EFFECTS OF L-ASPARAGINASE ON PROTEIN AND GLYCOPROTEIN SYNTHESIS

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1. Introduction

The anti-neoplastic enzyme L-asparaginase inhibits the growth of certain malignant cells by interference with cell nutrition [1,2]. We have recently reported [3] that L-asparaginase inhibits the incorporation of labeled L-fucose and D-glucosamine into glycoproteins of L5178Y murine leukemia cells. Three questions arise with respect to this finding: 1) Is the inhibition of glycoprotein synthesis a consequence of a general inhibition of all protein formation? 2) Can the observed results be explained on the basis of the known glutaminase activity [4] of asparaginase preparations? 3) Is glycoprotein formation inhibited by L-asparaginase in cell lines resistant to the anti-neoplastic action of the enzyme? In the present study, we have provided evidence to indicate that L-asparaginase preferentially inhibits glycoprotein formation in asparaginase-sensitive cell lines in a manner unrelated to the glutaminase activity of the enzyme.

For this study, we have employed the L5178Y murine leukemia, which has an absolute requirement for L-asparagine, in culture [5], and a subline, L5178Y/ASP, derived from L5178Y by selection for asparaginase resistance. Incorporation of labeled L-leucine, L-fucose and D-glucosamine into protein and glycoprotein was measured, and the inhibitory effects of L-asparaginase were compared with those of cycloheximide, a potent inhibitor of protein synthesis [6].

L-Asparaginase inhibited incorporation of fucose and glucosamine into L5178Y glycoproteins as rapidly as did cycloheximide, but inhibited incorporation of leucine into proteins less rapidly than cycloheximide. The inhibitory action of the enzyme was not related to contaminating glutaminase activity, during short-term incubations. Asparaginase did not effect protein and glycoprotein formation by L5178Y/ASP cells, except at very high enzyme levels.

2. Materials and methods

The L5178Y and L5178Y/ASP cell lines were provided by Mr. I. Wodinsky, Arthur D. Little Inc., Cambridge, Massachusetts. The latter cell line was derived from L5178Y by selection for resistance to 100 I.U./kg of L-asparaginase *in vivo*. Both cell lines were carried in CDF_1 mice by weekly transplant of 10^6 cells.

L-Asparaginase (360 U./mg) was obtained from the National Cancer Institute, and was manufactured by Merck, Sharpe and Dohme, Inc. L-Leucine-1-^{14}C, D-glucosamine-U-^{14}C, L-fucose-U-^{3}H, L-asparagine-4-^{14}C and L-glutamine-U-^{14}C were purchased from New England Nuclear Corp., cycloheximide (crystalline) from Mann Research Laboratories, Fischer's medium (10 X) from Grand Island Biological Col, and *N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer from Calbiochem Corp.

Cells were removed from animals on day seven after transplant, and were collected in cold 0.9% NaCl. Erythrocytes were removed by osmotic shock, and the cells were washed with HEPES-buffered saline [7]. The cells were suspended in modified Fischer's medium [8] with 7.65 g/l HEPES replacing the $NaHCO_3$. The suspension, which contained 500 mg of cells (wet weight) per 10 ml, was filtered through Miracloth (Calbiochem Corp.).

Cell suspensions were incubated in the modified Fischer's medium containing specified levels of cycloheximide, L-asparaginase and radioactive precursors, at 37°, with occasional shaking. At intervals, 0.2 ml portions of the suspension were removed, quickly chilled and the cells collected by centrifugation at 250 *g* for 40 sec. The resulting pellets were resuspended in 1 ml of cold 5% trichloroacetic acid, and the cells were collected, by centrifugation, after 3 min. After two more acid washes and one ether-ethanol (1:2, v/v) wash the precipitate was washed with water, and dispersed in 50 μl of water + 450 μl of Nuclear Chicago Solubilizer (NCS). After solution was complete, a 400 μl portion of this mixture was diluted with 10 ml of a toluene-based liquid phosphor for measurement of radioactivity by liquid scintillation counting. After incorporation and hydrolysis, the labeled material in the case of L-leucine-1-^{14}C was 100% leucine; the fucose-U-^{3}H, 100% fucose; and the glucosamine, 70% glucosamine, 20% galactosamine and 10% *N*-acetylneuraminic acid [3].

Incubation mixtures contained 10 mg/l of L-asparagine or 200 mg/l of glutamine, levels which are found in Fischer's medium [8]. Tracer levels of labeled L-asparagine or L-glutamine were added. Total incubation volumes were 200 μl. At intervals, 10 μl portions were removed, and subjected to electrophoretic separation in 0.05 M barbital buffer at pH 8. Carrier amino acids were added to facilitate identification of the appropriate spots by the ninhydrin color reaction. The spots were removed from the paper, and radioactivity was determined by liquid scintillation counting.

3. Results and discussion

Cell suspensions were mixed with 0.02–200 U./ml of L-asparaginase and warmed to 37°. After 5 min, labeled L-leucine (final level = 200 μM), D-glucosamine (10 μM) or L-fucose (1 μM) was added. Cell samples were taken at 0, 10, 20 and 30 min after addition of radioactive precursors. Incorporation of radioactivity into the acid-insoluble fractions represented incorporation of leucine into protein, and of fucose and glucosamine into glycoprotein [3]. The rate of such incorporation proceeded linearly for at least 20 min in control assay. The data of fig. 1 represent inhibition of leucine and glucosamine incorporation as a function of the L-asparaginase concentration. Experiments utilizing L-fucose yielded data similar to that obtained with glucosamine. Precursor incorporation into L5178Y cells was profoundly inhibited, even by 0.02 U./ml levels of the enzyme. Higher levels produced some additional inhibition. In contrast, precursor incorporation in L5178Y/ASP was unaffected until enzyme levels reached 200 U./ml.

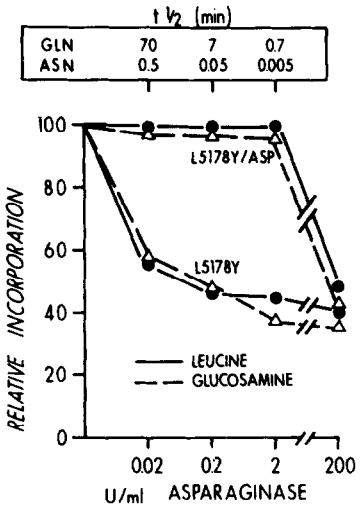

Fig. 1. Glycoprotein and protein synthesis by L5178Y and L5178Y/ASP cells. Levels of L-asparaginase in the medium are specified. The data indicate relative incorporation of leucine and glucosamine over a 20 min interval. The rates of loss of glutamine (GLN) and asparagine (ASN) from the medium as function of the L-asparaginase concentration, are also shown.

The rates of hydrolysis of L-asparagine and L-glutamine in Fischer's medium were measured, and the time required for loss of 50% of the initial levels of these amino acids is shown, in fig. 1, as a function of the L-asparaginase level. The data indicate that inhibition by L-asparaginase of leucine and glucosamine incorporation over a 20 min interval is not related to removal of glutamine from the medium.

In other studies, we found that omission of glutamine from Fischer's medium did not inhibit the incorporation of glucosamine or leucine into macromolecules in L5178Y or L5178Y/ASP cells under conditions specified above.

Incorporation of leucine and glucosamine by

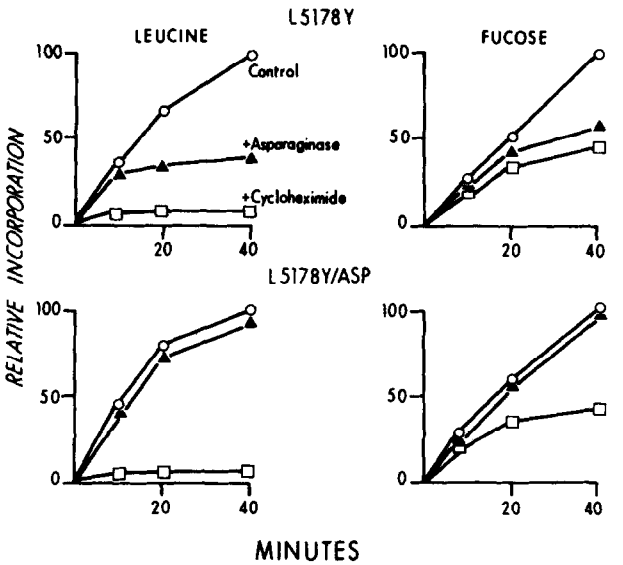

Fig. 2. Glycoprotein and protein synthesis by L5178Y and L5178Y/ASP cells. Cells were incubated with 2 U./ml of L-asparaginase (▲), 10 μg/ml of cycloheximide (□) or with neither (○), along with labeled leucine or fucose, as described in the text. Results are expressed relative to the 40 min control value, taken as 100%.

L5178Y/ASP cells was affected at 200 U./ml of L-asparaginase. This might be related to the capacity [3] of high levels of this enzyme to cleave the glycopeptide bond between carbohydrate and the amide nitrogen of L-asparagine found in certain glycoproteins [9]. Alternatively, use of such high concentrations of the enzyme may cause the enzyme to enter the intracellular space and act on intracellular asparagine pools.

In another study, L5178Y cell suspensions were incubated with labeled precursors together with 10 μg/ml of cycloheximide or 2 U./ml of asparaginase. This level of cycloheximide was the minimum which promptly stopped incorporation of leucine into protein. Incorporation of precursors into an acid-insoluble fraction was measured. The results (fig. 2) show that cycloheximide abolished leucine incorporation at once, but that leucine incorporation continued for a short time in the presence of asparaginase. Presumably, proteins not containing asparagine were formed, together with some proteins utilizing endogenous stores of this amino acid. Fucose incorporation was inhibited at approximately equal rates by cycloheximide or asparaginase. These results were not changed when glucosamine was employed as the glycoprotein precursor. The combination of asparaginase + cycloheximide was not more effective than cycloheximide alone. These data suggest that considerable incorporation of leucine but not of fucose into cycloheximide-inhibitable protein continues after administration of asparaginase to L5178Y cells.

These studies were repeated, using the L5178Y/ASP cell line as a control. L-asparaginase did not inhibit incorporation of either fucose or leucine into macromolecules, at 2 U./ml (fig. 2).

We conclude that L-asparaginase inhibits a cycloheximide-sensitive incorporation of fucose into glycoprotein more rapidly than the enzyme inhibits cycloheximide-sensitive leucine incorporation into protein. Over short time periods, this inhibition is not related to glutaminase activity of asparaginase preparations. The latter enzyme inhibited neither protein nor glycoprotein in an asparaginase-resistant cell line, except at very high asparaginase levels. Although interference with glycoprotein synthesis would be expected as a result of a general inhibition of protein formation [1,2], a preferential early inhibition of glycoprotein formation could explain the rapid dissolution of cell membranes caused by L-asparaginase [10].

Acknowledgments

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References

[1] J.D. Broome, Trans. N.Y. Acad. Sci. 30 (1968) 690.
[2] D.A. Cooney and R.E. Handschomacher, Ann. Rev. Pharmacol. 10 (1970) 421.
[3] H.B. Bosmann and D. Kessel, Nature 226 (1970) 850.
[4] H.K. Miller and M.E. Bahr, Biochem. Pharmacol. 18 (1969) 2225.
[5] E.E. Haley, G.A. Fischer and A.D. Welch, Cancer Res. 21 (1961) 532.

[6] F.O. Wettstein, H. Noll and S. Penman, Biochim. Biophys. Acta 87 (1964) 525.
[7] C. Shipman Jr., Proc. Soc. Exptl. Biol. Med. 130 (1969) 305.
[8] G.A. Fischer and A. Sartorelli, Methods Med. Res. 10 (1964) 247.
[9] A. Neuberger, A. Gottschalk and R.D. Marshall, Glycoproteins, ed. A. Gottschalk (American Elsevier, New York, 1966) p. 273.
[10] L.H. Sobin and J.G. Kidd, J. Exptl. Med. 123 (1966) 55.