or leukocyte contamination must be considered. Fluoride stimulation resulted in an increase in the production of cyclic-AMP by the cells of the buffy coat of the cat (1·3 nmoles/mg protein), mouse (3·7 nmoles/mg protein) and rat (4·0 nmoles/mg protein) as compared with that of the erythrocyte ghosts of the mouse (0·38 nmoles/mg protein) and the rat (0·18 nmoles/mg protein). With rat platelets, fluoride stimulation of adenyl cyclase resulted in C-AMP production of 1·2 nmoles/mg protein. It is clear that the specific activity of the leukocyte and platelet adenyl cyclase is greater than that of the erythrocyte ghosts. It should be noted, however, that no norepinephrine stimulation could be detected with leukocyte and platelet preparations suggesting that these cells are responsive to some other hormones. In addition, microscopic examination of the erythrocyte preparations demonstrated that while platelet clumps with some trapped leukocytes were visible in washed erythrocyte fractions, none were visible in the ghost preparation. Instead, one obtains a pellet which possesses a fluoride but not norepinephrine-stimulated adenyl cyclase and is largely composed of platelets with some trapped leukocytes. We can safely say, therefore, that the fluoride and especially the norepinephrine-stimulated adenyl cyclase of the rat and mouse erythrocyte ghosts preparations reside in the plasma membranes of the erythrocytes and are not derived from contaminating platelets or leukocytes.

It is clear, therefore, that under the conditions of these experiments both NE- and NaF-stimulated adenyl cyclase can be found in non-nucleated erythrocytes such as the mouse and rat. Both of these species have an active sodium pump but the barely detectable adenyl cyclase in the human, cat and dog makes it impossible to support the concept that the presence of this enzyme might be correlated with that of an active sodium pump.

It remains to be demonstrated whether the adenyl cyclase in rat and mouse erythrocytes has an active role to play or only represents a residue from a functional past. Further characterization of the enzyme of the rat erythrocyte is underway.

Biochemical Pharmacology
Hoffman-La Roche Inc.
Nutley, N. J. 07110, *U.S.A.*

HERBERT SHEPPARD
CHARLES BURGHARDT

REFERENCES

1. L. M. KLAINER, Y. M. CHI, S. L. FRIEDBERG, T. W. RALL and E. W. SUTHERLAND, *J. biol. chem.* **237**, 1239 (1962).
2. O. M. ROSEN and S. L. ROSEN, *Biochem. biophys. Res. Commun.* **31**, 82 (1968).
3. E. W. SUTHERLAND, T. W. RALL and T. MENON, *J. biol. Chem.* **237**, 1220 (1962).
4. B. WEISS and E. COSTA, *J. Pharmac. exp. Ther.* **161**, 310 (1968).
5. G. KRISHNA, B. WEISS and B. B. BRODIE, *J. Pharmac. exp. Ther.* **163**, 379 (1968).

Biochemical Pharmacology, Vol. 18, pp 2578–2580. Pergamon Press. 1969. Printed in Great Britain

l-Asparaginase resistance in human leukemia—Asparagine synthetase

(*Received* 21 *March* 1969; *accepted* 2 *May* 1969)

l-ASPARAGINASE inhibition of rodent tumors has been related to *l*-asparagine depletion of tumor cells that require an exogenous source of *l*-asparagine.[1,3] It has since been demonstrated that a mechanism of *l*-asparaginase resistance in nonsensitive murine tumors is the presence of an *l*-asparagine synthetic pathway, mediated by the enzyme asparagine synthetase.[4,10] Studies on the control of *l*-asparagine biosynthesis in mammals have shown product inhibition of asparagine synthetase, a lack of substrate stimulation of asparagine synthetase by NH_4Cl or *l*-aspartic acid, and markedly increased asparagine

synthetase levels after *l*-asparaginase treatment. On the basis of this data it has been postulated that derepression of enzyme is the primary control mechanism for asparagine synthetase in resistant mammalian cells.[9,10] The nearly complete absence of asparagine synthetase in murine tumor cells sensitive to the inhibitory effect of *l*-asparaginase represents a biochemical difference between sensitive and resistant tumor cells which is currently being exploited for therapy. We have studied asparagine synthetase levels in 18 patients with leukemia who were subsequently treated with *l*-asparaginase, in order to determine whether this enzyme may play a role in human leukemic cell resistance to *l*-asparaginase.

E. coli l-asparaginase was obtained through the courtesy of Merck, Sharp & Dohme or through National Cancer Institute contract No. PH-43-65-1056 with Squibb & Co. It was given at a dose of 200 I.U. per kg daily to eighteen patients with leukemia, all of whom had pretreatment determinations of asparagine synthetase. The usual duration of treatment was 3 weeks; however, a single patient with chronic granulocytic leukemia was treated only 3 days because of the coincident development of both toxicity and response. The following patients were treated, with the number of patients with

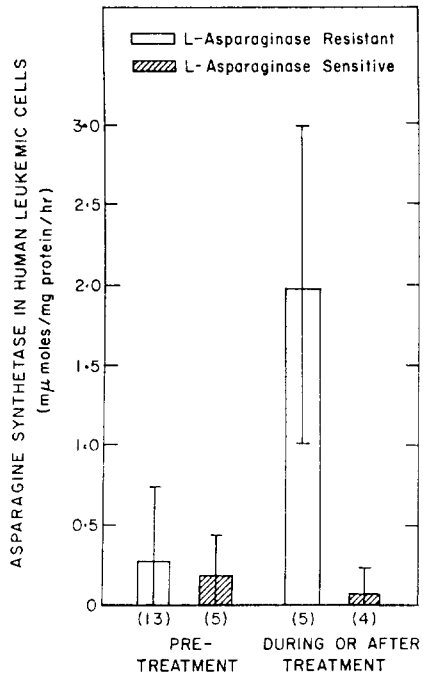

FIG. 1. Asparagine synthetase levels in eighteen patients with leukemia determined prior to therapy with *l*-asparaginase and in nine cases during or after therapy. For each group the bar gives the mean level of asparagine synthetase expressed as mμmoles asparagine formed per mg protein per hr of incubation. The number in parentheses below each bar represents the number of patients in each group, and the bracket with each bar shows one standard deviation.

leukemia resistant to *l*-asrapaginase (R) or sensitive (S) being indicated in parentheses: acute lymphoblastic leukemia (5R, 4S); acute granulocytic leukemia (3R); chronic granulocytic leukemia (4R, 1S); and chronic lymphocytic leukemia (1R). The nine patients with repeat determinations during or after therapy included the following: acute lymphoblastic leukemia (2R, 3S); chronic granulocytic leukemia (2R, 1S); chronic lymphocytic leukemia (1R).

Cells were obtained from bone marrow aspirates or from peripheral blood specimens obtained by

plasmapheresis. Leukemic cells were separated by Dextran sedimentation or by differential centrifugation, and washed an average of five times with 3 to 4 vol. of a balanced salt solution. Thorough washing of cells was particularly critical during *l*-asparaginase therapy, since even trace contamination with *l*-asparaginase could obliterate the *l*-asparagine formed in the subsequent assay. Acetone powder extracts were prepared and incubated in duplicate or triplicate for 30 min at 37° in a 1·0-ml incubation medium containing 100 μmoles Tris at pH 7·8, 8 μmoles $MgCl_2$, 20 μmoles *l*-glutamine, 8 μmoles disodium ATP, 2 μmoles *l*-aspartic acid, 0·4 μc ^{14}C-*l*-aspartic acid (u.l., New England Nuclear Corp.), and 1 to 4 mg of enzyme protein.[11] The reaction was terminated by 1·0 ml 10% trichloroacetic acid, the pH was readjusted to 5–7 with 5 N KOH, and 0·5 mg *l*-asparagine carrier was added. ^{14}C-*l*-asparagine formed was then separated from ^{14}C-*l*-aspartic acid by eluting the reaction mixture through an Alumina column (Fisher Co.) with 0·5 N acetic acid. This represents a modification of an earlier method,[12] in that acetic acid is used in lieu of water as the wash solution. This change was made because of our repeated inability to elute ^{14}C-*l*-asparagine (Nuclear-Chicago) with water alone, compared to 80 to 85% elution of ^{14}C-*l*-asparagine and $< 2\%$ elution of ^{14}C-*l*-aspartic acid with 0·5 N acetic acid. Radioactivity was determined in a liquid scintillation spectrometer, and results expressed as mμmoles asparagine formed per mg protein per hr of incubation. Confirmation of *l*-asparagine synthesis in this system was demonstrated by a 100 per cent reduction in elutable radioactivity after incubation of reaction product with *E. coli* *l*-asparaginase.

The results of this study are summarized in Fig. 1. It can be seen that asparagine synthetase in leukemic cells was nearly undetectable prior to therapy regardless of subsequent response to *l*-asparaginase treatment. The 4 patients in whom an antileukemic effect occurred had no change in asparagine synthetase with therapy; however, there was a 7-fold increase in the mean level of asparagine synthetase in the five patients who were unresponsive to treatment. The difference between asparagine synthetase levels in the sensitive and resistant patients, determined during or after *l*-asparaginase, was highly significant ($t < 0{\cdot}005$).[13]

We conclude from this data that *l*-asparaginase resistance in human leukemic cells is at least in part related to the capacity for *l*-asparagine biosynthesis via asparagine synthetase. Furthermore, the fact that this enzymatic difference between sensitive and resistant cells could not be demonstrated prior to therapy underlines the importance of studying such mechanisms under the stress of treatment.

Acknowledgements—We thank M. R. Paul for expert technical assistance and Drs. B. G. Leventhal and R. Graw for their help in these studies.

Solid Tumor Service
Medicine Branch
National Cancer Institute
Bethesda, Md. 20014, *U.S.A.*

C. M. Haskell
G. P. Canellos

REFERENCES

1. J. D. Broome, *J. exp. Med.* **118**, 121 (1963).
2. L. H. Sobin and J. G. Kidd, *Proc. Soc. exp. Biol. Med.* **119**, 325 (1965).
3. L. H. Sobin and J. G. Kidd, *J. exp. Med.* **123**, 55 (1966).
4. M. K. Patterson, Jr. and G. R. Orr, *Biochem. biophys. Res. Commun.* **26**, 228 (1967).
5. J. D. Broome and J. H. Schwartz, *Biochim. biophys. Acta* **138**, 637 (1967).
6. M. K. Patterson, Jr. and G. R. Orr, *J. biol. Chem.* **243**, 376 (1968).
7. B. Horowitz, B. Madras, A. Meister, L. J. Old, E. A. Boyse and E. Stockert, *Science N.Y.* **160**, 533 (1968).
8. J. D. Broome, *J. exp. Med.* **127**, 1055 (1968).
9. M. D. Prager and N. Bachynsky, *Biochem. biophys. Res. Commun.* **31**, 43 (1968).
10. M. D. Prager and N. Bachynsky, *Archs Biochem. Biophys.* **127**, 645 (1968).
11. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
12. S. P. Bessman, in *Methods in Enzymology* (Eds. S. P. Colowick and N. O. Kaplan), p. 575. Academic Press, New York (1957).
13. G. H. Weinberg and J. A. Schumaker, *Statistics—An Intuitive Approach*, Wadsworth, Belmont, California (1962).