resistance was obtained by dividing the D10 value of L-asparaginase-resistant cells by the D10 value of the L-asparaginase-sensitive cells

These results indicated that cross-resistance was not involved in L-asparaginase resistance in L5178Y D10/R cells, at least by this criterion. This conclusion was substantiated by SDS-PAGE analysis of the two cell types, which did not demonstrate any evidence for a high molecular weight protein unique to the L5178Y D10/R cells (data not shown).

Finally, both sensitive and resistant cells were analyzed by Western blot analysis with an antiserum prepared against bovine pancreatic asparagine synthetase (Fig. 5). Both cell lines contained immunoreactive proteins of approximately 65 and 63 kD, which is the same molecular weight reported for purified asparagine synthetase from bovine pancreas (Pfeiffer et al., 1987).

In spite of the presence of this immunoreactive material in both cell types, asparagine synthetase activity is undetectable in the sensitive cells (Table 5). It can also be seen that, of the four enzymes involved in asparagine metabolism for which activity was assayed in the two cell lines, asparagine synthetase was the only one whose activity was found to differ significantly between the sensitive and resistant cells. None of the other enzymes demonstrated any

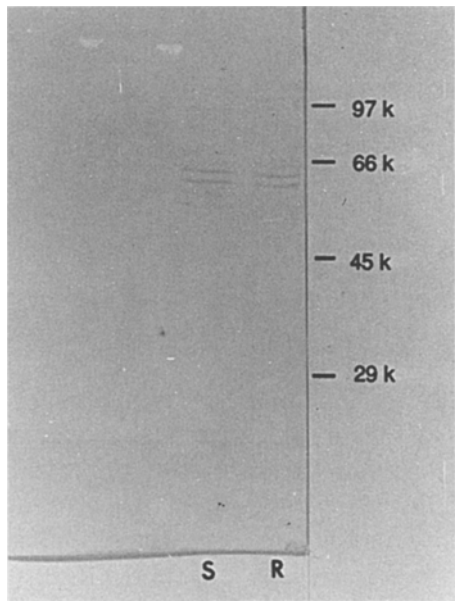

Fig. 5. Analysis of L5178Y D10 and L5178Y D10/R cell extracts for the presence of asparagine synthetase by Western blot. A cell extract from the indicated cell type was analyzed by electrophoresis on a 5–15% polyacrylamide gradient gel in the presence of SDS. After electrophoresis, the sample was transferred to nitrocellulose and analyzed by Western blotting with a rabbit antiserum specific for bovine pancreatic asparagine synthetase, as described in Materials and methods. Extract corresponding to 1×10^6 cells was loaded in each lane. *S* denotes extract from L5178Y D10/5 cells; *R* denotes extract from L5178Y D10/R cells

Table 5. Kinetic parameters of enzymes from L5178Y D10 and L5178Y D10/R tumor cell extracts[a]

	L5178Y D10 (Sen)		L5178Y D10/R (Res)		Res. vs. Sen ratio[b]	
Enzymes	Km_{app}	$Vmax_{app}$	Km_{app}	$Vmax_{app}$	Km_{app}	$Vmax_{app}$
Asparagine synthetase	nd[c]	nd	0.75 ± 0.06	0.32 ± 0.08	nd	>45
L-asparaginase	12.5 ± 1.3	0.95 ± 0.09	13.3 ± 0.5	1.00 ± 0.11	1.06	1.05
Glyoxylate-asparagine aminotransferase[d]	0.33 ± 0.05	0.17 ± 0.02	0.36 ± 0.04	0.17 ± 0.03	1.09	1.00
Serine hydroxymethyl transferase[e]	1.47 ± 0.21	1.67 ± 0.15	1.45 ± 0.11	1.72 ± 0.07	0.99	1.03

[a] Each number in this table represents the mean of at least three separate determinations. Km_{app} is given in mM, $Vmax_{app}$ in μmol per gram protein min

[b] Except for asparagine synthetase, none of these ratios were statistically different from one ($P > 0.02$). Significance was determined by Student's two-tailed t-test

[c] *nd* not detectable

[d] The activity of this enzyme was measured using glyoxylate as the substrate

[e] This enzyme was assayed in the glycine to serine direction

differences in either Km or Vmax between the two cell types. These results suggest that expression of asparagine synthetase activity in L5178Y D10 cells is regulated by alteration of the protein in either the sensitive or resistant cells. The immunoreactive material in the sensitive cells presumably corresponds to enzyme which has either not been activated or whose activity has been inactivated or suppressed by an as-yet-undefined mechanism.

Discussion

The purpose of this project was to examine possible mechanisms which might be responsible for conversion from the L-asparaginase-sensitive to -resistant phenotype in L5178Y murine leukemia cells, in light of the mechanisms that have been described for other tumor cell systems. To do this meaningfully, it was necessary to use sensitive and resistant cells that were as genetically homogeneous as possible. This would allow minimization of the background contributed by genetic anomalies unrelated to L-asparaginase sensitivity which had accumulated in the sensitive and resistant cell lines over the past 30 years. In addition, the L-asparaginase-resistant L5178Y cells obtained from the DCT Tumor Bank demonstrated multiple phenotypes, including a population having the appearance of a hairy cell leukemia, which can arise from B lymphocytes (Fu et al., 1974), and another population which contained only metacentric chromosomes, instead of the telocentric arrangement which has been reported for DBA/2 mice (Committee on Standardized Nomenclature for Mice, 1972; Schendl, 1971). Therefore, an L-asparaginase-resistant cell line, designated L5178Y D10/R, was isolated by growth of cloned L5178Y D10 mouse lymphoma cells in the presence of L-asparaginase at a concentration that was cytotoxic to over 60% of the cells in the initial sensitive population. The resistant cell line was over 1000X more resistant to L-asparaginase than the parental sensitive cells. This has allowed an investigation into possible modes of resistance shortly after the emergence of the L-asparaginase-resistant phenotype.

Tolerance to gradually increasing increments of L-asparaginase was not observed. Instead, the degree of resistance was the same, regardless of the concentration of L-asparaginase used for selection of resistant cells. The resistant phenotype was stable up to 11 months in culture with or without exogenous asparagine. If a conversion to auxotrophy occurred in only a small percentage of cells, a gradual and detectable increase in the L-asparaginase-sensitive population might be expected, as the growth rate of L-asparaginase-sensitive cells has been reported to be one and one-half times faster than that of L-asparaginase-resistant cells (Colofiore et al., 1973).

Karyotypic analysis of L-asparaginase-resistant cells showed no evidence of large homogeneously staining regions, which is a phenotypic property associated with stable gene amplification. Unstable amplification is frequently associated with double minute chromosomes (Schimke et al., 1981), which were also not observed here, suggesting that neither stable nor unstable gene amplification had occurred. In addition, the intensities of the bands obtained by Southern blot hybridization with a cDNA probe specific for the human asparagine synthetase gene were approximately the same in both cell lines, indicating that the structural gene was present at the same copy number in the two cell types, regardless of

their resistance phenotype. It exists probably as a single copy, based on the faint hybridization intensity, although this may be attributed in part to the lack of complete homology between the human cDNA and the murine gene. Presently, the degree of identity between the mouse gene and human cDNA is unknown. Andrulis et al. (1987) have found the extent of identity between asparagine synthetase cDNA from CHO and human cells to be 86% and that of the protein to be over 95%, suggesting that the gene is well conserved.

It is interesting that, not only is the gene copy number the same in the two cell lines, but the number and sizes of the fragments generated by restriction endonuclease digestion also appear to be similar. This implies that there has been no chromosomal rearrangement that could lead to activation of the asparagine synthetase gene in resistant cells, or, conversely, that could disrupt the integrity of the structural gene in sensitive cells, thus rendering it inactive. Since a heterologous probe was used for Southern blot analysis, it is not certain that each of the detectable bands corresponds to a fragment of the asparagine synthetase gene. Regardless, the important observation is still valid, which is that there is no unique fragment in either cell line that suggests a translocation has occurred.

There are several underrepresented regions on the Southern blot manifested as fragments seen in DNA from sensitive but not resistant cells. Although it cannot presently be ruled out that these represent heterozygous or homozygous deletions, it is more likely that either the probe does not hybridize strongly to some fragments and is more easily removed in the wash procedure, or that slightly more sensitive DNA was used for Southern blot analysis. This latter alternative is supported by the fact that the intensity of each band seen with the BamHI-digested DNA from sensitive cells is consistently 1.4 times greater than that of the corresponding band from the resistant cells.

No evidence for conferred cross-resistance in the asparagine synthetase-producing L5178Y D10/R cells was indicated by cytotoxicity of the cells to different drugs implicated in the multidrug-resistance phenotype. While the asparaginase-resistant cells are approximately 1000X more resistant to L-asparaginase, they demonstrated sensitivity to the same concentrations of colchicine, vincristine, actinomycin D, and puromycin as did the sensitive cells. Cross-resistance to all these drugs is usually seen as a consequence of overexpression of the P-glycoprotein (Gerlach et al., 1986; Kartner et al., 1983). In addition, SDS-PAGE demonstrated no high molecular weight proteins unique to the resistant cells.

Although genomic fragments were not detectably different, this does not rule out point mutations or small deletions which might not detectably alter mobility of the fragments in gels. This is especially relevant in light of the presence of material in both sensitive and resistant cells that reacts with an antiserum prepared against bovine pancreatic asparagine synthetase. This is different from L-asparaginase-sensitivity in Jensen rat sarcoma cells, which have been reported to contain neither material that reacts with asparagine synthetase-specific antibodies nor asparagine synthetase-specific mRNA (Andrulis et al., 1987), and suggests that asparaginase resistance might arise in malignant cells by more than one mechanism.

It is possible that the material in the sensitive cells represents a protein that shares only a limited number of epitopes with asparagine synthetase without necessarily having asparagine synthetic activity. However, the similar size of the immunoreactive material from both sensitive and resistant cells indicates considerable structural similarity of the material from the two sources. Interestingly, an asparagine synthetase-specific monoclonal antibody that reacted with asparagine synthetase from a number of cell types possessing asparagine synthetase activity, including L5178Y D10/R cells (Pfeiffer et al., 1987), did not detect any immunologically reactive material in the L5178Y sensitive cells (N. Pfeiffer, personal communication), indicating that the enzyme from sensitive cells may have an altered conformation from that of resistant cells, and, as such, has lost its enzymatic activity.

In summary, none of the previously described mechanisms for resistance of cells to chemotherapeutic agents, such as gene amplification, P-glycoprotein expression, or gene inactivation by methylation, seems to play a role in the acquisition of L-asparaginase resistance in L5178Y murine leukemia cells. Instead, the mechanism of resistance presumably involves differences in enzyme structure between the L-asparaginase-sensitive and -resistant cells, in that both cell types contain immunoreactive material detectable with an asparagine synthetase-specific antiserum. Additional analyses into the mechanism that allows for the intracellular presence of this material in the absence of detectable enzymatic activity are currently underway.

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