Reversal by L-Glutamine of the Inhibition of Lymphocyte Mitosis Caused by *E. coli* Asparaginase[1] (34535)

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L-Asparaginase has been reported to inhibit the mitotic response of lymphocytes to phytohemagglutinin (PHA) if added directly to the medium (1), or in cell suspensions prepared from patients being treated with this drug (2). Motosis of lymphocytes in medium without L-asparagine or L-aspartic acid has also been noted (3). We have reported that *E. coli* L-asparaginase is a potent inhibitor of lymphocyte mitosis and secondary antibody formation *in vitro,* while comparable amounts of the L-asparaginase of agouti serum are ineffective (4).

The bacterial enzyme is known to have a broader substrate specificity than agouti serum, acting on L-glutamine as well as L-asparagine (5). This study expanded the previous observations and provided evidence that the *E. coli* preparation's effect on lymphocyte mitosis is a function of its glutaminase activity.

Material and Methods. Methods of human lymphocyte culture and quantitation of their mitotic activity have been described in detail elsewhere (4). Briefly, cultures consisted of approximately 2 million cells in 4 ml of medium (minimal essential medium with 20% autologous plasma). Mitotic stimulus was provided by the addition of 0.1 ml of phytohemagglutinin (PHA-M, Difco Laboratories, Detroit, Mich.), 5 μg of tuberculin (PPD, Parke Davis and Co., Detroit, Mich.), or by the mixed lymphocyte reaction (6). *E. coli* L-asparaginase (30 IU/mg; Sigma Chem. Co., St. Louis, Missouri) was added to tubes as indicated.

Twenty-four hr prior to harvesting 0.1 μCi of ^{14}C thymidine (sp act 54.1 mCi/mmole) was added to triplicate tubes. At the end of the incubation period, the cells were sedimented, washed with 0.85% NaCl, cold trichlorocetic acid, and cold methanol. After overnight drying at room temperature, the material was dissolved with Hyamine (Packard Instrument Co., Downers Grove, Ill.), transferred to vials containing Liquifluor (Packard), and counted in a Packard Tri-Carb scintillation counter.

Results. The effects of 1 IU/ml of *E. coli* enzyme on lymphocytes stimulated by PHA and by antigens is shown in Table I. *E. coli* L-asparaginase blocks thymidine incorporation into cells stimulated by PPD and by histocompatability antigens in the mixed lymphocyte reaction, as well as their response to PHA.

Reversibility of the effect of L-asparaginase on lymphocyte mitosis is demonstrated in Table II. When the bacterial enzyme is present in the final incubation medium, nucleotide incorporation is blocked. Removal of the enzyme by changing the medium at the end of 24 hr results in restoration of normal lymphocyte reactivity.

Failure of agouti serum to inhibit lymphocyte mitosis, together with the observed mitotic activity of cells in asparagine poor medium (3), suggested that the effect of the *E. coli* enzyme might be due to its activity on substrates other than L-asparagine. The bacterial preparation has glutaminase as well as asparaginase activity (5). Direct addition of L-asparagine and L-glutamine to medium containing active enzyme and lymphocytes did not overcome the mitotic inhibition. Ex-

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TABLE I. Effect of L-Asparaginase on Mitotic Response of Lymphocytes.[a]

Cell source	Mitotic stimulus	Enzyme added: None	Enzyme added: L-Asparaginase[b]
Normal donor	PHA[c]	15,500	2
Tuberculin-sens. donor	PPD[d]	6139	8
Normal donors: AB + MS	Mixed lymphocyte[e]	4967	0

[a] Mitotic response of lymphocytes expressed as cpm (counts per minute) of ^{14}C thymidine incorporated during the final 24 hr of a 120-hr incubation period. Cultures contained approximately 2 million lymphocytes in 4 ml of medium (minimal essential medium with 20% autologous plasma). Results are the average of triplicate samples.

[b] L-Asparaginase (Sigma), *E. coli*, 30 IU/mg; 1 IU/ml was added to each tube.

[c] PHA-M, phytohemagglutinin (Difco): 0.1 ml was added to each tube.

[d] PPD, tuberculin (Parke Davis): 5 μg were added to each tube.

[e] Equal volumes of cell suspensions from donors AB and MS were added to each tube.

periments were therefore performed with medium preincubated for 24 hr with the *E. coli* enzyme and then heated at 67° for 3 hr to destroy the enzyme's activity. The results are shown in Table III.

At a concentration of 0.5 IU/ml active, enzyme blocked thymidine incorporation by lymphocytes stimulated with PHA. Neither L-asparagine nor L-glutamine could reverse this. Preincubation of medium with heat-inactivated enzyme did not inhibit the mitotic response. However, in medium which was preincubated with active enzyme followed by heat inactivation, there was complete inhibition of lymphocyte mitotic activity. Inhibition was not reversed by addition of L-asparagine to the medium but was reversed by addition of L-glutamine.

Discussion. The glutaminase activity of *E. coli* L-asparaginase was shown to account for its inhibition of HeLa cell proliferation (7). Our results indicate that its effect on lymphocyte mitosis is similarly mediated. The glutaminase activity of purified *E. coli* enzyme is 2% of its asparaginase activity (5), while the L-glutamine concentration in human plasma far exceeds that of L-asparagine (8). Therefore, despite its effect on antigenically-stimulated lymphocytes *in vitro* *E. coli* asparaginase would not appear to be a potentially useful *in vivo* immunosuppressive agent.

TABLE II. Reversibility of Inhibition of the Lymphocyte Response[a] to PHA by L-Asparaginase.

Additives to initial medium	Additives to medium after change at 24 hr[b]: None	PHA[c]	L-Asparaginase[d]	PHA + L-asparaginase
None	89	16,549	15	10
PHA[c]	17,669	17,919	214	168
L-Asparaginase[d]	51	7025	11	9
PHA + L-asparaginase	4623	5309	10	10

[a] Mitotic response of lymphocytes expressed as cpm of ^{14}C thymidine incorporated during the final 24 hr of a 96-hr incubation period.

[b] Twenty-four hr after initiation of the culture, the medium was removed, the cells were washed, and they were resuspended in fresh medium with additives as indicated.

[c] PHA-M, 0.1 ml, added to each tube.

[d] L-Asparaginase, *E. coli*; 1 IU/ml added to each tube.

TABLE III. Response of Lymphocytes to PHA[a] in Medium Preincubated with L-Asparaginase.

L-Asparaginase[b]		Added amino acid			
Preincubated with medium[c]	Added with lymphocytes	None	L-Asparagine[d]	L-Glutamine[e]	L-Asparagine + L-glutamine
None	None	20,586	26,305	19,090	18,308
	0.5 IU/ml	87	91	131	129
0.5 IU/ml inactivated[f]	None	16,873	23,102	18,295	19,454
0.5 IU/ml	None	701	237	9068	9382

[a] Mitotic response expressed as cpm of ^{14}C thymidine incorporated into lymphocytes during the final 24 hr of a 96-hr incubation period. PHA-M, 0.1 ml, was added to each tube.

[b] L-Asparaginase, *E. coli.*

[c] Medium (minimal essential medium with 20% pooled human serum) was preincubated at 37° for 24 hr, then heated to 67° for 3 hr before the lymphocytes were added.

[d] L-Asparagine, 0.1 mg, added to each tube with the lymphocytes.

[e] L-Glutamine, 2 mmoles, added to each tube with the lymphocytes.

[f] L-Asparaginase was heated 67° for 3 hr before adding to the medium for preincubation.

The role that L-glutamine plays in the transformation process remains to be clarified. Glutamine starvation is known to reversibly reduce protein and RNA synthesis and to diminish polyribosomal activity of liver cells (9). The alterations of lymphocyte metabolism in glutamine-free medium seem to be similarly profound.

Lymphocyte mitosis is also reversibly inhibited by mycoplasmal arginase (4, 10). Thus, L-glutamine and L-arginine are essential for transformation of the small lymphocyte, while L-asparagine is not required. The former amino acids are included in a list of "essential" amino acids for many mammalian cell cultures (11). However, the nutritional requirements for transformation and mitosis of the lymphocyte have not been determined.

The small lymphocyte is reported to be enzymatically poorer than transformed lymphocytes (12) and other mammalian cells. Its nutritional requirements may therefore be unique. Manipulation of environment by enzymes or other means might inhibit lymphocyte mitosis without damaging other cells. A precise characterization of the essential medium components for lymphocyte transformation and mitosis should provide tools for the further study and control of immune reactions.

Summary. Inhibition of lymphocyte mitosis by *E. coli* L-asparaginase can be prevented by addition of L-glutamine to enzyme-treated culture medium. Like arginine, glutamine appears to be essential for lymphocyte transformation and mitosis, while exogenous asparagine is not required.

1. Astaldi, G., Burgio, G. R., Krc, J., Genova, R., and Astaldi, A. A., Lancet **1**, 423 (1969).

2. McElwain, T. J. and S. K. Hayward, Lancet **1**, 527 (1969).

3. Dartnall, J. A. and Baikie, A. G., Lancet **1**, 1098 (1969).

4. Simberkoff, M. S., Thorbecke, G. J., and Thomas, L., J. Exp. Med. **129**, 1163 (1969).

5. Campbell, H. A., Mashburn, L. T., Boyse, E. A., and Old, L. J., Biochemistry **6**, 721 (1967).

6. Hirschhorn, K., Bach, F., Rapaport, F. T., Converse, J. M., and Lawrence, H. S., Ann. N. Y. Acad. Sci. **120**, 303 (1964).

7. Kim, J. H., Boyse, E. A., Old, L. J., and Campbell, H. A., Biochim. Biophys. Acta **158**, 476 (1968).

8. "Biochemists Handbook," (Long, C., ed.) p. 851–852. Van Nostrand, Princeton, N. J. (1961).

9. Eliasson, E., Bauer, G., and Hultin, T., J. Cell Biol. **32**, 287 (1967).

10. Copperman, R. and Morton, H. E., Proc. Soc. Exp. Biol. Med. **123**, 790 (1966).

11. Eagle, H., Science **130**, 432 (1959).

12. Gough, J. and Elves, M. S., Acta Haematol. **37**, 42 (1967).

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