Asparagine Biosynthesis in Human KB Tumor Cells: Inhibitor Studies with Asparagine and Glutamine Antagonists

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SUMMARY

Resistance to L-asparagine depletion in tumor cells has been correlated with asparagine biosynthesis mediated by the enzyme asparagine synthetase. In this study, we describe certain characteristics of the asparagine synthetase of the KB human tumor cell carried in tissue culture. The KB enzyme is similar to that of other mammalian tumors in terms of cofactor requirements and inhibition by L-asparagine antagonists. The in vitro effect of glutamine antagonists on enzyme activity was determined. 6-Diazo-5-oxo-L-norleucine and azotomycin were shown to be effective inhibitors, but azaserine was relatively ineffective. The implications of combining such drugs with the antineoplastic agent L-asparaginase are discussed.

INTRODUCTION

Tumor inhibition by the antineoplastic agent L-asparaginase results from L-asparagine depletion, and resistance is related to synthesis of endogenous L-asparagine via the enzyme asparagine synthetase (1). Because of the importance of asparagine synthetase in tumor cell resistance to L-asparaginase, it has been studied in rodent tumors (3, 8, 14, 16), regenerating rat liver (15), guinea pig tissues (7), and man (6). These studies have shown that mammalian asparagine synthetase promotes the synthesis of L-asparagine from L-aspartic acid in the presence of a source of ammonia, a divalent cation (usually Mg²⁺), and ATP. Studies on the control of asparagine biosynthesis in mammals have shown product inhibition of asparagine synthetase (3, 14), a lack of substrate stimulation of asparagine synthetase in vivo by NH₄Cl or L-aspartic acid (16), and markedly increased asparagine synthetase levels following L-asparaginase therapy in vivo (6, 16). In contradistinction to these studies, relatively little has been reported regarding the effect of inhibitors on the asparagine synthetase derived from mammalian tissues, either in vitro or in vivo. In particular, there have been no reports of asparagine synthetase inhibition by clinically useful glutamine antagonists, except for a single instance in which azaserine inhibition in vitro was studied (14). In order to determine the effect of asparagine and glutamine antagonists on asparagine synthetase, we have studied the in vitro effect of a variety of such compounds on an enzyme preparation derived from the KB human tumor line maintained in tissue culture (4).

MATERIALS AND METHODS

KB human tumor cells grown in suspension culture were maintained at cell counts of 50,000 to 400,000/ml in Eagle’s minimal essential medium, supplemented with 5% horse serum, L-glutamine (0.3 g/liter), penicillin, and streptomycin. Cells were harvested at 24- to 48-hr intervals, washed twice with cold lactated Ringer’s solution, rapidly frozen in a Dry Ice-acetone bath, and stored at —20°. Acetone powder extracts were prepared and incubated as 3 to 5 parallel samples for 30 min in a 1.0-ml incubation medium containing 100 μmoles Tris at pH 7.8, 1 μmole EDTA, 10 μmoles mercaptoethanol, 8 μmoles MgCl₂, 20 μmoles L-glutamine, 8 μmoles disodium ATP (Sigma Chemical Co., St. Louis, Mo.), 2 to 8 μmoles L-aspartic acid, 0.4 to 0.8 μCi L-aspartic acid⁻¹⁴C (uniformly labeled; New England Nuclear Corp., Boston, Mass.) and 1 to 4 mg enzyme protein. The reaction was terminated by 1.0 ml 10% trichloroacetic acid, L-asparagine⁻¹⁴C was separated from L-aspartic acid⁻¹⁴C by elution through an alumina column with 0.5 N acetic acid, and radioactivity was measured by scintillation counting as previously described (6). The results were expressed as μmoles asparagine formed/mg protein/hr.

For the studies of drug inhibition, the incubation medium contained 4 μmoles L-aspartic acid and 0.8 μCi L-aspartic acid⁻¹⁴C/1.0 ml. The following chemicals were studied: DL-BAH¹ (Sigma); L-asparagine (General Biochemicals Corp., Chagrin Falls, Ohio); and D-asparagine (Calbiochem, Los Angeles, Calif.). The following drugs² were studied: azotomycin (NSC 56654); azaserine (NSC 742); duazomycin A (NSC 51097); DON (NSC 7365); DONV (NSC 117613); and puromycin (NSC 3055); The results were expressed as a percentage of the uninhibited control value ± S.E., expressed as a percentage.

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1 The abbreviations used are: DL-BAH, DL-aspartic acid-β-hydroxamic acid; DON, 6-diazo-5-oxo-L-norleucine; DONV, 5-diazo-4-oxo-L-norvaline.
2Provided by Dr. Harry Wood, Cancer Chemotherapy National Service Center, Bethesda, Md. 20014.
RESULTS

Twenty-five experiments were conducted with the KB enzyme system, and the mean asparagine synthetase level found was 28.4 ± 3.2 mmol asparagine/mg protein/hr (± S.E.). Deletion of MgCl₂, ATP, or L-glutamine abolished all asparagine synthesis in this system, and asparagine was not formed when the acetone powder was heated prior to incubation. Asparagine production was linear with time up to 90 min. When ammonium chloride was substituted for L-glutamine in the reaction at equimolar concentrations, asparagine synthesis occurred at 50% of the rate seen in a parallel control with L-glutamine as the amide donor. Studies were also performed to determine the concentration of L-aspartic acid required for zero-order kinetics. This was found to be 4 mM, and all of the inhibitor studies were therefore performed at that concentration of L-aspartic acid.

Asparagine synthetase levels were neither stimulated nor inhibited by puromycin in concentrations of 1.0 and 4.0 mM. This was studied because of reports that asparagine synthetase levels may be increased in certain murine tumors by inhibiting protein synthesis with puromycin (16). Our failure to demonstrate such an effect with KB cells and in L1210 murine leukemia (unpublished observations) has allowed us to study asparagine synthetase without the inclusion of puromycin in the incubation medium.

The results of incubation experiments with DL-BAH, L-asparagine, and DONV are shown in Chart 1. Equimolar concentrations of these 3 compounds resulted in 36, 72, and 88% inhibition of asparagine synthetase, respectively. In contrast are the results of repeated experiments with D-asparagine at concentrations of 0.5 and 1.0 mM, where no inhibition could be demonstrated. The effect of glutamine antagonists (2, 11, 18) is demonstrated in Chart 2. The inhibition achieved with 4 of these agents is as follows: 4 mM duazomycin A, 20%; 20 mM azaserine, 16%; 0.5 to 4.0 mM azotomycin, 42 to 81%; and 1.0 mM DON, 71%. In all of these studies, the standard errors were similar for the inhibited and uninhibited samples.

DISCUSSION

Asparagine synthetase is an important factor in clinical resistance to L-asparaginase; therefore, it seemed desirable to study a human neoplastic cell system with regard to inhibitors that may improve the clinical usefulness of L-asparaginase in man. Such a system was discovered in the KB human neoplastic cell line originally cultured by Eagle (4). In our preliminary experiments with this system, we found that it shares the cofactor requirements and in vitro enzymatic characteristics of most mammalian asparagine synthetases reported to date.

The first general class of potential inhibitors studied were those bearing a chemical resemblance to asparagine. The marked inhibition of asparagine synthetase by L-asparagine coincides with results reported in most of the other mammalian systems reported to date (3, 14). In addition to L-asparagine inhibition, definite inhibition was demonstrated with DL-BAH, a compound which has been shown to inhibit asparagine biosynthesis in Lactobacillus arabinosus (13). The other inhibitor in this group was DONV, an L-asparagine analog that probably has the additional characteristic of irreversibly binding to L-asparaginase binding sites (5).

The L-asparagine analogs used in this study have not been studied in therapeutical combination with L-asparaginase in man or in experimental animals, and it is unlikely that such combinations will be useful. The main reason for this prediction is that the L-asparaginase preparations in clinical use are in fact amidohydrolases (1) and would thus be expected to destroy such inhibitors before they could enter cells. Moreover, DONV has the additional effect of irreversibly binding with L-asparagine, thus leading to loss of L-asparaginase activity (5).

The second class of compounds studied were those which might inhibit asparagine synthetase by interfering with the
acquisition of the amido group from L-glutamine (2, 11, 18). All 4 of the glutamine antagonists studied inhibited asparagine synthetase; however, definite quantitative differences were demonstrated. Duazomycin A and azaserine were relatively ineffective inhibitors at the concentrations used, whereas marked inhibition was observed with DON and azotomycin. The inhibition achieved with 20 mM azaserine in our study was 16%, which is somewhat less than the 31% inhibition reported for the Novikoff hepatoma (14). There are no comparison data for the other 3 glutamine antagonists studied.

These findings may explain some of the results of recent studies in which L-asparaginase was combined with glutamine antagonists. Studies with azaserine in combination with L-asparaginase initially suggested synergism (12); however, more recent work with murine neoplasms (9) and human leukemia (10) have failed to demonstrate any additive therapeutic effect. These latter results may be explained by our finding that azaserine is a relatively poor inhibitor of asparagine synthetase in vitro. In contrast, both DON and azotomycin appear to increase the antineoplastic efficacy of L-asparaginase when used in combination against certain murine neoplasms (9, 17). These results may be explained by our finding that both DON and azotomycin inhibit asparagine synthetase strongly.

ACKNOWLEDGMENTS

We thank Miss Maria Paul for expert technical assistance and Dr. Patrick Henry for his generosity in providing the KB cells used to initiate this study.

REFERENCES

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