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Hypersensitivity of rat glioma sublines with acquired ACNU resistance to L-asparaginase

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Cell lines resistant to 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride (ACNU) show a high degree of collateral sensitivity to L-asparaginase. The mechanism for this phenomenon was investigated by comparing the nutritional requirements and asparagine synthetase activity of the resistant sublines to those of parent cells. Nine ACNU-resistant sublines were isolated from rat glioma 9L cells after incubation with various concentrations of ACNU in Ham's F-12 medium. The 9L cells grew independently of asparagine, developing well in asparagine-deficient Dulbecco's modified Eagle's medium. In contrast, the growth rates of all nine ACNU-resistant sublines decreased under the same conditions and required the addition of 10^{-4} M asparagine for maximum growth. Asparagine synthetase activity in the ACNU-resistant cells was much lower than in the 9L cells, suggesting that the requirement for asparagine in the resistant sublines was due to reduced activity of this enzyme. A growth-inhibition assay showed that the ACNU-resistant sublines were more sensitive to L-asparaginase than 9L cells by up to 2×10^5-fold. These results suggest that L-asparaginase therapy has the potential to become a new approach for treating acquired ACNU resistance.

KEY WORDS • **drug resistance** • **nitrosourea** • **brain neoplasm** • **mutagenesis** • **auxotrophy** • **asparagine**

CHEMOTHERAPY plays a valuable role in the management of many malignancies, but the overall success rate for treatment of most solid tumors remains quite low. In addition, the initial response of many tumors to chemotherapy is too often followed by a very short remission period and failure to respond to subsequent therapy. This phenomenon has been ascribed to the acquisition of drug resistance by tumor cells and is a major reason for the failure of chemotherapy.[10] We previously developed nine 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride (ACNU)-resistant sublines from rat glioma 9L cells by selection procedures with ACNU-containing Ham's F-12 medium in order to investigate the mechanism of resistance to this drug.[23] Much research has been done on the resistance to chloroethylnitrosourea (CENU) compounds including ACNU, but most of these studies have investigated deoxyribonucleic acid (DNA) repair systems.[24,29,33] Increased activity of O^6alkylguanine-DNA alkyltransferase (O^6-AT), a DNA repair enzyme, has been proposed as a major mechanism of CENU resistance.[24,34] Agents that deplete intracellular O^6-AT, such as methylnitrosourea or O^6-methylguanine, are known to reverse CENU resistance[4,33] but are difficult to use clinically owing to their strong mutagenic properties and other side effects.[34]

We have observed that ACNU-resistant cells do not grow well in Dulbecco's modified Eagle's medium (DMEM), while the growth rate of wild-type 9L cells is not altered. This difference in the nutritional requirements of wild and ACNU-resistant cells led us to examine the biological basis of the phenotypic change acquired by the resistant cells. In this paper, we report on the auxotrophy for asparagine of ACNU-resistant cell lines derived from 9L cells and on an approach for overcoming ACNU resistance by the use of L-asparaginase.

Materials and Methods

Cell Lines and Culture Conditions

The 9L rat glioma cell line[3] was developed in Fischer 344 rats by the administration of 1-methyl-1-nitrosourea. Sublines resistant to ACNU (R_1, R_3, R_{12}, RA5, RA10, RA20, RA40, RA60, and RA80) were isolated

TABLE 1

Biological characteristics of 9L cells and nine ACNU-resistant sublines

Characteristic	Cell Line									
	9L	R_1	R_3	R_{12}	RA5	RA10	RA20	RA40	RA60	RA80
ACNU resistance index*	1.0	10.0	21.0	25.2	15.5	17.4	13.7	13.3	14.5	13.7
population doubling time (hrs)†	22.4	27.5	21.7	21.7	23.5	23.7	25.2	25.9	24.7	26.8

* The resistance index was calculated as the concentration required to inhibit 37% of cells (IC_{37}) for ACNU-resistant cells divided by that for 9L cells.

† Values are the means of three experiments. The standard deviation was always within a 10% range. Except for the R_1 subline, no values were significantly different from the value for 9L cells ($p < 0.05$).

from 9L cells *in vitro* by multiple or single selection procedures with ACNU, as described previously.[23] These cell lines were maintained at our laboratories in Ham's F-12 medium supplemented with 10% fetal calf serum (FCS) and streptomycin (100 μg/ml). The cells were grown in monolayer cultures at 37°C in a humidified chamber with 5% CO_2 and 95% air, and were subcultured every 3 days.

Assay of ACNU and L-Asparaginase Toxicity

Resistance to ACNU was defined by a resistance index of the concentration required to inhibit 37% of cells (IC_{37}) divided by that for the parent 9L cells, as described previously.[23] The cytotoxic effect of L-asparaginase was quantified as follows. First, 1×10^5 cells were seeded into 35-mm plastic dishes and, 4 hours later, the medium was changed to DMEM supplemented with 10% dialyzed FCS and 10^{-4} M asparagine containing various concentrations of L-asparaginase. Cells were then cultured under the same conditions as described above. Every 24 hours thereafter, cells from triplicate dishes were sampled separately and counted by the nigrosin dye exclusion test.[18] The minimum concentration of L-asparaginase (kU/ml) that inhibited the growth of the cells completely was employed as an index of the cells' sensitivity to L-asparaginase.

Effect of Nutritional Constituents on Cell Growth

Dulbecco's modified Eagle's medium (which does not contain aspartic acid, asparagine, alanine, proline, cyanocobalamine, thymidine, hypoxanthine, putrescine, linoleic acid, or lipoic acid) was used with 10% dialyzed FCS as the control medium. Then, 1×10^5 exponentially growing cells were plated in 35-mm plastic dishes. After 4 hours, when the cells had become fixed to the plates, the culture medium was replaced by DMEM containing one of the following: aspartic acid (1×10^{-4} M), asparagine (2.5×10^{-5} M to 1×10^{-2} M), alanine (1×10^{-4} M), proline (3×10^{-4} M), cyanocobalamin (10^{-6} M), thymidine (3×10^{-6} M), hypoxanthine (3×10^{-5} M), putrescine (10^{-6} M), linoleic acid (3×10^{-7} M), or lipoic acid (10^{-6} M). Viable cells were counted every 24 hours thereafter by the same method as described above.

Assay of Asparagine Synthetase Activity

Asparagine synthetase activities were measured by determining the amount of [^{14}C]-L-asparagine formed from [^{14}C]-L-aspartic acid (specific activity 228 mCi/mmol) as described by Goldfarb, *et al.*[9] Some modifications were made: The separation of asparagine from aspartic acid was carried out by high-performance liquid chromatography on a column of LS-215,* eluted with 4% isopropyl alcohol in 0.16 M lithium citrate (pH 5.0) at a flow rate of 0.3 ml/min at 70°C. The radioactivity in the asparagine fraction was determined with a scintillation counter, and asparagine synthetase activity was measured as nanomoles of L-asparagine formed per milligram of protein per hour. Protein determinations were performed using the method of Lowry, *et al.*[20] Statistical analysis of asparagine synthetase activity and the population doubling time of the cell lines was performed using Student's t-test.

Results

Biological Characteristics of 9L and ACNU-Resistant Cells

The sensitivity to ACNU of the nine resistant cell lines in F-12 medium was expressed as a resistance index, determined by dividing the values for each ACNU-resistant subline by that of the 9L cells (Table 1). The index was lowest (10.0) in R_1 cells and highest (25.2) in R_{12} cells. The resistance index values of the nine cell lines did not change in a 12-month period after their isolation. The population doubling time was 22.4 hours for 9L cells and was significantly different only for R_1 cells ($p < 0.05$).

Effect of Asparagine on Cell Growth

The effects of various concentrations of asparagine in DMEM supplemented with 10% dialyzed FCS on cell growth are shown in Fig. 1. The parent 9L cells showed essentially no difference in growth regardless of the presence of asparagine, while the growth of the nine ACNU-resistant cell lines was much lower in the absence of asparagine. In particular, growth was sup-

* Chromotography system manufactured by Toyo Soda Company, Ltd., Tokyo, Japan.

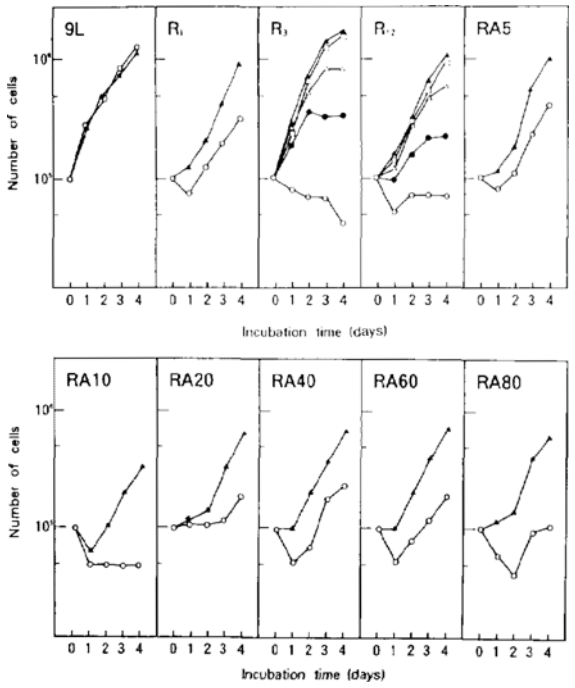

FIG. 1. Effect of asparagine (ASN) on the growth of 9L cells and nine ACNU-resistant sublines. Symbols denoting ASN concentrations added: *open circle* = none; *closed circle* = 2.5×10^{-5} M; *open triangle* = 5×10^{-5} M; *closed triangle* = 10^{-4} M; *square* = 1×10^{-2} M. Cells (1×10^5) were seeded in plastic dishes in ASN-free Dulbecco's modified Eagle's medium with 10% dialyzed fetal calf serum. After cell attachment, the medium was exchanged for one containing the indicated concentrations of ASN.

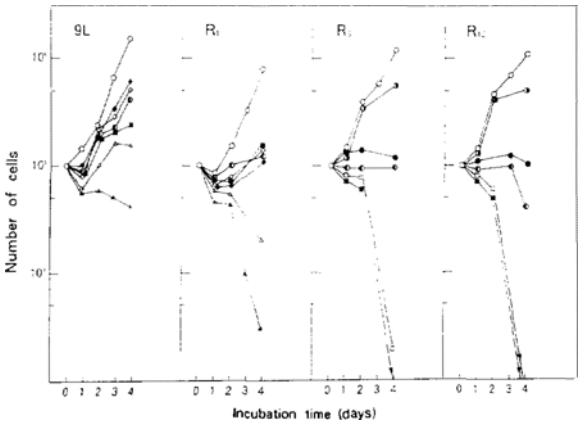

FIG. 2. Growth curves of 9L cells and three ACNU-resistant sublines after treatment with L-asparaginase (L-ASP). Symbols denoting L-ASP concentrations added (kU/ml): *open circle* = none; *open/closed circle* = 10^{-6}; *closed circle* = 5×10^{-6}; *closed/open circle* = 10^{-5}; *closed square* = 10^{-4}; *open diamond* = 10^{-3}; *closed diamond* = 10^{-2}; *open triangle* = 10^{-1}; *closed triangle* = 10^{0}. Cells (1×10^5) were seeded in plastic dishes in asparagine-free Dulbecco's modified Eagle's medium (DMEM). After cell attachment, the medium was changed to DMEM with 10^{-4} M asparagine and dialyzed 10% fetal calf serum containing the indicated concentrations of L-ASP (L-ASP at 1 kU is equivalent to the production of 1 M NH_3 when L-ASP decomposes asparagine at 37°C for 1 minute).

pressed completely for R_3, R_{12}, RA10, and RA80 cells. Addition of 10^{-4} M asparagine to cultures of the nine ACNU-resistant cell lines increased growth markedly in all cases. For R_3 and R_{12} cells, the effects of the addition of 2.5×10^{-5}, 5×10^{-5}, and 10^{-2} M asparagine were also examined. In both cell lines, the growth rate increased in proportion to the increase of the asparagine concentration up to 10^{-4} M, then showed a plateau. On the other hand, addition of the other amino acids, cyanocobalamine, thymidine, hypoxanthine, putrescine, linoleic acid, or lipoic acid to the culture medium had no affect on the growth curves of either 9L cells or the nine resistant cell lines (data not shown).

Asparagine Synthetase Activity

The asparagine synthetase activity levels for each cell line are presented in Table 2. Compared to the activities of 9L cells (10.45 nmol/mg protein/hr), all the ACNU-resistant cell lines had a lower activity ($p < 0.05$), with especially low levels being seen in R_3 (0.10 nmol/mg protein/hr), R_{12} (0.12 nmol/mg protein/hr), RA10 (0.20 nmol/mg protein/hr), and RA80 cells (0.23 nmol/mg protein/hr).

Sensitivity to L-Asparaginase

Growth inhibition of 9L, R_1, R_3, and R_{12} cells by L-asparaginase (an inhibitor of asparagine synthetase) is shown in Fig. 2, and the sensitivity to this enzyme is compared with the asparagine synthetase activity in Table 2. The minimum concentration of L-asparaginase that inhibited growth completely was 10^0 kU/ml for the parental 9L cells, while for R_1, R_3, and R_{12} cells the levels were 10^{-1}, 10^{-5}, and 5×10^{-5} kU/ml, respectively. Therefore, the ACNU-resistant R_1, R_3, and R_{12} cells had acquired a collateral increase in sensitivity to L-asparaginase of 10-, 10^5-, and 2×10^5-fold, respectively.

Discussion

Asparagine Dependence of ACNU-Resistant Cell Lines

The substances present in Ham's F-12 medium and not contained in DMEM include: $CuSO_4$, $Ca(NO_3)_2$, $MgCl_2$, cyanocobalamine, hypoxanthine, thymidine, putrescine, linoleic acid, lipoic acid, proline, asparagine, aspartic acid, and alanine.[12,21] We examined the effect of all of these substances, except the first three inorganic salts, on the growth of ACNU-resistant cells. The addition of asparagine to DMEM markedly increased the growth rate of all nine ACNU-resistant cell lines, whereas that of wild-type 9L cells remained unchanged. The other nine substances did not affect the growth of any cell lines. Thus, we concluded that these ACNU-resistant cell lines required asparagine for maximum growth.

Asparagine is a nonessential amino acid that is synthesized from aspartic acid by asparagine synthetase and is decomposed to aspartic acid by asparaginase.[5] Four types of asparagine auxotrophic mutants have

TABLE 2

Asparagine synthetase activity and sensitivity to L-asparaginase of 9L cells and nine ACNU-resistant sublines

Characteristic	Cell Line									
	9L	R_1	R_3	R_{12}	RA5	RA10	RA20	RA40	RA60	RA80
asparagine synthetase activity* (nmol/mg protein/hr)	10.45 ± 2.10	6.50 ± 0.87†	0.10 ± 0.03§	0.12 ± 0.02§	1.68 ± 0.20‡	0.20 ± 0.04§	1.24 ± 0.22‡	2.15 ± 0.34‡	4.80 ± 0.57†	0.23 ± 0.03§
sensitivity to L-asparaginase‖ (kU/ml)	10^{0}	10^{-1}	10^{-5}	5×10^{-5}	ND	ND	ND	ND	ND	ND

* Asparagine synthetase activity is given as nmoles asparagine formed/mg protein/hr. Values are the means ± standard deviations of three to four determinations.

† $p < 0.05$, compared to 9L cells (Student's t-test).

‡ $p < 0.01$, compared to 9L cells (Student's t-test).

§ $p < 0.005$, compared to 9L cells (Student's t-test).

‖ Sensitivity is expressed as the concentration of L-asparaginase (kU/ml) required to completely inhibit cell growth. ND = not determined.

been reported: asparagine synthetase-deficient mutants;[9,16] asparagyl-transfer ribonucleic acid synthetase-deficient mutants;[32] succinate dehydrogenase activity-deficient mutants;[27] and nicotinamide adenine dinucleotide (NADH) coenzyme Q reductase activity-deficient mutant.[7] The nine cell lines were not affected by the addition of aspartic acid, so the enzyme activity of asparagine synthetase was measured. All nine cell lines had a far lower level of activity than did the 9L cells. The R_3, R_{12}, RA10, and RA80 cells, for which growth was suppressed completely in the absence of asparagine, had particularly low asparagine synthetase activities, while the other cell lines with less growth suppression had relatively higher activities. These data suggest that a reduced level of asparagine synthetase of the resistant cell lines plays a major role in the auxotrophic phenotype of these cells, although the other three explanations for the asparagine dependence of ACNU-resistant cells mentioned above are also possible.

Mechanism of Asparagine Dependence and L-Asparaginase Sensitivity

Like other alkylating agents,[8,26] ACNU produces mutagenic activity as well as cytotoxic activity.[2] Abe, *et al.*,[1] have demonstrated that ACNU induces sister chromatid exchanges both *in vivo* and *in vitro*. Horie, *et al.*,[15] have reported that mammalian cells pretreated with ACNU developed mutant lines resistant to structurally and functionally unrelated drugs in proportion to the concentration of ACNU. The ACNU-resistant cells used in the present study were isolated by selection procedures using various concentrations of ACNU in single- or multi-step methods without prior exposure to strong mutagens such as N-methyl-N'-nitro-N-nitrosoguanidine or ethylmethane sulfonate. The concentration of ACNU used for selection varied from 5 to 120 μg/ml but, irrespective of the concentration used, all nine ACNU-resistant cell lines isolated had a lower asparagine synthetase activity than the wild-type 9L cells. These results strongly suggest that ACNU specifically acts to reduce asparagine synthetase activity, probably via its mutagenic effects. The antitumor activity of L-asparaginase was first reported by Kidd,[19] and it has been used clinically in the treatment of lymphocytic leukemia.[14] This compound acts by depleting the cellular asparagine content, and the sensitivity of tumor cells to it therefore depends on the asparagine synthetase activity of the target cell.[22] We examined the sensitivity of R_1, R_3, and R_{12} cells and found that all three ACNU-resistant sublines showed collateral sensitivity to L-asparaginase, with the R_3 and R_{12} cells possessing a particularly high degree of sensitivity. These data also suggest that the phenotypic basis of the requirement for asparagine in these cell lines is their low asparagine synthetase activity.

Counteracting ACNU Resistance

Recently, two possible approaches for overcoming drug resistance have been considered. One is to use compounds that reverse the biochemical changes causing drug resistance in combination with antineoplastic agents. This kind of approach includes the use of calcium ion blockers to suppress P-glycoprotein expression in multidrug resistance,[30] buthionine sulfoximine (a glutathione synthetase blocker) for melphalan resistance,[11] or methylnitrosourea to deplete O^6-AT in the case of CENU resistance.[33] Another approach is to use different antineoplastic agents to which the resistant tumor cells have developed collateral sensitivity.[13] However, the collateral increase in sensitivity of drug-resistant cells is generally reported to be less than fivefold according to *in vitro* studies,[17,25,31] so this has not seemed a very promising approach. On the other hand, the three ACNU-resistant cell lines examined were highly sensitive to L-asparaginase, probably due to their low asparagine synthetase activity. The other six resistant cell lines may also show a high sensitivity to L-asparaginase because their asparagine synthetase activities were much lower than that of the wild 9L cells. Therefore, L-asparaginase therapy may become a new approach for treating ACNU resistance. To further examine the potential usefulness of such therapy, the rate of mutations of the locus for asparagine synthetase induced by ACNU and the sensitivity to L-asparaginase

should be studied in a large number of human cell lines,[6,28] since these results are preliminary and do not involve human tissue.

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